Jpn. J. Cancer Res. 86, 1080–1085, November 1995

Inhibition of Tumor Cell Haptotaxis by Sodium D-Glucaro-δ-lactam (ND2001)

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We used the Boyden chamber system to investigate the mechanism by which the antimetastatic agent sodium D-glucaro-δ-lactam (ND2001) inhibits tumor cell invasion, and by establishing what ND2001 did not achieve, we were able to pinpoint the areas in which it was successful as an inhibitor. ND2001 did not inhibit cell adhesion of a highly metastatic B16 melanoma variant (the B16 variant) to the reconstituted basal membrane Matrigel, nor did it affect the production or activity of basal membrane-degrading type IV collagenase, but, in the Boyden chamber, ND2001 inhibited cell migration of the B16 variant toward a chemoattractant, laminin, on the lower surface of a Matrigel-free filter set (haptotaxis). Lewis lung carcinoma (3LL) cells that had been treated with ND2001 also exhibited hardly any haptotaxis, although the cells showed no alteration in behavior during cell adhesion to Matrigel. Since ND2001 did succeed in inhibiting the pulmonary metastases of the B16 variant and 3LL, we infer that inhibition of the metastases by ND2001 in these tumors is likely to be due to the inhibition of haptotactic migration.

Key words: Antimetastatic agent — Haptotaxis — Invasion — Metastasis — ND2001

We have previously reported the effectiveness of sodium D-glucaro-δ-lactam (ND2001), a derivative of the antibiotic nojirimycin A, as an agent capable of inhibiting experimental tumor metastasis.[1] Although ND2001 did not exhibit any cytotoxicity or obvious antitumor activity, it did inhibit spontaneous pulmonary metastases of three tumor lines, i.e., a highly metastatic B16 melanoma variant (the B16 variant), 3LL[4] and rat KDH-8 liver carcinoma.[1] We infer that, because the metastases were reduced by *in vitro* treatment of these tumor cells with ND2001, ND2001 inhibited the metastases by altering the properties of the tumor cells through some direct action other than cytotoxicity.[1] Moreover, because this agent inhibited tumor cell invasion when used in the Boyden chamber method, we infer that ND2001 inhibited metastases by inhibiting the extravasation of the tumor cells.[1] The tumor cell invasion in this system consists of a series of stages: adhesion of tumor cells to the reconstituted basal membrane Matrigel, degradation of Matrigel by the cells, and cell migration toward a chemoattractant, laminin, coated on the lower surface of the filter.[2,3] We have now examined the effects of ND2001 on each stage of the process to elucidate the mechanism by which this agent inhibits tumor metastasis.

MATERIALS AND METHODS

Tumor cell lines and culture The following tumor cell lines were used: a highly metastatic B16 melanoma variant (the B16 variant),[1] another B16 variant B16-BL6,[4] 3LL, PC-13 human large-cell lung carcinoma,[5] PC-14 human poorly differentiated lung adenocarcinoma,[5,6] YS-K human ovarian cancer[5] and T-24 human urinary bladder carcinoma.[7] Cell lines were cultured on DMEM supplemented with 10% FCS (Flow Labs, McLean, VA) at 37°C in a humidified 5% CO_2-95% air atmosphere.

Cell adhesion Tumor cells were cultured for 12 h in the presence of 100 μg/ml of ND2001, harvested by treatment with PBS containing 0.08% sodium citrate at 37°C for 5 min, washed with the culture medium, and suspended in the culture medium. One hundred μl of the suspension was put into each well of 96-well tissue culture plates (C. A. Greiner und Söhne GmbH, Frickenhausen, Germany) with or without a Matrigel coating. After incubation for the indicated time, each well was washed three times with the medium, then the number of cells attached to the bottom of the well was determined by the modified MTT method.[8,9] Absorbance of the MTT reaction mixture was measured at 577 nm with the reference absorbance at 630 nm.

Phagokinetic displacement Apparent random movement of cells was measured essentially according to the method of Albrecht-Buehler *et al.*[10] Gold colloid consisting of

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[4] Abbreviations: 3LL, Lewis lung carcinoma; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

1.3 m*M* $AuCl_4H$, 10.6 m*M* Na_2CO_3 and 0.009% formaldehyde was overlaid on the bovine serum albumin- or laminin-coated cover glass. The cover glass was rinsed with PBS and then with DMEM. Cells cultured in the presence of ND2001 for 48 h were put on the cover glass and incubated on the DMEM medium supplemented with ND2001, and 0.1% or 10% FCS (measurement medium) for measuring the phagokinetic displacement. The cells on each cover glass were fixed with 3.5% formaldehyde in PBS, dried, and overlaid on a slide. The number of cells showing movement was then counted for fixed areas under a light microscope (×200); we also counted the number of cells which had not moved.

