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An Antibody-Tumor Model for the Targeting of CA125-producing Gynecologic Malignancies

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By immunizing a mouse with HOUA-1 cells established from an endometrial cancer patient, two murine monoclonal antibodies designated 196-14 and 196-28 were generated, which were reactive with ovarian cancer-associated antigen CA125, originally defined by OC125 antibody. Antigenic determinants of these antibodies, although overlapping each other, were different from that of OC125 and the combined use of ^{125}I-labeled 196-14 and OC125-coated beads markedly increased the sensitivity of measuring CA125 antigen. Both radioiodinated and ^{111}In-labeled 196-14 localized well in CA125-producing human ovarian cancer tissues OVA-5 xenografted in nude mice. The biodistribution of radioiodinated 196-14 was quite different from that of ^{111}In-labeled 196-14. Radioiodine was cleared faster from the OVA-5 tumor, making a clear contrast to the prolonged retention of ^{111}In in the tumor. Initial tumor uptake of radioiodinated 196-14 was the same as that of ^{111}In-labeled 196-14 but decreased thereafter, due to the dehalogenation of radioiodinated antibody in the tumor. This antibody-tumor model seems to be suitable for examining the usefulness of monoclonal antibody-conjugates in the diagnosis and therapy of CA125-producing endometrial or ovarian cancers.

Key words: CA125 — Radiolabeled monoclonal antibody — Endometrial cancer — Ovarian cancer

MoAbs[6] against various cancer-associated antigens have been produced and widely used in the evaluation of histology, serodiagnosis and the localization of tumors.[1-3] The sensitivity and specificity of serodiagnosis and tumor uptake of injected MoAb-conjugates are highly dependent on the binding affinity and specificity of the MoAbs employed.[3] An endometrial adenocarcinoma cell line, designated HOUA-1, was established from a 55-year-old endometrial cancer patient[4] and two MoAbs designated 196-14 and 196-28 have been generated by immunization of a BALB/c mouse with HOUA-1 cells. MoAbs 196-14 and 196-28 are reactive with ovarian cancer-associated antigen CA125, originally defined by OC125 Ab, produced by immunizing mice with ovarian cancer cells.[5,6] However, these MoAbs have been shown to bind to epitopes different from that of OC125 Ab with high affinities. In the present paper, we describe the application of radiolabeled 196-14 and 196-28 for measuring CA125 antigens and for carrying radionuclides to CA125-producing tumor tissues xenografted in nude mice.

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[6] Abbreviations used in this paper: MoAb, monoclonal antibody; Ab, antibody; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; PB, phosphate buffer; DTPA, diethylenetriaminepentaacetic acid; IRMA, immunoradiometric assay.

MATERIALS AND METHODS

HOUA-1 cells HOUA-1 cells were obtained from a 55-year-old Japanese woman who underwent radical hysterectomy and lymphnode resection on Feb. 6, 1982. A portion of the endometrial tumor, a mixed type of well, moderately and poorly differentiated adenocarcinoma, was rinsed, finely minced and digested with 600 Pronase Unit Dispase/ml (Gohdo-Shusei Co., Tokyo) for 30 min at 37°C. After centrifugation at 900*g* for 10 min, the sediment was resuspended in Ham's F-12 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 15% fetal calf serum and 0.03% L-glutamine. The suspension, placed in 6 cm plastic dishes, was incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.[4] The cells were dissociated using 0.25% trypsin solution and cultured after 1:2 dilution.

The cultured cells were observed with a phase-contrast microscope. They were fixed with 95% ethanol solution and stained with Papanicolaou, PAS, mucicarmine, alcian blue and peroxidase solution. After staining of the cells with 3% Giemsa solution, a G-band karyotype was analyzed. Histograms of chromosome number were prepared based on more than 50 metaphase plates.

Ovarian cancer tissue OVA-5 Human ovarian cancer tissue OVA-5, used as a tumor model, was originally

obtained from a 50-year-old woman at surgery.[7] Tumor tissue was cut into pieces of 3 to 5 mm^3 and transplanted subcutaneously into the dorsal area of nude mice. The histology of serially transplanted nude mice was serous cystadenocarcinoma and immunohistochemical study indicated CA125 expression on surface membranes.

