Changes in CA125 release and surface expression caused by drugs in uterine cervix adenocarcinoma cells

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The effect of drugs on the release of CA125 antigen and the binding of anti-CA125 monoclonal antibody (MoAb) to malignant cells was evaluated in vitro. TMCC-1, uterine cervical adenocarcinoma cells, were exposed to dexamethasone (DEX), sodium n-butyrate (NaB), dibutyryl cyclic AMP (dbcAMP), retinoic acid (RA), calcitriol (VD3), and interferon-γ (IFN-γ). NaB, RA and VD3 increased CA125 release per cell and ^{125}I-labeled anti-CA125 MoAb binding to the cells. DEX also increased the ^{125}I-labeled anti-CA125 MoAb binding to the cells, and CA125 antigen release per cell was also slightly increased. IFN-γ suppressed both CA125 release and ^{125}I-labeled MoAb binding. A combination of DEX, VD3 and RA and increased the binding of MoAb to TMCC-1 cells, but the amount of bound MoAb was not significantly different from that obtained by single drug treatment. DbcAMP had no significant effect on enhancing MoAb binding. Drugs can increase the binding of anti-CA125 MoAb to malignant cells and they may be applied to increase the tumor uptake of radiolabeled MoAbs in vivo.

Key words: CA125, TMCC-1, monoclonal antibody, ovarian cancer, VD3

INTRODUCTION

THOUGH A LOT OF TRIALS for the imaging and therapy of malignant tissues with radiolabeled monoclonal antibodies (MoAbs) have been reported, the application in vivo is still in the struggling stage.[1,2] To increase the tumor uptake of radiolabeled MoAbs, the enhancement of cancer-associated antigens on the cell surface may be effective, and for that purpose a pharmacological approach has been investigated.[3] In our previous report,[4] the effect of dexamethasone (DEX), interferon-γ (IFN-γ) and sodium butyrate (NaB) on CA125 expression in 8 cell lines was examined and it was observed that not all cells be-

haved the same way. CA125 expression and ^{125}I-labeled anti-CA125 MoAb binding to the cells were increased by NaB, which is known as a differentiator, in 3 cell lines and decreased by DEX in the same 3 cell lines. IFN-γ increased them in one cell line but decreased them in two. These discrepancies among cells should be noted in the application of drug administration because they might modify the antigen expression in immunotherapy. To further assess the effect of drugs on CA125 antigen expression and MoAb binding, we used TMCC-1, uterine cervix adenocarcinoma cells which highly express CA125 antigen. The effect of calcitiol (VD3), an activated form of vitamin D_3, and that of retinoic acid (RA) are of particular interest, as both drugs are known as differentiators.

MATERIALS AND METHODS

Cells

TMCC-1 cells[5] derived from uterine cervix adeno-

carcinoma highly express CA125 antigen on the cell surface and release CA125 antigen into the culture medium supernatant.

Drugs
Dexamethasone (DEX; 9α-fluoro-16α-methylprednisolone, Sigma, St. Louis, MO), sodium *n*-butyrate (NaB; $C_4H_7NaO_2$; Kanto, Tokyo, Japan), dibutyryl cyclic adenosine $3',5'$monophosphate (dbcAMP; Sigma), and retinoic acid (RA; all trans, Sigma) were commercially obtained. Calcitriol (VD3; 1.25-dihydroxyvitamin D_3, Rosch, Basel, Switzerland), an active form of vitamin D_3, was provided by Rosch Japan. Recombinant human interferon-γ (IFN-γ; produced by Genentech, South San Francisco, CA) was supplied by Toray.