Type IV collagenase activity Type IV collagenase was assayed by the procedure described previously.[11] B16-BL6 cells were cultured for 76 h, washed twice with a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM/F12) supplemented with 1% FCS and cultured on the medium for 24 h. The cells were washed twice with DMEM/F12 and incubated on the medium for 24 h. ND2001 (100 μg/ml) was added to the above-mentioned culture media for examination of its effect on the production of type IV collagenase. The culture medium was collected by centrifugation at 1,000*g* at 4°C for 10 min, and the supernatant was centrifuged at 10,000*g* at 4°C for 10 min. The supernatant was concentrated by using a Centricon 30 (Amicon, Danvers, MA). The type IV collagenase in the supernatant was detected by gelatin-zymography.[11] The effect of ND2001 on the activity of type IV collagenase was analyzed by staining it with 0.1% Coomassie blue after incubating the electrophoresed gelatin gel in 50 m*M* Tris-HCl (pH 7.5) containing 100 μg/ml of ND2001, 0.15 *M* NaCl, 10 m*M* $CaCl_2$ and 0.05% NaN_3 at 37°C for 40 h.

Haptotaxis assay Measurement of haptotaxis was performed according to the Boyden chamber method described previously.[2,3] Twenty-four-well Transwell cell culture chambers (Costar, Cambridge, MA) were used, in which the lower surface of the Nuclepore filter was coated with 10 μg of laminin (Collaborative Research Inc., Bedford, MA), a cell-attracting substance. Cells treated with 100 μg/ml of ND2001 were detached by treating them with 0.08% sodium citrate, washed twice with DMEM containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), and resuspended in the same medium. A 100-μl aliquot was placed in the upper compartment of the Transwell chamber, while 600 μl of DMEM containing 0.1% bovine serum albumin or 3T3-conditioned medium[2] was placed in the lower compartment. The chamber was incubated at 37°C in a humidified 5% CO_2-95% air atmosphere. After incubation, cells on the upper surface of the filter were removed, while cells which had moved to the lower surface were fixed, stained, and counted in 5 fixed areas (0.3 mm^2) under a light microscope (×200). Each assay was carried out in duplicate, and the numbers were averaged. For each tumor cell line, the experiment was repeated twice.

RESULTS

Effects of ND2001 on tumor cell adhesion The number of the B16 variant cells adhering to the bottom of the culture plate increased linearly for 30 min and reached a maximum within 1-h incubation (Fig. 1A). Pretreatment of the cells with ND2001 at 100 μg/ml for 12 h had no effect on cell viability, and the number of the treated cells adhering to the culture plate was almost equal to the

