Immunoscintigraphy of Human Tumors Transplanted in Nude Mice with Radiolabeled Anti-ras p21 Monoclonal Antibodies

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Anti-ras p21 monoclonal antibody (RASK-3) was used for immunoscintigraphy of human cancer cell lines in nude mice. Iodine-125-labeled RASK-3 was injected into nude mice with either human colon cancers (FCC-1 or BM-314) or lung cancer (KNS-62). Clear images were obtained in all three cancers 7 days after the injection of antibody. No localization of ^{125}I-labeled control monoclonal antibody was observed. The ratio of tissue/blood radioactivity and % ID/g in the tumor were significantly higher than other organs by Day 8. The specific localization index examined by ^{131}I-RASK-3 and ^{125}I-control monoclonal antibody was also higher in the tumor than in other tissues. In the in vitro study, binding of RASK-3 to tumor cells increased significantly by treatment of cells with either lysolecithin or periodate-lysine-paraformaldehyde, which confirmed the intracellular localization of ras p21. The mechanism by which anti-ras p21 antibodies accumulate in tumor sites could be the necrotic changes in tumor cells or changes in membrane permeability of non-necrotic cells. These results provide a strong rationale for the utilization of ras p21 as a target antigen in the imaging of a variety of human cancers.

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Transfection of DNA derived from human cancer cells into NIH3T3 cells has demonstrated activated ras genes with transforming activities (*1–3*). These activated ras genes were subsequently found to possess point mutations at particular sites in the DNA sequences (*4–7*). The presence of these activated ras genes was observed in various human cancers and the frequency of these mutated genes varied depending on their histologic type (*4–8*). Many cancers, however, were found to also express higher levels of ras gene transcripts or ras gene product p21 than their normal counterparts (*9–11*).

The apparent accumulation of ras gene product p21 in cancer cells led us to investigate whether they could be an appropriate target for tumor imaging. If it is the case, then tumor imaging by anti-ras p21 antibodies would be widely applicable since an elevation of p21 levels has been noted in a variety of human cancers (*12–15*). We report here our analysis in utilizing anti-ras p21 monoclonal antibody for detecting human cancers heteroplanted into nude mice.

MATERIALS AND METHODS

Antibodies

The preparation of anti-ras p21 monoclonal antibody RASK-3 reactive with all N-, Ha-, and Ki-ras has been previously described (*15*). RASK-3 was purified from ascites by the use of a protein A column MAPS II (Bio Rad Lab., Richmond, CA). The anti-human platelet antibody HPL-1 was used as a control monoclonal antibody (*16*).

The RASK-3 and the HPL-1 were labeled with iodine-131 (^{131}I) and iodine-125 (^{125}I) by the iodogen method (*17*). As for the reactivity of radiolabeled RASK-3, immunoblotting assays were performed with radiolabeled RASK-3, which showed the specific binding of RASK-3 to 21,000 dalton molecules. The specific radioactivities were ~259 MBq/mg for both RASK-3 and HPL-1.

Cell Lines

Two human colon cancer cell lines, BM-314 (kindly provided by Dr. Kohzoh Imai, Sapporo Medical College, Sapporo, Japan) and FCC-1 (*18*), and a human lung cancer cell line, KNS-62 (*19*), were maintained in culture in RPMI 1640 with 10% fetal bovine serum.

Immunoscintigraphy and Measurements of the Tissue/Blood Radioactivity Ratios

BALB/c nude mice were subcutaneously inoculated into the left thigh with 1×10^7 cells of one of the human tumor cell lines. Three weeks after inoculation, the mice were injected through the tail vein with 2.22 MBq of ^{125}I-labeled RASK-3 (experimental group) or ^{125}I-labeled HPL-1 (control group). The mice were given 0.1% potassium iodide in their

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drinking water for a week prior to the injection of antibodies in order to block thyroid uptake of inorganic iodide. Total-body scans of the anesthetized mice were performed with a gamma scintillation camera (Sigma 410, Ohio Nuclear, Solon, Ohio) at 3, 5, and 7 days after injection. The scintigraphies were obtained with 5,000 counts per mouse using the same color scale throughout the study. The mice were killed 8 days after the injection of antibodies, and various organs and tumors were removed and immediately weighed. The radioactivity was determined using a gamma scintillation counter (Auto Well Gamma System, ARC-300, Aloka) and the results were expressed as tissue (cpm/g)/blood (cpm/g) radioactivity ratios.