Monoclonal antibodies A female BALB/c mouse was immunized by intraperitoneal injection of 1×10^7 HOUA-1 cells (passage 50). Three days after the third injection, extracted spleen cells (1×10^8) were fused with mouse myeloma NS-1 cells (1×10^7) using 50% polyethylene glycol #4000 (Merck Co., Rahway, NJ), then seeded into five 96-well microculture plates (Corning Glass Works, Corning, NY).[8] Hybridomas were grown in hypoxanthine-aminopterin-thymidine medium for 10 days. Culture supernatants of hybridomas were screened using an ELISA test,[9] and clones were selected which reacted with HOUA-1 cells but failed to react with human lymphocytes. Selected hybridomas, cloned by the limiting dilution method, were injected intraperitoneally into BALB/c mice primed with pristane (Tokyo Kasei Co., Tokyo). MoAbs were purified by applying the obtained ascites to a Protein A affinity column (Bio-Rad Laboratories, Richmond, CA).

Isotype-matched anti-human thyroglobulin MoAb, designated 59A, was used as the control Ab. Unlabeled OC125 Ab was obtained from ascitic fluid kindly supplied from R.C. Bast Jr. (Duke University, Durham, NC).

Radiolabeling of monoclonal antibodies MoAbs were radioiodinated using the chloramine-T method.[10] Purified MoAbs (40 μg) in 0.3 *M* PB, pH 7.5, and ^{125}I (11.1 MBq) for protein labeling (Amersham International, Buckinghamshire, UK) were mixed with 2.5 μg of chloramine-T (Nakarai Chemicals, Kyoto) dissolved in 0.3 *M* PB. After 5 min of reaction, radiolabeled MoAbs were separated from free radioiodine by Sephadex G-50 gel chromatography. The specific activity of the ^{125}I-labeled MoAb was about 185 MBq/mg. ^{125}I-labeled OC125 was obtained from the commercially available CA125 RIA kits (Centocor Co., Malvern, PA).

MoAbs were labeled with ^{111}In using DTPA as a bifunctional chelating agent.[11] In brief, MoAb solution (1 mg/ml) in 0.1 *M* $NaHCO_3$ was mixed with cyclic DTPA anhydride at a DTPA-to-MoAb ratio of 8 for 1 h at room temperature, and then unconjugated DTPA was separated by Sephadex G-50 gel chromatography. DTPA-conjugated MoAb in 0.2 *M* citrate buffer was mixed with ^{111}In chloride (Nihon Mediphysics, Takarazuka) and allowed to stand for 1 h at room temperature. The conjugation ratio of DTPA to MoAb, calculated as previously described,[11] was 0.88 and the labeling efficiency was more than 95% without the purification step.

Cell binding assay ^{125}I-labeled MoAbs were incubated with increasing concentrations of HOUA-1 or SHIN-3 cells (human ovarian serous cystadenocarcinoma cell line established by Y. Kiyotsuka, Nara Medical College, Kashihara) in 5.7×46 mm microcentrifuge tubes for 1 h at 4°C. After centrifugation at 10,000*g*, the supernatant was aspirated and the tubes were cut. The radioactivity bound to the cells was counted in an auto-well gamma counter.

^{125}I-labeled MoAbs and increasing amounts of unlabeled MoAbs were incubated with 1×10^6 HOUA-1 cells for 1 h at 4°C, and the radioactivities bound to the cells were counted. Data were used to produce Scatchard plots from which affinity constants were calculated.[12]

Immunoradiometric assay of CA125 ^{125}I-labeled MoAbs (60,000 cpm/100 μl), standard CA125 antigens of CA125 RIA kits (100 μl) and OC125-coated beads were incubated for 20 h. After washing of the beads, bound radioactivities were measured and standard curves were plotted. To determine whether various MoAbs recognized the same epitopes on CA125 antigen or not, a competitive inhibition assay was performed. CA125 antigens (100 μl) were incubated with OC125-coated beads for 4 h. After washing of the beads to remove the unbound antigen, ^{125}I-labeled MoAbs (100 μl) and increasing concentrations of unlabeled MoAbs (100 μl) were added and incubated for 20 h. Bound radioactivities were then measured.