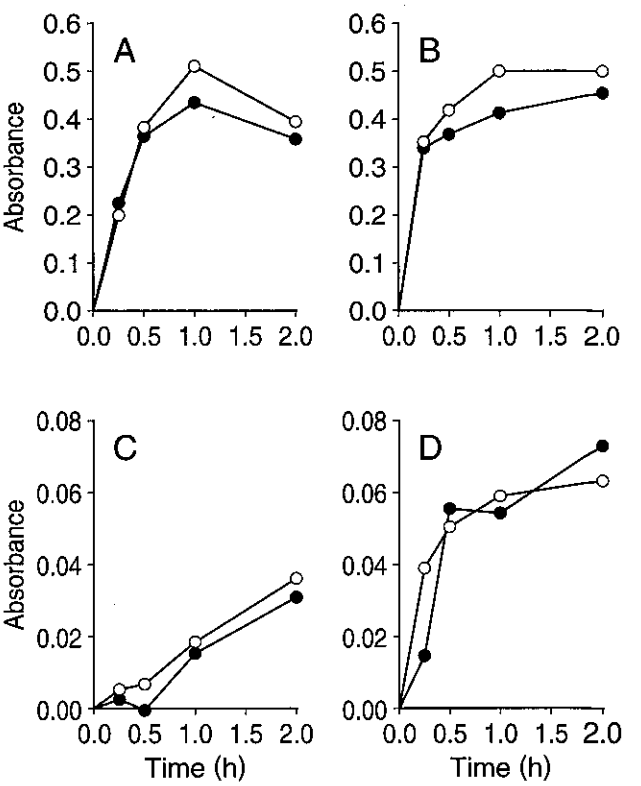

Fig. 1. Effect of ND2001 on adhesion of tumor cells. The B16 variant (A and B) or 3LL (C and D) cells were cultured in the presence of 100 μg/ml of ND2001, detached from the culture dishes, washed and suspended in the culture medium. One hundred μl of the suspension was put into each well of a 96-well tissue culture plate without Matrigel coating (A and C) or with Matrigel coating (B and D). After incubation, the number of cells adhering to the bottom was measured by the modified MTT method. Absorbance of the MTT reaction mixture was measured at 577 nm, together with the reference absorbance at 630 nm. ○, cells cultured in the absence of ND2001 and assayed in the absence of ND2001; ●, cells cultured in the presence of ND2001 (100 μg/ml) and assayed in the presence of ND2001 (100 μg/ml).

number of the control cells (no pretreatment) (Fig. 1A). Nor did ND2001 affect adhesion of cells to the reconstituted basement membrane Matrigel (Fig. 1B).

3LL cells became attached more slowly to the well bottom than did the B16 variant cells, and the adhesion increased linearly for 2 h. ND2001 did not affect the adhesion of 3LL (Fig. 1C). The number of 3LL cells attached to the Matrigel increased linearly for 30 min and reached a maximal number within 1 h, at a lower count than that recorded for the B16 variant. Nor was adhesion of 3LL to Matrigel affected by ND2001 (Fig. 1D). Observation under a light microscope revealed no effect of ND2001 on the morphology of attached cells of the B16 variant or the 3LL cells.

Effects of ND2001 on phagokinetic displacement of cells Phagokinetic displacement of the B16 variant cells pretreated with ND2001 for 48 h was not affected by ND2001 in the measurement system, in which two concentrations of FCS were used for the medium (Table I). The movement of cells treated with ND2001 was almost the same as that of non-treated control cells. On the other hand, the movement of the B16 variant cells was strongly inhibited by a peptide, RGDS or GRGDS, used as positive control substances, by direct co-incubation without pretreatment. RGDS (500 μg/ml) inhibited the cell movement during 7-h incubation (data not shown).

Inhibition of type IV collagenase B16-BL6 cells treated with ND2001 showed almost the same activity of type IV collagenase (92 kDa gelatinase, MMP-9) (Figs. 2A and 2B, lane 3) as that of non-treated cells (Figs. 2A and 2B, lane 2). ND2001 did not inhibit the enzymatic activity,

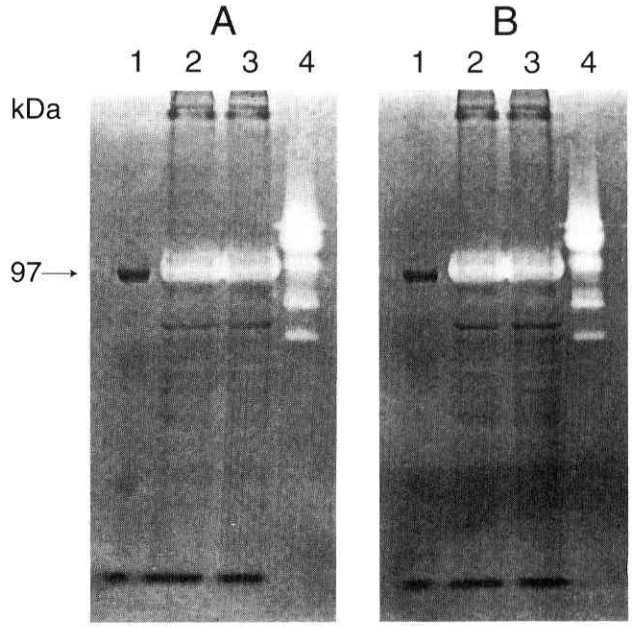

Fig. 2. Effect of ND2001 on type IV collagenase. B16-BL6 cells were cultured sequentially on three culture media in the presence of 100 μg/ml of ND2001, then harvested by centrifugation and the supernatant was used for a type IV collagenase assay. (A) A zymogram after incubation with 100 μg/ml of ND2001. (B) A zymogram after incubation when free of ND2001. 1, Molecular weight markers (97 kDa); 2, non-treated cells; 3, ND2001-treated cells; 4, type VIII collagenase (standard sample).