Measurements of Percent Injected Dose per Gram and Specific Localization Index

Mice bearing tumor (FCC-1) were also injected with 2.22 MBq of ^{131}I-labeled RASK-3 and ^{125}I-labeled HPL-1. These mice were also given potassium iodide. The mice were killed 3 and 8 days after the injection of antibodies. Various organs as well as the tumors were removed and immediately weighed. The radioactivity was determined and the results were expressed as a % of injected dose per gram (tissue (cpm/g)/injected dose (cpm) × 100) and specific localization index. Specific localization indices were calculated as follows:

$$\text{Specific localization index} = \frac{\text{Tissue } ^{131}\text{I (cpm/g)}/^{125}\text{I (cpm/g)}}{\text{Blood } ^{131}\text{I (cpm/g)}/^{125}\text{I (cpm/g)}}.$$

In Vitro Treatment of Tumor Cells with Lysolecithin and Periodate-Lysine-Paraformaldehyde (PLP)

Tumor cells (1×10^6) were incubated with 3 ml of 50 μg/ml lysolecithin (lysophosphatidyl-choline, type 1, Sigma, St. Louis, MO) at 4°C for 5 min. The treatment was stopped by adding phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.02% sodium azide. Effect of lysolecithin was assessed by trypan blue staining under a microscope.

Cells were treated with PLP at room temperature for 6 hr. Cells were washed with PBS and suspended in RPMI 1640.

Indirect Immunofluorescence Assays (*20, 21*)

Tumor cells with or without lysolecithin treatment were incubated with 2 μl of RASK-3 or HPL-1 (1 mg/ml in PBS) at 4°C for 30 min. After washing with PBS containing 1% BSA and 0.02% sodium azide, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G was added to the cells and incubated at 4°C for 30 min. All specimens were analyzed on an EPICS-C flow cytometer (Coulter Electronics, Hialeah, FL).

Radioimmunobinding Assays

Tumor cells (5×10^5) with or without treatment by PLP were incubated with ^{125}I-labeled RASK-3 or ^{125}I-labeled HPL-1 (30,000 cpm) in culture tubes at room temperature for 30 min. Cells were washed three times with 1 ml Hanks solution, and the radioactivity of cells was determined using a gamma scintillation counter.

Tumor cells (5×10^5) with or without prior PLP treatment were incubated with various concentrations of non-radioactive RASK-3 or HPL-1. Cells were then incubated with ^{125}I-labeled RASK-3 (30,000 cpm) in culture tubes at room temperature for 30 min. Cells were washed three times with 1 ml Hanks solution, and the radioactivity of cells was determined using a gamma scintillation counter (Auto Well Gamma System, ARC-600, Aloka).

RESULTS

Immunoscintigraphy by RASK-3

Three to 4 wk after the inoculation of 1×10^7 tumor cells of FCC-1, BM-314, or KNS-62, the mean tumor diameter was usually between 10 mm to 15 mm with no evidence of metastasis. Mice were injected with either ^{125}I-labeled RASK-3 (to the experimental group) or ^{125}I-labeled anti-human platelet monoclonal antibody, HPL-1 (to the control group) as described in Materials and Methods.

On the third and fifth days no clear tumor image was obtained in either group. On the seventh day, however, a radioactive area corresponding to the tumor was clearly observed in each mouse of the experimental group with one of three tumor cell lines. In the control group, tumor images were non-visible (Fig. 1).

In Vivo Distribution of Antibodies

The tissue/blood radioactivity ratios in FCC-1 inoculated mice on the eighth day after injection of antibodies are shown in Figure 2. The ratio in the tumor injected with ^{125}I-RASK-3 was significantly higher than those of other normal tissues. It was also several times higher than the ratio in the tumor injected with ^{125}I-HPL-1. In addition, the ratios in various organs injected with ^{125}I-RASK-3 were slightly, but consistently, higher than those in the corresponding organs in the group injected with ^{125}I-HPL-1. The same results were observed in BM-314 or KNS-62 inoculated mice. The tissue/blood radioactivity ratios in BM-314 tumors and KNS-62 tumors injected with ^{131}I-RASK-3 were 2.1 and 1.8, respectively. They were significantly higher than the ratios in other organs injected with ^{131}I-RASK-3 and the ratios in tumors injected with ^{125}I-HPL-1.