***In vivo* biodistribution study and immunoscintigraphy** Human ovarian serous adenocarcinoma tissue, designated OVA-5, was serially transplanted into nude mice which secreted large amounts of CA125 antigen into the circulation.[7] The solution containing potassium iodide was administered to mice beginning one day before the injection of ^{125}I-labeled MoAbs antibodies till the end of the experiment to inhibit the uptake of released radioiodine into the thyroid. Nude mice, bearing 0.3 to 0.5 g of OVA-5 tumor, were injected with 37 kBq of radiolabeled Abs into the tail vein. The dose of Ab administered was adjusted to 10 μg per mouse by the addition of unlabeled corresponding Ab. At designated times after injection, groups of mice were killed by ether inhalation. Organs were removed and weighed, and radioactivities were counted. Biodistribution data were expressed both as percentages of injected dose per gram of tissue and tumor-to-normal tissue ratios. Radiolabeled control 59A Ab was also injected into tumor-bearing mice. The localization index was derived from the ratio of specific to nonspecific activity in the tumor divided by the same ratio in the blood.

For imaging of nude mice, 1.85 MBq of ^{131}I- or ^{111}In-labeled 196-14 Ab was administered intravenously at a total Ab dose of 50 μg. At 24, 48 and 96 h after injection, mice were anesthetized by intraperitoneal pentobarbital injection and imaged using a gamma-camera equipped with a pin-hole collimator.

RESULTS

Characteristics of HOUA-1 cells HOUA-1 cells were spindle-shaped, columnar and polygonal epithelial cells arranged in a pavement or jigsaw puzzle-like manner. They revealed a piling-up tendency without contact inhibition and showed anaplastic and pleomorphic features. The cytoplasms were stained with PAS but were negative to mucicarmine and alcian blue. HOUA-1 cells grew well and more than 150 serial passages were performed within three years. The doubling time was about 50 h. The chromosome number of HOUA-1 cells was widely distributed, and cells were aneuploid.

Monoclonal antibodies Among many hybridomas obtained by fusing mouse myeloma NS-1 cells and spleen cells of a mouse immunized with HOUA-1 cells, two hybridomas were selected which secreted antibodies designated 196-14 and 196-28. Both MoAbs were of the IgG_1 subclass and reacted with HOUA-1 cells. Furthermore, these two MoAbs were also reactive with lung adenocarcinoma and ovarian serous adenocarcinoma cells, which are known to express CA125 antigen (data not shown). From immunohistochemical studies, these two MoAbs were also reactive with human endometrial and ovarian cancer tissues, showing the same reactivity as OC125 Ab (data not shown).

Both ^{125}I-labeled 196-14 and 196-28 specifically bound to HOUA-1 and SHIN-3 cells with the affinity constants of 1.08×10^9 M^{-1} and 0.66×10^9 M^{-1}, respectively. Higher bindings of 196-14 and 196-28 were observed than that of ^{125}I-labeled OC125 obtained from a commercially available CA125 kit (Fig. 1). Furthermore, the bindings of ^{111}In-labeled 196-14 and OC125 antibodies to endometrial and ovarian cancer cells were similar to those of the ^{125}I-labeled antibodies. ^{125}I-labeled 196-14 and 196-28 showed excellent binding to CA125 antigen captured on OC125-coated beads (Fig. 2). The combined use of ^{125}I-labeled 196-14 and OC125-coated beads mark-