Table I. Effect of ND2001 on Phagokinetic Displacement of the B16 Variant

Experiment	Measurement medium: Addition (μg/ml)		Measurement medium: FCS (%)	Cell no. (total 100 cells): Moved	Cell no. (total 100 cells): Did not move
1[a]	None		0.1	39	61
	ND2001	100[b]	0.1	27	73
	ND2001	300[c]	0.1	35	65
	RGDS	500	0.1	7	93
	GRGDS	500	0.1	9	91
2[d]	None		10	70	30
	ND2001	100[b]	10	89	11
	ND2001	300[c]	10	83	17
	RGDS	500	10	51	49
	GRGDS	500	10	26	74

a) Movement in 72 h.
b) Cells cultured in the presence of 100 μg/ml of ND2001 were used.
c) Cells cultured in the presence of 300 μg/ml of ND2001 were used.
d) Movement in 20 h.

Table II. Inhibition by ND2001 of Haptotaxis of the B16 Variant and 3LL

Experiment	Cell line	Treatment	Haptotaxis: Cell no./mm^2	Haptotaxis: %
1	B16 variant	None	193.0	100
		ND2001	99.0	51.3
2	B16 variant	None	70.7	100
		ND2001	38.0	53.7
3	3LL	None	48.7	100
		ND2001	14.3	29.4
4	3LL	None	50.3	100
		ND2001	17.0	33.8

Tumor cells were cultured in the presence of ND2001 (100 μg/ml) for 1 day (Experiment 1) or 3 days (Experiments 2, 3 and 4), harvested, washed and used for the haptotaxis assay. One hundred μl of cell suspension (3×10^4 cells for Experiment 1; 1×10^5 cells for Experiments 2, 3 and 4) was placed in the upper compartment of the Transwell chamber. Numbers of the cells which moved to the lower surface of the filter in 2.5 h (Experiments 2 and 3) or 3 h (Experiments 1 and 4) were counted by the procedure described in "Materials and Methods."

Table III. Inhibition by ND2001 of Haptotaxis of Human Tumor Cell Lines

Experiment	Cell line	Treatment	Haptotaxis	
			Cell no./mm^2	%
1	PC-13	None	39.0	100
		ND2001	12.0	30.8
2	PC-13	None	29.0	100
		ND2001	15.3	52.8
3	PC-14	None	22.0	100
		ND2001	7.3	33.2
4	PC-14	None	107.7	100
		ND2001	46.3	43.0
5	YS-K	None	11.7	100
		ND2001	4.7	40.2
6	YS-K	None	116.0	100
		ND2001	87.3	75.3
7	T-24	None	118.0	100
		ND2001	63.7	54.0
8	T-24	None	100.7	100
		ND2001	61.0	60.6

Tumor cells were cultured in the presence of ND2001 (100 μg/ml) for 1 day (Experiments 1, 3, 5, 6, 7 and 8), 2 days (Experiment 2) or 3 days (Experiment 4), harvested, washed and used for the haptotaxis assay. One hundred μl of cell suspension (1.5×10^5 cells for Experiments 1, 2, 3, 5, 6, 7 and 8; 1.35×10^5 cells for Experiment 4) was placed in the upper compartment of the Transwell chamber. The chemoattractant was laminin for Experiment 4 or laminin plus conditioned medium for Experiments 1, 2, 3, 5, 6, 7 and 8. Numbers of the cells which moved to the lower surface of the filter in 1.5 h (Experiments 3 and 8), 2 h (Experiments 5, 6 and 7), 3 h (Experiment 2), 4 h (Experiment 1) or 6 h (Experiment 4) were counted by the procedure described in "Materials and Methods."

since incubation of the gel after electrophoresis with ND2001 did not affect the gelatinolytic activity of type IV collagenase (Figs. 2A and 2B, lane 2).

Inhibition of haptotaxis with ND2001 In the Transwell chamber, however, ND2001 inhibited haptotaxis of the B16 variant as well as invasion[1] of the cells into the Matrigel set (Table II). ND2001 also inhibited haptotaxis of 3LL cells (Table II). To effect these inhibitions, pretreatment with ND2001 was needed. In addition to the cell lines taken from the animal tumors, ND2001 also inhibited haptotaxis of cells of human origin: PC-13 large-cell lung carcinoma, PC-14 poorly differentiated lung adenocarcinoma, YS-K ovarian cancer and T-24 urinary bladder carcinoma (Table III).