Cell culture
All the cell cultures were performed in 25 cm^2 plastic flasks with 10 m*l* of culture medium (RPMI1640, Nissui, Tokyo) containing 10% fetal calf serum at 37°C in 5% CO_2. TMCC-1 cells (2×10^6) were seeded to each flask, and preincubated for 24 hr. The culture medium was changed every 24 hr to measure CA125 concentrations. During the first 24 hr after preincubation, cells were cultured in medium without drugs. Then cells were cultured the next 5 days with DEX (10^{-9}, 10^{-7}, 10^{-6} M), NaB (10^{-7}, 10^{-5}, 10^{-4} M), dbcAMP (10^{-8}, 10^{-6}, 10^{-5} M), RA (10^{-8}, 10^{-6}, 10^{-5} M), VD3 (10^{-10}, 10^{-8}, 10^{-7} M), or IFN-γ (10, 10^2, 10^3 U/m*l*). Each culture was done in duplicate. The first day after drug addition was designated as "day 1" and the final day as "day 5". DEX, VD3 and RA were first dissolved in ethanol and diluted with culture medium to below 0.1% of the concentration of ethanol. The medium containing each drug was freshly prepared on the first day of drug addition and stored at 4°C. The harvested medium was centrifuged at 500 g for 10 minutes and the supernatant was stored at $-40°C$. After exposure to each drug, the cells were harvested with 10 m*l* of phosphate buffer saline (Nissui Pharmaceutical Co., Ltd., Tokyo) with 0.02% ethylendiamine tetraacetic acid (Nakarai Co., Ltd.), and the number of cells in each culture was counted with a a hemacytometer. Cell viability after drug treatment was assessed by Trypan blue (Merck, Darmstadt, Germany) staining. The effect of each drug combination was studied by mixing DEX, VD3 and RA. The least effective doses of each drug enhancing ^{125}I-labeled MoAb binding to TMCC-1 cells were combined.

Measurement of CA125
To determine the CA125 value for each culture supernatant, we used CA130 assay kits (Daiich, Tokyo).

CA130 kits consist of beads coated with 145-9 MoAb and ^{125}I-labeled 130-22 MoAb, both of which recognize epitopes on CA125 antigen, but are different from OC125.[6,7] Duplicate measurement was done with one step assay for each duplicate culture. Values obtained with the CA130 kit correlated well with CA125 values obtained with commercially available kits.[8] Samples (100 μl) and ^{125}I-labeled 130-22 MoAb (5×10^4 cpm/100 μl) were incubated with beads coated with 145-9 MoAb for 4 hr at room temperature. Each sample was measured in duplicate. The beads were washed three times with 3 m*l* of distilled water and the radioactivity of each bead was measured with a scintillation counter. The CA125 value was obtained from the standard curve plotted by standard sample value.

Cell binding assay
Cells treated with each concentration of drug were suspended in various numbers of cells ranging from 1×10^4 to 2×10^6 cells per 100 μl phosphate buffered saline. Then each sample was incubated for 2 hr with ^{125}I-labeled 145-9 MoAb (5×10^4 cpm/100 μl) at 4°C in a 0.4 m*l* microtube. After incubation, the tubes were centrifuged at 10,000 g for 5 min, and the radioactivity of each pellet was measured in a well-type γ-counter. The binding percentage of the radioactivity added was calculated. For Scatchard plot analysis, 2×10^5 cells were incubated with unlabeled 145-9 MoAb ranging from 12.5 ng to 10 μg and ^{125}I-labeled 145-9 MoAb for 2 hr at 4°C. The numbers of MoAb binding sites and affinity constants were determined.[9]

Statistics
Data were expressed as the mean$\pm$SD. The effect of drugs on the CA125 value of the culture supernatant, number of cells at harvest and binding of ^{125}I-labeled anti CA125 MoAb to cells were expressed as a percentage of the control. The statistical significance of the difference between drug treated and non treated cells was determined by the two sample t-test or Welch's test based on the results of tests for equal variance.

RESULTS

TMCC-1 cells were grown as an adherent line. In the repeated experiment, 2×10^6 TMCC-1 cells suspended in 10 m*l* of RPMI1640 culture medium without any drugs in a 25 cm^2 flask increased from $1.3 \pm 0.2 \times 10^7$ to $1.6 \pm 0.2 \times 10^7$ cells per flask after 7 days of culture. On day 7, cells were confluent in the flask. CA125 release of control cells on the last day varied from $8.2 \pm 0.4 \times 10^2$ to $1.3 \pm 0.1 \times 10^3$ U/m*l* and its viability was found to be $85.3 \pm 7.0\%$ by

Table 1 Effect of each drug on cell number, CA125 release and ^{125}I-labeled 145–9 MoAb binding into culture medium