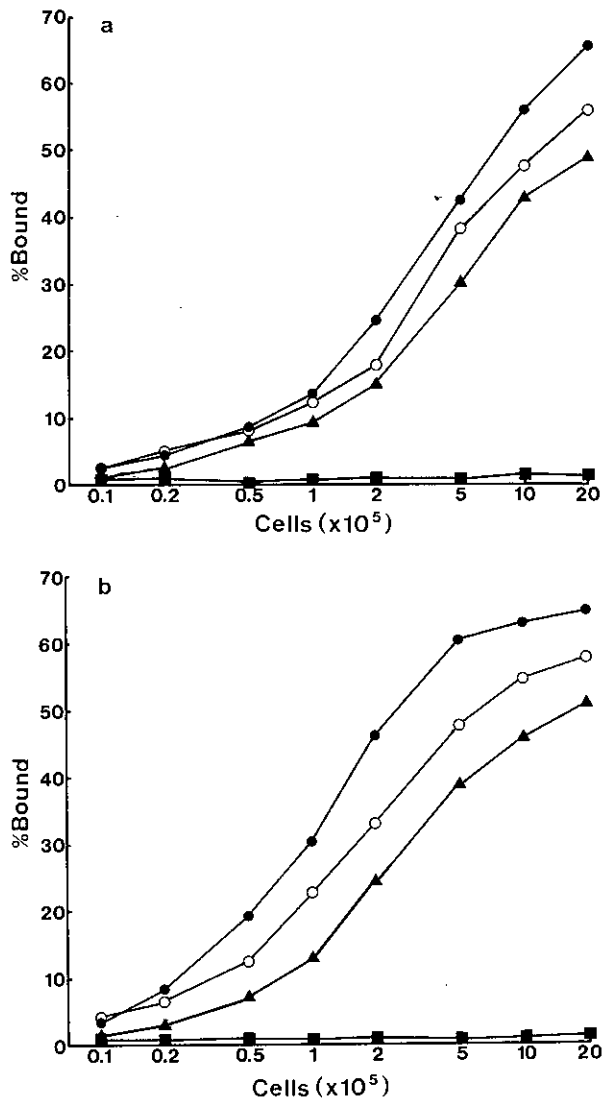

Fig. 1. Cell-binding curves of ^{125}I-labeled MoAbs. ^{125}I-labeled MoAb (●, 196-14; ○, 196-28; ▲, OC125; ■, control 59A) was incubated with increasing concentrations of HOUA-1 (a) or SHIN-3 (b) cells for 1 h at 4°C. After centrifugation, radioactivity bound to cells was counted. ^{125}I-labeled OC125 was obtained from commercially available kits.

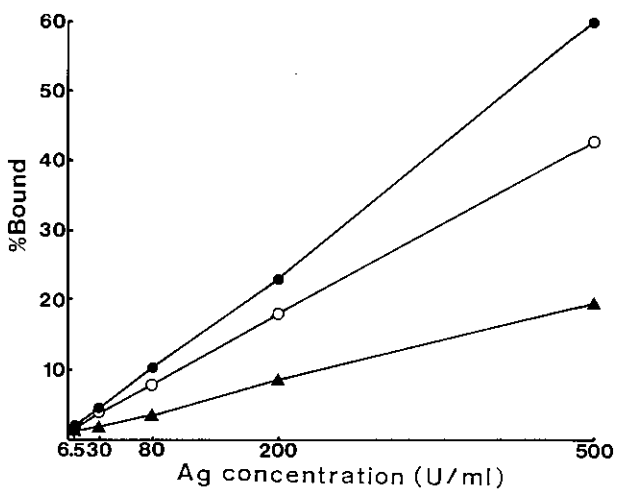

Fig. 2. Standard curves of IRMA of CA125. ^{125}I-labeled MoAb (●, 196-14; ○, 196-28; ▲, OC125), standard CA125 antigen and an OC-125-coated bead were incubated for 20 h. Standard CA125 antigen and OC-125-coated beads were obtained from commercially available kits.