Considering these uses of RASK-3 for the imaging of various tumors, we further analyzed the binding characteristics of RASK-3 to one of three lines, FCC-1. With ^{131}I-RASK-3 and ^{125}I-HPL-1, the % ID/g was evaluated in FCC-1 (Table 1). The value for tumors injected with ^{131}I-RASK-3 (4.98 ± 1.38) was significantly higher than that of other tissues or blood. It was also several times higher than the value in tumors injected with ^{125}I-HPL-1 (1.27 ± 0.53). The specific localization indices in tumors, liver, spleen, and colon were also examined three and eight days after injection of ^{131}I-RASK-3 and ^{125}I-HPL-1 (Fig. 3). The values for tumors increased and became much higher than those in the liver, spleen, or colon by Day 8.

In Vitro Binding of RASK-3 to Tumor Cells

Binding of RASK-3 to lysolecithin or PLP-treated target cells was examined in vitro with non-labeled and

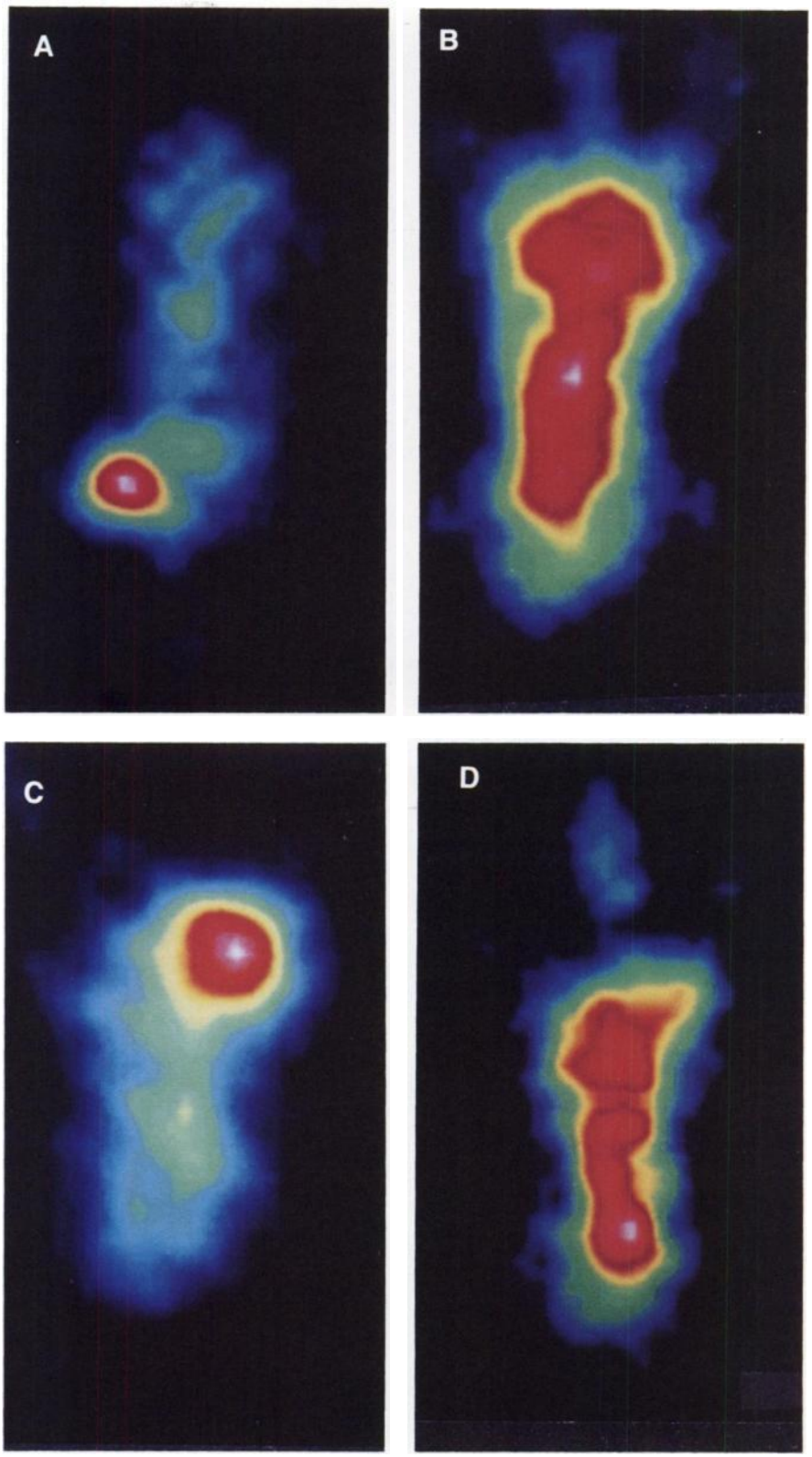

FIGURE 1
Immunoscintigraphy (nude mice, 3 mice in each group) of three heterotransplanted human tumor cell line (FCC-1, BM-314, and KNS-62) with ^{125}I-labeled antibodies. (A, C, and E) Total-body scans of mice 7 days after i.v. injection of ^{125}I-labeled RASK-3 (experimental group). (B, D, and F) Total-body scans of mice 7 days after i.v. injection of ^{125}I-labeled HPL-1 (control group). (A and B) FCC-1; (C and D) BM-314; and (E and F) KNS-62. The contrast of the control images in Figures 1B, D, and F has been increased relative to the contrast of the experimental images. There is no accumulation of radiolabeled control antibodies to tumor tissues even with the increased contrast.