Drug	Concentration	Cell number[a] (%)[b]	CA125 (%)[b]	CA125 release per cell (×10^{-4}U/cell)	% Cell Binding[b,c] 5×10^4 (Cells)	1×10^5 (Cells)	2×10^5 (Cells)
Control		100.0	100.0	7.6±0.2	100.0	100.0	100.0
DEX	10^{-6}	68.9±18.5*[d]	55.6±7.0*	8.8±0.2*	169.1±20.7*	169.2±15.7*	128.7±8.4*
	10^{-7} (M)	62.3±19.8*	69.8±8.3*	8.5±0.4*	179.0±16.8*	160.9±16.1*	131.8±9.5*
	10^{-9}	106.3±21.3	111.8±12.2	9.3±1.0	100.0±15.4	118.8±11.2*	105.4±8.6
NaB	10^{-4}	65.9±22.6*	131.4±7.4*	13.7±0.1*	147.1±4.6*	132.3±5.8*	111.1±3.4*
	10^{-5} (M)	106.2±29.1	110.2±8.1	7.3±0.4	122.5±3.9*	123.0±5.4*	117.2±3.8*
	10^{-7}	97.7±23.3	108.9±8.7	7.7±0.4	103.4±3.6	107.7±4.7	103.0±3.9
VD3	10^{-7}	57.3±24.5*	110.5±12.6	11.8±0.1*	161.8±15.6*	147.9±13.2*	130.4±12.1*
	10^{-8} (M)	93.4±28.7	97.4±5.8	7.1±0.4	129.3±11.0*	124.1±11.1*	120.0±12.2*
	10^{-10}	72.2±21.8	92.0±9.6	7.8±0.4	128.6±11.0*	103.5±9.3	111.6±10.2
RA	10^{-5}	64.2±18.5*	101.7±5.7	15.6±0.6*	129.0±15.2*	134.5±11.3*	123.4±11.3*
	10^{-6} (M)	83.6±24.5	98.9±12.0	7.9±1.0	139.4±11.4*	134.7±11.4*	121.1±11.7*
	10^{-8}	82.1±14.8	86.5±7.8	8.6±0.7	123.6±12.4*	110.2±9.8	108.4±13.6
IFN-γ	10 (U/ml)	41.1±14.4*	28.5±8.3*	4.8±0.6*	26.0±6.4*	26.8±1.2*	30.1±0.8*
	10^2	23.3±5.7*	17.4±1.4*	5.2±0.3*	N.D.[e]	N.D.	N.D.
bdcAMP	10^{-5}	100.0±6.0	111.2±6.0*	8.4±0.2*	103.3±10.4	95.6±6.2	96.4±5.3
	10^{-6} (M)	87.5±16.6	119.3±13.5*	10.0±0.9*	115.2±15.0	100.0±11.7	96.7±5.4
	10^{-8}	100.0±17.5	120.0±7.4*	9.1±0.3*	118.3±16.0	111.9±7.2	90.7±7.0

[a] Cell number was counted after exposure to each drug for 5 days.
[b] Results were expressed as percentage of control.
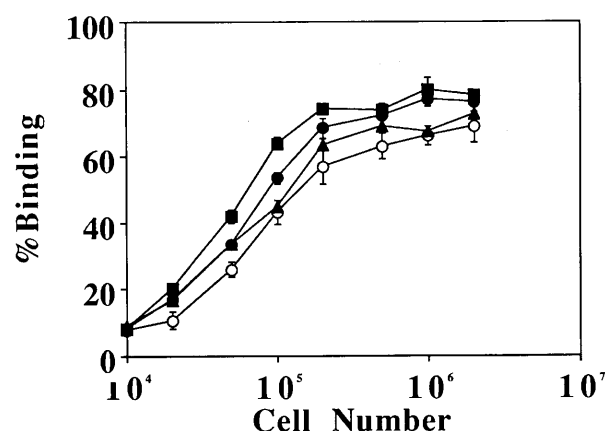[c] each number of cells were suspended in 100 μl volume and incubated with 145–9 MoAb for 2 hr.
[d]*; Significantly different from control by 2-sample t-test (n=4, p<0.05).   [e] N.D.; Not done
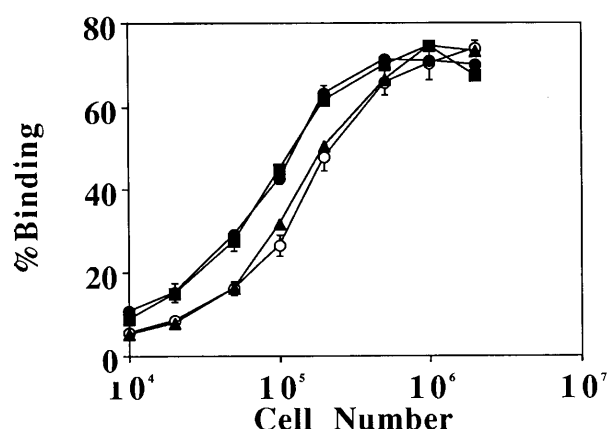
Fig. 1 Increase of ^{125}I-labeled 145–9 MoAb binding to TMCC-1 cells after treatment with DEX. TMCC-1 cells (2×10^6) were seeded and incubated without any drugs for 2 days and then exposed to each DEX concentration for 5 days. Culture medium was replaced every 24 hr. Cells were incubated with ^{125}I-labeled 145–9 MoAb for 2 hr and radioactivity bound to cells was measured. DEX concentrations are control (○), 10^{-9} M (▲), 10^{-7} M (●) and 10^{-6} M (■); SD (±) is shown by bars.