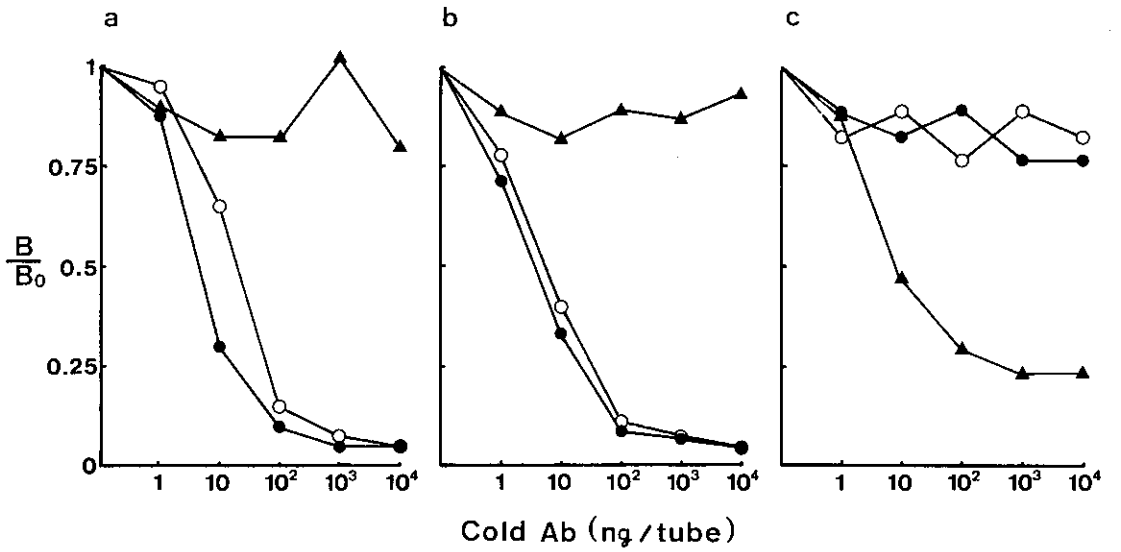

Fig. 3. Competitive inhibition assay. After CA125 antigen had been captured on the beads coated with OC125, increasing amounts of unlabeled MoAb (●, 196-14; ○, 196-28; ▲, OC125) were incubated with ^{125}I-labeled MoAb (a, ^{125}I-labeled 196-14; b, ^{125}I-labeled 196-28; c, ^{125}I-labeled OC125).

edly increased the sensitivity of the homologous assay, where OC125 was used as the ^{125}I-labeled probe and as an immunoadsorbent. These bindings were completely inhibited by both unlabeled 196-14 and 196-28, whereas unlabeled OC125 did not inhibit the binding at all (Fig. 3). The inverse was the case when OC125 antibody was used as the ^{125}I-labeled probe, indicating that the epitopes of 196-14 and 196-28 are different from that of OC125.

***In vivo* biodistribution study** The biodistribution of MoAb 196-14 in nude mice bearing OVA-5 xenografts was examined, since it has a higher binding affinity constant to CA125 antigen than that of 196-28. Serum CA125 levels of nude mice bearing the OVA-5 tumor ranged from 150 to 300 U/ml, depending upon the size of tumors.

Both radioiodinated and ^{111}In-labeled 196-14 were well localized in xenografted OVA-5 tumors. ^{125}I-labeled 196-14 was cleared from the circulation rapidly and the tumor-to-blood ratio reached 1.8 as early as 12 h after injection. However, radioiodine was also cleared from the tumor rapidly and the peak tumor-to-blood ratio was obtained 24 and 48 h after injection and then decreased (Table I). Peak tumor-to-liver, -to-lung and -to-muscle ratios were 13.7, 7.4 and 28.7, respectively. Tumor uptake of 196-14 was much higher than that of control Ab and localization indices at 24, 48 and 96 h after injection were 16.6, 14.8 and 6.4, respectively.

Peak tumor uptake of ^{111}In-labeled 196-14 Ab was the same as that of the radioiodinated antibody, but it retained its peak value until 48 h and then decreased (Table II). The tumor-to-blood ratio was 2.9 at 24 h but increased to 19.3 at 96 h after injection. Localization indices at 24, 48 and 96 h after injection were 5.6, 7.7 and 16.3, respectively. However, high deposition in the liver and kidney was seen with ^{111}In-labeled 196-14.