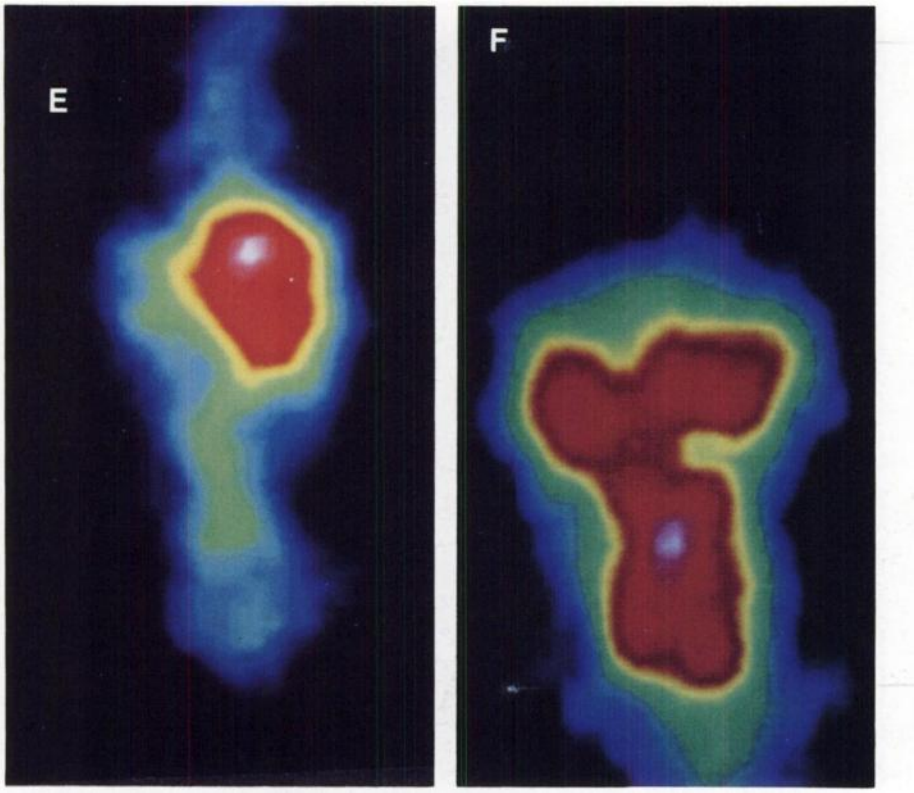

FIGURE 1 *continued*

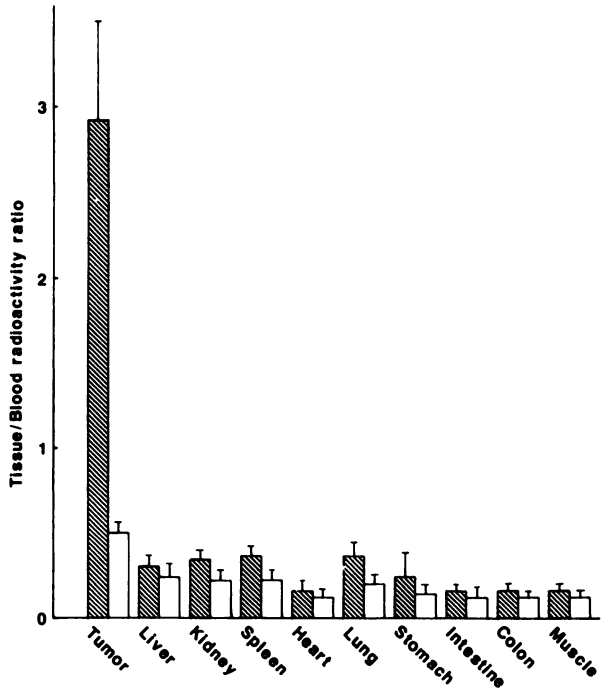

FIGURE 2
The tissue/blood radioactivity ratios in the various organs 8 days after injection of ^{125}I-labeled RASK-3 or ^{125}I-labeled HPL-1. Values shown are the mean ± s.d. of the results obtained from FCC-1 bearing mice (n = 3).

^{125}I-labeled monoclonal antibodies. The treatment of cells with these reagents are known to enhance cellular permeability. Cells treated with lysolecithin and incubated with RASK-3 showed significantly higher fluorescence intensities than the lysolecithin untreated cells (Fig. 4).

Cells were similarly treated with PLP, and the binding of ^{125}I-labeled RASK-3 to these tumor cells was also analyzed. As shown in Figure 5, the binding of

TABLE 1
Distribution of Labeled Monoclonal Antibodies in Nude Mice with Xenogeneic Human Cancers (FCC-1)

	n = 7	
	RASK-3	HPL-1
Tumor	4.98 ± 1.38*	1.27 ± 0.53
Liver	0.45 ± 0.07	0.46 ± 0.01
Kidney	0.52 ± 0.10	0.49 ± 0.12
Spleen	0.53 ± 0.11	0.46 ± 0.08
Colon	0.29 ± 0.06	0.25 ± 0.06
Blood	1.57 ± 0.23	1.87 ± 0.30

* mean %ID/g on Day 8 ± s.d.

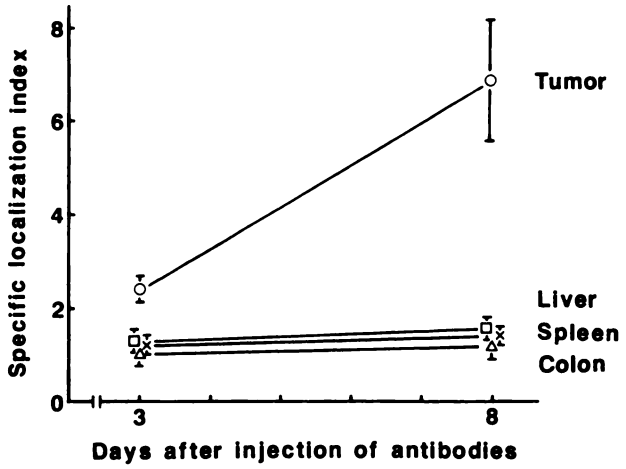

FIGURE 3
The specific localization index in tumor, liver, spleen, and colon 3 and 8 days after injection with ^{131}I-labeled RASK-3 and ^{125}I-labeled HPL-1. Values shown are the mean ± s.d. of the results obtained from FCC-1-bearing mice (n = 3).

^{125}I-labeled RASK-3 to tumor cells significantly increased with PLP treatment. The binding of ^{125}I-labeled RASK-3 to tumor cells was significantly decreased by prior incubation of FCC-1 cells with non-labeled RASK-3, but not HPL-1 (Fig. 6).

DISCUSSION

Recent immunohistochemical analysis of various human cancer tissues indicate that the expression of ras oncogene/proto-oncogene is significantly increased in a wide variety of human cancers (*12–15*). Our initial trials of imagings with three different tumor cell lines, FCC-1 (colon cancer), BM-314 (colon cancer), and KNS-62 (lung cancer) demonstrated clear tumor images in all three cell lines, suggesting the use of ras p21 as a target antigen. Analysis of tissue/blood radioactivity ratio or % ID/g showed significant accumulation of radioisotopes in tumor tissues by Day 8 after the inoculation of antibodies. With the three human tumor cell lines, the mean tumor/blood radioactivity ratio ranged from 1.8 to 2.9, whereas the maximal tissue/blood radioactivity ratio with various normal tissues were under 0.6. The mean % ID/g in tumors (FCC-1) was 4.60, and was significantly higher than those in various normal tissues. The specific localization index (tumor: 6.49) showed that this binding was antigen specific.