Fig. 2 Augumentation of ^{125}I-labeled 145–9 MoAb binding to TMCC-1 cells after treatment with VD3. The cells were treated in the same way as shown in Fig. 1. VD3 concentrations are control (○), 10^{-8} M (▲), 10^{-6} M (●) and 10^{-5} M (■); SD (±) is shown by bars.

Trypan blue staining on the final day (day 5).

The effect of 6 drugs on the number of cells, CA125 release on the final day and binding of 145–9 MoAb are summarized in Table 1. Among the 6 drugs examined, the effect of DEX was of interest. CA125

release from TMCC-1 cells was suppressed by DEX at a concentration of 10^{-7} M, but CA125 release per cell was slightly increased since the cell growth was also suppressed. The binding of ^{125}I-labeled 145–9 MoAb was significantly increased (Fig. 1). Cell viability was 89.3±7.1% following 10^{-7} M DEX treatment with no significant difference from control cells. NaB (10^{-4} M) increased both CA125 release

Table 2 Effect of combination treatment on cell number, CA125 release and ^{125}I-labeled MoAb binding into culture medium

| Drug Combination (M)	Cell number[a] (%)[b]	CA125 (%)[b]	CA125 release per cell ($\times 10^{-4}$ U/ml)	% Cell Binding[b,c]		
				5×10^4 (Cells)	1×10^5 (Cells)	2×10^5 (Cells)
Control	100	100.0	6.1 ± 0.3	100.0	100.0	100.0
DEX (10^{-7})+VD3 (10^{-8})	$76 \pm 15^{*d}$	$87.4 \pm 6.4^*$	$9.5 \pm 0.5^*$	$150.6 \pm 13.3^*$	$126.9 \pm 13.5^*$	107.6 ± 6.0
DEX (10^{-7})+RA (10^{-6})	107 ± 16	101.5 ± 7.2	$7.8 \pm 0.4^*$	$133.2 \pm 11.5^*$	$123.8 \pm 10.3^*$	$114.8 \pm 5.6^*$
DEX (10^{-7})+VD3 (10^{-8})+ RA (10^{-6})	108 ± 21	$119.6 \pm 6.0^*$	$9.1 \pm 0.1^*$	$157.5 \pm 15.3^*$	$119.0 \pm 10.3^*$	109.8 ± 5.6

[a] Cell number was counted after exposure to each combination of drugs for 5 days.
[b] Results were expressed as percentage of control.
[c] Each number of cells was suspended in 100 μl volume and incubated with 145-9 MoAb for two hr.
[d]*; Significantly different from control by 2-sample t-test (n=4, p<0.05).
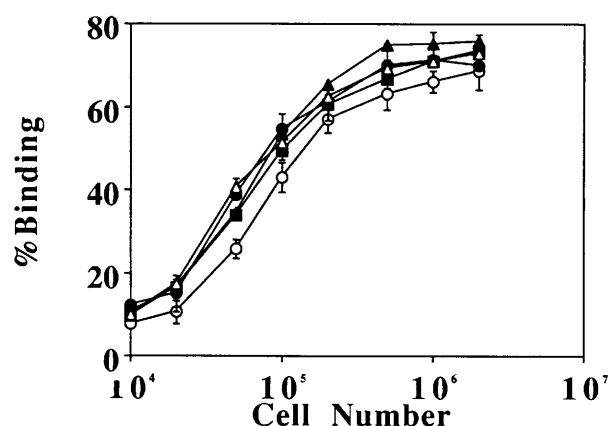
Fig. 3 Effect of drug combinations on binding of ^{125}I-labeled 145-9 MoAb to TMCC-1 cells. Cells exposed to DEX (10^{-7} M) alone (■), DEX (10^{-7} M)+VD3 (10^{-8} M) (●), DEX (10^{-7} M)+RA (10^{-6} M) (▲), DEX (10^{-7} M) +VD3 (10^{-8} M)+RA (10^{-6} M) (△) and control cells cultured without any drugs (○); SD (±) is shown by bars.