Scintigraphy of nude mice confirmed the biodistribution studies. ^{131}I- and ^{111}In-labeled 196-14 Abs clearly visualized xenografted OVA-5 tumor in nude mice from 24 h after injection (Fig. 4). Liver and kidney were prominent with ^{111}In-labeled Ab.

DISCUSSION

CA125 is an ovarian cancer-associated antigen recognized by OC125 and is clinically used, not only for serodiagnosis but also as a target for imaging and therapy of patients with ovarian cancer.[13-17] However, CA125 antigen is not specific for ovarian cancer and the elevation of serum CA125 levels has also been reported in patients with endometrial cancer and benign diseases such as endometriosis, pleuritis and ascites.[18,19] ^{125}I- or ^{111}In-labeled OC125 specifically binds to HOUA-1 cells derived from a patient with endometrial cancer, indicating the expression of CA125 antigen on the cell surfaces. Therefore, it is not surprising that two MoAbs generated by immunization with HOUA-1 cells are found to be reactive with CA125 antigen, although OC125 was generated by immunizing with ovarian cancer cells. The antigenic nature of CA125 is still under investigation. When untreated culture medium of HOUA-1 cells was

Table I. Biodistribution of ^{125}I-Labeled 196-14 and 59A in Nude Mice Bearing Human Ovarian Cancer OVA-5 Xenograft

	3 h	12 h	24 h	48 h	96 h
Blood					
196-14	30.70±2.67	11.55±1.93	2.27±0.30	1.09±0.05	0.70±0.14
59A	—	—	13.95±0.61	11.63±2.19	7.79±1.91
Liver					
196-14	10.27±1.57	6.13±1.15	1.66±0.74	0.43±0.05	0.21±0.04
59A	—	—	3.58±0.25	3.20±0.71	2.51±0.73
Kidney					
196-14	8.65±0.75	3.54±0.57	0.80±0.18	0.40±0.03	0.24±0.03
59A	—	—	3.94±0.20	3.40±0.66	2.43±0.72
Lung					
196-14	9.63±1.06	4.61±0.75	1.71±0.23	0.86±0.15	0.36±0.07
59A	—	—	5.84±0.40	4.41±0.76	3.37±0.74
Muscle					
196-14	0.78±0.15	0.71±0.01	0.47±0.08	0.23±0.08	0.09±0.03
59A	—	—	0.90±0.04	0.84±0.09	0.55±0.11
Tumor					
196-14	9.44±0.32	20.36±2.05	12.57±2.41	6.02±2.07	1.85±0.13
59A	—	—	4.68±0.50	4.38±0.99	3.41±1.21

Data are shown as percentages of injected dose per gram of tissue, mean±SD of 3 to 4 mice.

Table II. Biodistribution of ^{111}In-Labeled 196-14 and 59A in Nude Mice Bearing Human Ovarian Cancer OVA-5 Xenograft

	3 h	12 h	24 h	48 h	96 h
Blood					
196-14	34.35±1.17	15.75±1.96	6.79±1.31	3.12±0.51	0.49±0.13
59A	—	—	10.51±0.46	6.09±0.87	4.29±0.21
Liver					
196-14	17.13±2.70	21.51±4.46	19.98±3.10	19.78±2.30	19.16±1.47
59A	—	—	20.38±1.31	16.52±2.05	17.04±0.16
Kidney					
196-14	13.52±1.18	17.21±1.81	17.17±0.97	17.12±1.47	12.89±1.87
59A	—	—	16.23±0.71	17.74±0.69	14.30±0.08
Lung					
196-14	10.75±1.00	7.05±0.46	4.62±0.73	3.61±0.79	2.04±0.41
59A	—	—	5.83±0.57	4.28±0.57	3.44±0.18
Muscle					
196-14	0.91±0.13	0.99±0.08	0.99±0.06	0.90±0.09	0.64±0.14
59A	—	—	1.00±0.07	0.82±0.04	0.71±0.03
Tumor					
196-14	13.87±1.49	17.97±2.52	19.12±0.66	19.80±3.08	9.61±4.03
59A	—	—	5.35±0.25	4.97±0.31	5.17±0.56

Data are shown as percentages of injected dose per gram of tissue, mean±SD of 3 to 4 mice.

subjected to Sephacryl S-300 gel chromatography (Pharmacia LKB Biotechnology, Uppsala), CA125 activity was eluted in the void volume (more than 900 kilodaltons) (data not shown).