These figures, taken together, may indicate the usability of RASK-3 for tumor imagings when compared with previous experiments by us and others using anti-carcinoembryonic antigen (CEA) antibody (*22–26*) and anti-alpha/fetoprotein (AFP) antibody (*27,28*). The % ID/g and specific localization index in tumors by anti-CEA antibody were almost equivalent to those using RASK-3, although the tumor/blood radioactivity ratio with anti-CEA antibody was somewhat higher than that

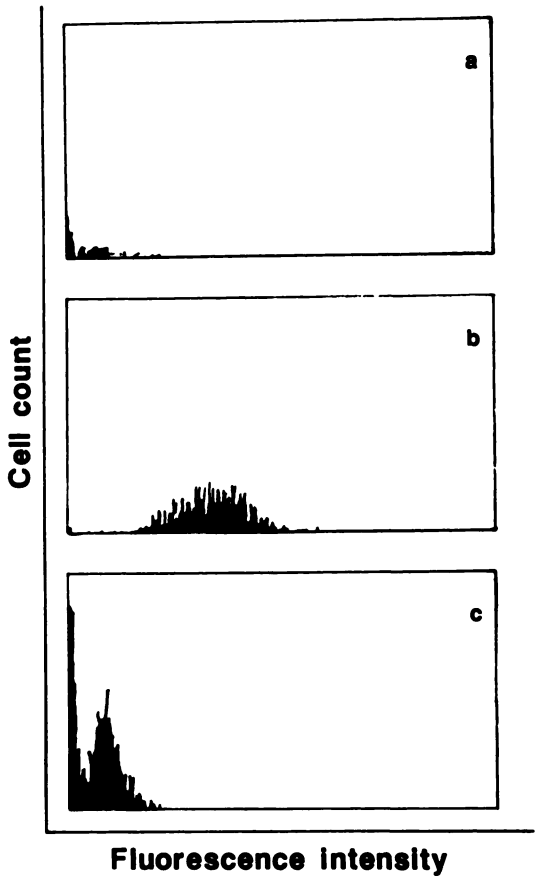

FIGURE 4
Reactivities of RASK-3 with tumor cells treated with lysolecithin. FCC-1 was treated with lysolecithin, and reactivities of RASK-3 with these cells were assayed by flow cytometry. (A) RASK-3 without lysolecithin treatment. (B) RASK-3 with lysolecithin treatment. (C) HPL-1 with lysolecithin treatment.

with RASK-3. The tumor/blood radioactivity ratio with anti-AFP antibody was even lower than that with RASK-3. Slightly, but significantly higher tissue/blood radioactivity ratios were obtained in various normal tissues with anti-ras p21 antibodies than with control monoclonal antibody HPL-1. It is known that a variety of normal cells also express ras p21 (*9–11*). Since the anti-ras p21 monoclonal antibodies (RASK-3) used in this study also react with murine c-ras p21, inoculated antibodies may have bound to normal tissues. The accumulation of anti-ras p21 monoclonal antibody was nevertheless much higher in tumor tissues giving a clear image. It should also be stressed that the use of RASK-3 for imaging, which is non-species-specific, may provide an excellent experimental tool proximating its use in humans.

The mechanism by which anti-ras p21 antibodies accumulate in tumor sites is unclear. The ras p21 is known to localize intracellularly (*29,30*), being hardly detectable on the surface of viable cells. In the present study, RASK-3 was found to specifically bind to tumor cells only after the treatment of cells with lysolecithin