and 145-9 MoAb binding to TMCC-1 cells. VD3 did not increase the CA125 concentration of culture supernatant. However, because cell growth was suppressed by 10^{-7} M of VD3, CA125 release per cell seems to have increased. At the same time, VD3 (10^{-8} M) significantly increased 145-9 MoAb binding to TMCC-1 cells (Fig. 2). The effect of RA on TMCC-1 cells was similar to that of VD3. RA at a concentration of 10^{-5} M increased the CA125 release per cell, and a lower concentration (10^{-6} M) of RA increased the binding of ^{125}I-labeled 145-9 MoAb to the cells. IFN-γ was strongly cytostatic or toxic to TMCC-1 cells. IFN-γ (10 U/ml) decreased both CA125 release and MoAb binding to TMCC-1 cells. The cell viability after treatment with 10 U/ml of IFN-γ was $77.9 \pm 13.8\%$ and it was below 50% at higher concentrations of IFN-γ. DbcAMP slightly increased the CA125 concentration in the culture supernatant, but did not significantly increase 145-9 MoAb binding.

The number of binding sites of non treated TMCC-1 cells was 4.2×10^5/cell and the affinity constant was 1.4×10^{-9} M^{-1}. The binding site was increased to 5.9×10^5/cell by 10^{-6} M of DEX, and to 6.4×10^5 by 10^{-7} M of VD3. Affinity constants were not significantly changed by these drugs. The increase in the number of binding sites is compatible with increased binding of 145-9 MoAb to TMCC-1 cells by these drugs.

The effects of combination treatment are summarized in Table 2. DEX (10^{-7} M) was included in all groups and VC3 (10^{-8} M) or RA (10^{-6} M) was added or both. Compared to non-treated cells, CA125 release was suppressed by the combination of DEX and VD3, and not significantly affected by the combination of DEX and RA, but increased by the combination of DEX, VD3 and RA. Both CA125 release per cell and the binding of 145-9 MoAb was increased by all of these three combinations (Fig. 3), but these effects do not seem to be significantly different from each other or from treatment with DEX, VD3 or RA alone.

DISCUSSION

Because early detection of ovarian cancer is not easy, and ovarian cancers are highly metastatic, the application of radioimmunodetection and therapy by means of CA125 antigen, which is highly expressed in ovarian cancer, is expected to play an important role. Various authors have already reported the administration of radiolabeled anti-tumor MoAbs in human studies.[10-12] Although the concentration of MoAbs in xenografts of nude mice was as high as 10% of the injected dose per gram of tumor,[13-15] it was below 0.01% in clinical human studies[10] and it does not seem easy to reach sufficient radiation doses for treatment. Generally, tumor tissue has been proven to have some heterogeneity of antigen expression.[16,17] That is, some cells of one tumor lesion highly express the antigen, but other do not express

it at all or express it at low levels. Though MoAbs themselves are highly specific to their antigens, the heterogeneity of antigen expression within tumor tissues may offset the efficacy of MoAb accumulation. Increasing the antigen density in tumors will augment the tumor accumulation of radiolabeled MoAbs and result in successful tumor detection and targeting therapy by radiolabeled MoAbs. Several drugs such as DEX,[18] NaB,[19,20] dbcAMP,[21] RA,[20] and IFN-γ[22-25] have been reported to regulate the expression of tumor associated antigen. To improve the targetting to the CA125 antigen, we investigated the effects of these drugs on the CA125 antigen expression and anti-CA125 MoAb binding to the cells.