Both 196-14 and 196-28 Abs showed the same reaction patterns with OC125 on ELISA, immunohistochemical and cell binding studies, indicating the recognition of CA125 by these Abs. Furthermore, rediolabeled 196-14

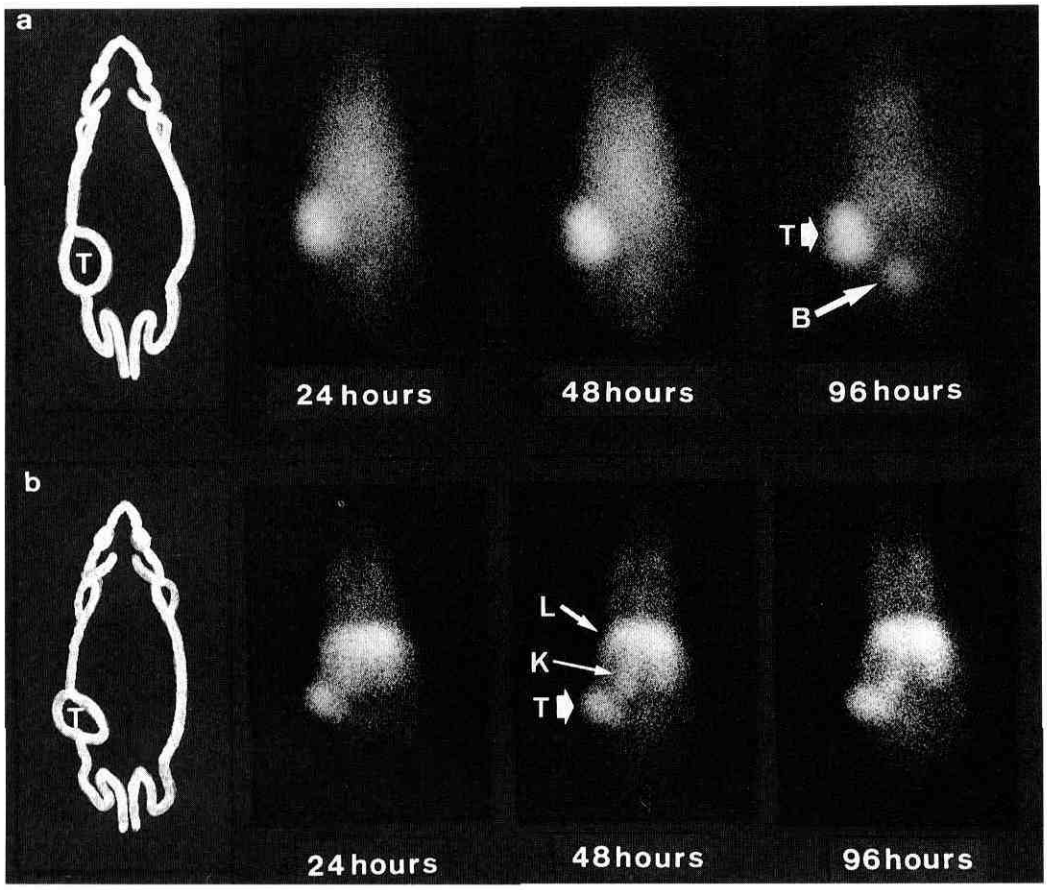

Fig. 4. Scintigrams of nude mice bearing OVA-5 xenografts. Images of mice were obtained at 24, 48 and 96 h after injection of ^{131}I-labeled (a) or ^{111}In-labeled 196-14 (b). T, tumor; B, bladder; L, liver; K, kidney.

and 196-28 bound specifically to the antigen captured on OC125-coated beads, indicating that their epitopes are located on the same antigen molecule. Competitive inhibition assays using 196-14, 196-28 and OC125 revealed that, although epitopes recognized by the former two MoAbs overlapped, 196-14 and 196-28 bound to determinants on CA125 antigen different from that of OC125. CA125 antigen molecule seems to have at least two separate epitopes.