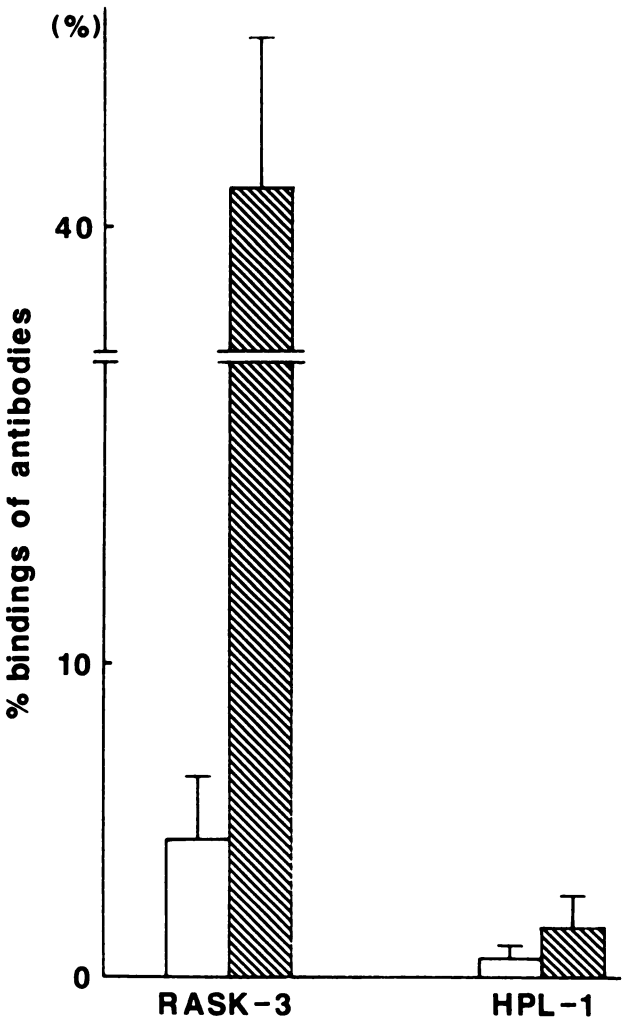

FIGURE 5
Bindings of RASK-3 and HPL-1 to tumor cells with (▨) or without (□) treatment with PLP; Values shown are the mean ± s.d. of the results obtained from FCC-1 cells (n = 3).

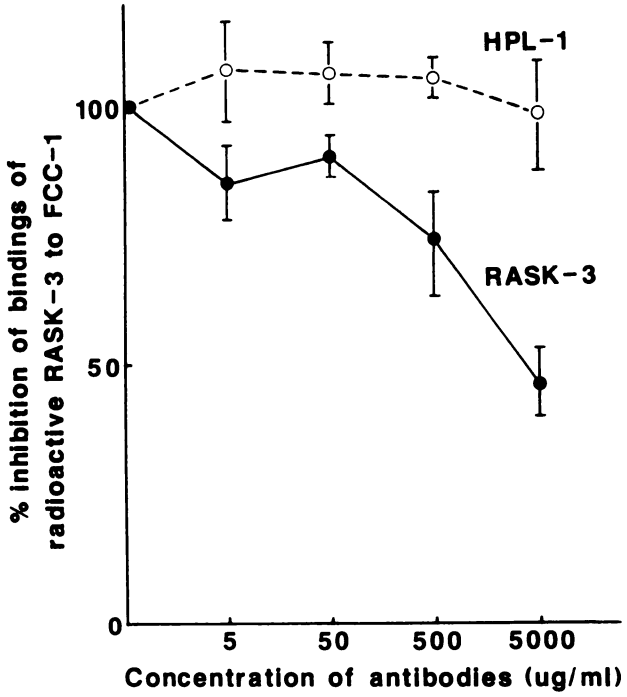

FIGURE 6
% inhibition binding of radioactive RASK-3 to FCC-1. Values shown are the mean ± s.d. of the results obtained from FCC-1 cells.

or PLP, which indicates the intracellular localization of ras p21 through cell membranes. Although, the possibility of the enhancement of cell surface reactivity is not completely excluded.

Recently, Welt et al. reported the imaging of heterotransplanted melanoma using antibodies reactive with an intracellular antigen (*31*). They speculated that their antibodies accumulated in the necrotic area of the transplanted tumor. In addition, antibodies reactive with intracellular myosin have been used to detect the site of myocardial infarction (*32*). In our study, massive tumor necrosis was not observed macroscopically but necrosis of tumor was found in part in hematoxylin eosin stained section by microscopic examination and grains were clearly found in necrotic as well as non-necrotic cells by autoradiography. However, there are several possible alternative explanations which include the shedding ras p21 by viable tumor cells, changes in membrane permeability by non-necrotic mechanisms, or the introduction of antigens by infiltrating phagocytic cells such as macrophages. It is also possible that minute amounts of ras p21 are expressed on the cell surface which are not sufficient to be detected by in vitro serologic assays.

The results obtained in this study encourage further investigation of the feasibility and optimum use of anti-ras p21 monoclonal antibodies for immunoscintigraphy. They also suggest the usefulness of antibodies directed toward other oncogene products, most of which are known to be intracellularly localized.

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