DISCUSSION

In the Boyden chamber method, adhesion to the Matrigel is the initial step of tumor cell invasion. The major components of the reconstituted basement membrane Matrigel are type IV collagen, laminin, fibronectin and heparan sulfate. Tumor cell adhesion to these components is inhibited by RGD-related substances,[12, 13] YIGSR-related substances,[13, 14] fragments of specific regions of fibronectin or laminin,[15] recombinant fibronectin fragments,[16] heparin,[16, 17] heparan sulfate[16] and sulfated chitin derivatives.[17] These substances interfere with the binding of the cell adhesion molecules found on tumor cell surfaces to the extracellular matrix components. Tunicamycin, an inhibitor of the synthesis of carbohydrate chains on cell surfaces, affects cell adhesion on fibronectin and laminin.[18] The above-mentioned agents, which interfere with or affect cell adhesion, inhibit tumor cell invasion and/or experimental metastases. Although ND2001 inhibited the invasion and metastases of the B16 variant and 3LL cells,[1] these cells treated with ND2001 adhered almost normally to the Matrigel.

Tumor cells invade the Matrigel by degrading a part or all of the gel components after adhesion occurs. Aminopeptidase inhibitors, such as bestatin, amastatin A and alfamenin B, inhibit tumor cell invasion into Matrigel,[3] while tissue inhibitors of metalloproteinases (TIMPs),[19] peptide 74[20] and razoxan,[21] inhibit type IV collagenase, which plays a major role in the degradation of the basement membrane,[22] and ertramustine[23] blocks type IV collagenase secretion. In contrast, ND2001 did not inhibit the production or activity of type IV collagenase. Heparin,[17, 24] chemically modified heparins,[25] sulfated chitin derivatives[17] and suramin[26] inhibit heparanase (endo-type β-glucuronidase), which hydrolyzes heparan sulfate at specific intrachain sites,[24] while ND2001 strongly inhibits exo-type β-glucuronidase.[27] It has been reported that many of the above inhibitors inhibit metastases of experimental tumors, and we are currently investigating whether ND2001 does or does not inhibit heparanase.

Since some tumor cells treated with ND2001 exhibited reduced cell haptotaxis, measured by eliminating only the Matrigel from the Boyden chamber system, we suppose that a stage in the process of tumor cell migration may be inhibited by ND2001. We know that there is apparent random movement (phagokinetic displacement) and migration toward an chemoattractant (haptotaxis) of tumor cells.[3, 10] The B16 variant or 3LL cells treated with ND2001 showed normal phagokinetic displacement.

What, then, is the particular property of tumor cells which is affected by ND2001, leading to the inhibition of haptotaxis? One possibility is that ND2001 inhibits the tumor cells' deformability. We know that the ability of tumor cells to change shape is essential for their migration during extravasation into the narrow spaces of partially degraded basal membrane or the extracellular matrix, so that if a tumor cell is unable to change its shape, it would be unable to pass through the pores

(diameter, 8 μm) of the filter used in the haptotaxis assay system. We also know that actins,[28,29] and vinculin[30] are related to a cell's ability to change shape, and that a reduction in the amount of A^X actin, a variant form of actin, is correlated with increased metastatic activity of tumor cells,[28,29] yet the B16 variant cells cultured in the presence of ND2001 did not exhibit any change in A^X actin content, or in the mobility of the actins on polyacrylamide gel electrophoresis, or in cell reactivity with anti-actin antibody when compared with the non treatment control (data not shown). This leaves us with the possibility that ND2001 inhibits the function of the putative receptors for the chemoattractant laminin, for, though ND2001 did not inhibit apparent random movement, it did inhibit migration in one direction. Since ND2001 is a specific and potent inhibitor of β-glucuronidase, ND2001 might have inhibited the receptor function for the chemoattractant by altering the presumable carbohydrate chains containing β-glucuronide essential for receptor behavior.

ND2001 inhibited the haptotaxis of animal tumor cell lines whose metastases[1] were inhibited by ND2001. Since the inhibition of haptotaxis by ND2001 has been observed not only in mouse tumor cells (melanoma and lung carcinoma) but also in four human tumor lines (lung, ovary and urinary bladder carcinomas), we need to elucidate the mechanism which inhibits haptotaxis and metastases and that is operative both in animal and human tumors. It may be possible to create new antimetastatic agents and tools to prevent tumor metastases and progression in the light of the results obtained.

(Received July 5, 1995/Accepted August 29, 1995)

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