It has been shown that the sensitivity of the serodiagnosis and tumor deposits of MoAb-conjugates administered intravenously are highly dependent on the binding affinity of the MoAbs employed.[3] Newly developed IRMA for the measurement of CA125 using ^{125}I-labeled 196-14 and OC125-coated beads showed a markedly higher % binding than the commercially available RIA kits where OC125 was employed both as an ^{125}I-labeled probe and an immunoadsorbent, because of the use of two MoAbs recognizing separate epitopes and/or the high binding affinity of 196-14. MoAb 196-14 was also proved to be an excellent antibody for tumor localization in CA125-producing human ovarian cancer tissues OVA-5 xenografted into nude mice. The size of the OVA-5 tumor correlated with the serum CA125 values of nude mice and the tumor was less necrotic than other CA125-producing tumors (data not shown). The OVA-5 tumor seems to be a good model for evaluating the usefulness of MoAb-conjugates in the diagnosis and therapy of CA125-producing tumors.

Among various radionuclides, ^{131}I and ^{111}In have been most widely used for the labeling of MoAbs for the diagnosis and therapy of cancers.[2, 15] However, different patterns of tumor accumulation were noted between radioiodinated and ^{111}In-labeled 196-14. Radioiodine was cleared rapidly from the tumor, whereas ^{111}In showed a prolonged retention in the tumor. Maximum tumor-to-normal tissue ratios of ^{125}I-labeled Ab were obtained at 24 to 48 h after injection, whereas those of ^{111}In-labeled Ab were obtained at 48 to 96 h after injection. The rapid loss of radioiodine from the tumor may be due to internaliza-

tion, active metabolism and dehalogenation of formed Ab-Ag complexes. Free radioiodine, thus generated, leaves the cells, whereas ^{111}In may be retained within the cells, as suggested in the imaging of cutaneous T-cell lymphoma using T101 Ab.[20]

As previously reported,[21] liver accumulation of ^{111}In-labeled Ab was remarkably higher than that of ^{125}I-labeled Ab. This is due in part to the intrinsic properties of ^{111}In-labeled Ab,[22] and in part to the effect of circulating CA125 antigen in which formed immune complexes are trapped in the reticuloendothelial tissue of the liver.[23] Metabolized ^{111}In may be trapped in the liver, whereas freed radioiodine may be released from the liver.[24] Circulating CA125 antigens also seem to be responsible for the rapid clearance of radiolabeled Ab from the circulation. In addition to the nature of antigens to be targeted, IgG or its fragments, radionuclides used for the labeling of MoAbs will also influence the biodistribution of MoAb-conjugates and the optimal time for imaging.

^{90}Y, which is a pure beta emitter with suitable nuclear properties for radioimmunotherapy, can be coupled to anti-tumor MoAbs through chelation with DTPA, as performed in ^{111}In-labeling.[25] Prolonged retention of ^{111}In-labeled 196-14 in the tumor indicates the potential of ^{90}Y-labeled 196-14 delivering a sufficient radiation dose to the tumor tissues. However, the high background radioactivity, especially deposits in liver and kidney, may restrict the administrable dose of radiopharmaceuticals and investigations are under way to decrease background radioactivity while preserving high tumor retention.[26, 27] ^{32}P has been administered intraperitoneally for the therapy of advanced ovarian cancer patients.[28] The results indicate that 196-14 will be useful for delivering radionuclides, chemotherapeutic agents or toxins selectively to cancer tissues.

In conclusion, HOUA-1 cell line has been established from a patient with endometrial cancer, and two MoAbs designated 196-14 and 196-28 have been generated which bind to CA125 antigen with high affinities. Both radioiodinated and ^{111}In-labeled 196-14 are localized well in human ovarian cancer tissue and seem to have a potential for *in vitro* and *in vivo* application in the diagnosis and therapy of CA125-producing endometrial or ovarian cancers.

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