The binding of ^{125}I-labeled MoAb to TMCC-1 cells increased after treatment with DEX, NaB, VD3 and RA. Of interest was the effect of DEX. The binding of ^{125}I-labeled MoAb increased and CA125 release per cell was slightly increased. In other cell lines studied in the previous study,[4] CA125 release and binding in the MoAb to cells were suppressed or not affected by DEX and changes in the surface expression of CA125 correlated well with changes in CA125 release. It has been reported that CA125 expression of OVCA433 cells, a nonmucinous epithelial ovarian cancer, was suppressed by DEX.[18] Therefore the effect of DEX on CA125 antigen expression and on the binding of radiolabeled MoAbs to cells is not always the same and it depends on the cell line. As Masuho et al. have suggested, CA125 antigen release may be associated with several different mechanisms,[26] and the manner of regulation of CA125 expression by DEX may differ among cell lines. The affinity constant of the anti-CA125 MoAb in relation to TMCC-1 cells after DEX treatment was not affected, but the number of binding sites increased approximately 40%. This is compatible with the change in the binding of ^{125}I-labeled anti-CA125 antibody.

Another noticeable finding of this study was the effect of VD3. VD3, is investiaged as one of the agents for differentiation therapy.[27] As far as we know, this is the first report on the effect of VD3 in enhancing CA125 expression and MoAb binding to cells. The mechanism of CA125 modulation by VD3 is unknown, and awaits further investigation.

Beside its effect of inducing cell differentiation, RA has other regulatory effects on cell proliferation, antigen expression, drug sensitivity and oncogene expression.[28,29] As for CA125 expression, Langdon et al. have reported a reduction in the percentage of cells staining with OC125 after treating PE04, an ovarian carcinoma cell line, with RA.[20] In this study, the binding of ^{125}I-labeled 145-9 MoAb was increased by an average of 30% by treatment with as low a concentration of RA as 10^{-6} M. Therefore,

the effect of RA on CA125 expression also seems to differ among cell lines.

NaB, a 4 carbon fatty acid, is a classic differentiator which induces differentiation of malignant cells, and changes cell growth rate, enzyme activity, and glycoprotein synthesis.[30] Some of the tumor associated antigen expressions of mammalian cells are also known to be modulated by NaB.[19,20] In this study, CA125 expression and binding of ^{125}I-labeled MoAb to TMCC-1 cells was increased by 10^{-4} M of NaB. In our previous study, 10^{-4} or 10^{-5} M of NaB had the same effect in 3 of the 8 cell lines examined. It is possible that this agent enhances MoAb binding to malignant cells presenting the CA125 antigen. Of interest was that VD3, RA and NaB, all of which have differentiating effects, affected CA125 antigen expression and MoAb binding to the cells. This phenomenon may indicate a relationship between CA125 antigen and the cell differentiation process.

DbcAMP is also reported to induce cell differentiation[31,32] and CA125 expression in several ovarian cancer cell lines also known to be increased by dbcAMP.[21] DbcAMP mildly increased CA125 release from TMCC-1 cells, but did not significantly increase 145-9 MoAb binding.

IFNs are known to regulate major histocompatibility antigens,[33] carcinoembryonic antigen[34] and TAG72 antigen.[35] Augmented localization of radiolabeled MoAb in tumors due to IFN-γ was also reported.[23,25] Marth et al. have reported that CA125 expression was increased by IFN-γ in 2 of 3 ovarian cancer cell lines.[36] In this study, IFN-γ decreased antigen expression and antibody binding. But, because IFN-γ was highly toxic to TMCC-1 cells, suppression of CA125 expression and MoAb binding by IFN-γ may be associated with cytotoxic processes.

The effects of combined treatment with DEX, VD3 and RA were not significantly different from single drug administration. But a combination of these drugs may be effective in increasing the total amount of antigens to be targetted in clinical radioimmunoimaging or radioimmunotherapy because malignant tissues have heterogeneity in their antigen expression and all cells do not always react to a particular drug in the same way.

In conclusion, we have demonstrated that drugs changed the CA125 antigen expression of TMCC-1 cells and ^{125}I-labeled MoAb binding to them *in vitro*. These drugs may be useful for application *in vivo* to increase the deposit of radiolabeled MoAbs in the tumor.

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