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Use of ^{111}In–L–LDL radiotracers to detect human pancreatic and mice melanoma tumors

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The present study was designed to evaluate the potential of labeled low-density lipoprotein with ^{111}In using a lipid chelating agent (bis(stearylamide) of diethylenetriaminepentaacetic acid: L) to detect pancreatic tumors and melanoma in mice by gamma-scintigraphy. We compare the biodistribution of radioactivity and scintigraphic images in nude mice heterotransplanted with human cancerous pancreatic duct cells (Capan-1) and in mice transplanted with murine tumor cells (B16 melanoma). Biodistribution studies showed that radioactivity was twice as high in the Capan-1 xenograft after injection of the radiolabel than after injection of radiometal alone, and 34-fold higher in the B16 tumor. On gamma-scintigraphic imaging, the Capan-1 tumor was just visible, whereas the B16 melanoma was clearly imaged. The lack of contrast of the Capan-1 tumor compared with the B16 melanoma could be due to a poor vascularization. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: indium; low-density lipoprotein labeling; radiotracer; tumor targeting; scintigraphic images

INTRODUCTION

The difficulty in eradicating pancreatic cancer is compounded by the fact that the disease is usually diagnosed in a late stage. Early diagnosis is hampered by the lack of a non-cytotoxic substance that targets malignant cells preferentially. Studies have therefore been focused on the development of synthetic vectors that can transport a radioelement or a drug selectively towards tumoral tissue. A major problem is to find the appropriate conditions for cell-specific targeting by drugs or markers. Monoclonal antibodies as natural vectors of diagnostic agents or cytotoxic drugs have been reported to target primary and metastatic human cancers.[1–5]

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Other workers have shown that low-density lipoproteins (LDLs) can be exploited to target certain cancerous tumors, which could prove of value in the diagnosis or therapy of cancer.[6–10] Receptor-mediated LDLs uptake is reported to be higher in tumor cells than in normal cells. The internalization and degradation of LDLs mediated by a receptor has prompted the use of LDL as a vector for the transport of therapeutic or diagnostic drugs to tumors,[11–15] particularly of the pancreas.

We recently proposed a new method for labeling LDL with ^{111}In using *N*,*N*″-bis(stearylamide)diethylenetriaminepenta-acetic acid as a lipid chelating anchor (L) to stabilize the radioelement on the LDL particles, producing the final radiotracer ^{111}In–L–LDL.[16] The lipophilic anchorage of the complex ^{111}In–L in the lipid layer of LDL was found not to modify greatly the structure of the Apo-B protein. Initial *in vitro* studies on the ^{111}In–L–LDL radiolabel confirmed that it did not prevent recognition by LDL receptors of cultured A594 human adenocarcinoma fibroblasts.[16] The presence of up to 30 In–L complexes per LDL particle did not alter the affinity of the LDL for its receptor. In the healthy mouse, *in vivo* studies showed that radioactivity was also strongly disseminated throughout the organism

after intravenous injection of ^{111}In in the form of citrate. However, when it was injected in the form of ^{111}In–L–LDL, the radioactivity became exclusively concentrated in the liver and almost undetectable in the rest of the animal.[17] This difference in biodistribution between free indium and the ^{111}In–L–LDL conjugate illustrates the vector role played by the LDLs.

We have also conducted *in vitro* studies on a human pancreatic duct cancer cell line, Capan-1, which over-expresses the LDL-receptor.[18] We followed the internalization of the tracer in cultured Capan-1 cells by electron microscopy and demonstrated its localization in the cytoplasm of the cancer cells. These *in vitro* findings prompted us to use Capan-1 xenografts as an *in vivo* model to develop a technique for detection of pancreatic cancers. We compared the results with those obtained on B16 melanoma cells that also over-express LDL-receptors.[17] In the present study, the ability of ^{111}In–L–LDL tracers to detect cancerous tumors by gamma-scintigraphy was examined in human pancreatic tumor (Capan-1) xenografts in nude mice and in B16 melanoma homografts in black mice. We show that the degree of vascularization of the tumor must be taken into account when using such methods for the detection of pancreatic cancers *in vivo*.

MATERIALS AND METHODS

Materials and abbreviations

All chemicals were of analytical grade, used without further purification unless otherwise indicated and were obtained from the following sources: 111-indium chloride ($^{111}InCl_3$) from NEN Research Products (USA); ^{125}I-albumin from Cis Bio international (^{125}I S4), indium(III) chloride ($InCl_3$) from Aldrich Chemical Co. (St Quentin Fallavier, France); bovine serum albumin (BSA) and tris(hydroxymethyl)aminomethane from Sigma Chemical Co. (St Louis, MO); Bio-Rad protein assay (Coomassie Blue) from Bio-Rad Laboratories (Ivry sur Seine, France); 20% ammonia solution (NH_4OH), trisodium citrate, dimethyl sulfoxide (DMSO), disodium hydrogen phosphate (Na_2HPO_4), ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), potassium bromide (KBr), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), solvents, acids, bases and other salts from Prolabo (Paris, France). NaCl solution (0.9%) was from Laboratoire Biosedra (Louviers, France). Minisart 0.45 μm filters were obtained from Sartorius (Palaiseau, France), fetal calf serum (FCS), fungizone, penicillin, phosphate-buffered saline (PBS) for cell culture, streptomycin, RPMI-1640 medium, trypsin EDTA mixture from Invitrogen (Cergy Pontoise, France) and plastic flasks from Nunc (Grand Island, NY). The experiments were carried out with xenografts of human pancreatic cancer cells of the Capan-1 line. B16 melanoma homografts were used for comparison.

Capan-1 xenografts

All animal experiments were carried out in conformity with the Declaration of Helsinki and the 'Guiding Principles in the Care and Use of Animals' approved in its revised form by the American Physiological Society, 1991. The xenografts were produced by subcutaneous injection of 5×10^6 5-day-old Capan-1 cells, in culture at the 441st passage, into leg of 4-week-old congenitally athymic female nu/nu mice (Swiss, IFFA CREDO, France) maintained in a pathogen-free environment. The Capan-1 cell line used in this study was established by Fögh[19] at the Sloan Kettering Institute for Cancer Research (NY) from a liver metastasis in a patient with an adenocarcinoma of the head of the pancreas. We received it at its 14th passage from the American Type Culture Collection (ATCC, Rockville, MD). It was maintained in RPMI-1640 medium supplemented with 15% FCS, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and fungizone (0.25 μg ml^{-1}). Cultures were carried out in 25 cm^3 plastic flasks. Trypsin (0.05%)–EDTA (0.02%) was used for the successive passages. The cells were then seeded at 2.5×10^5 cells/ml, and media were renewed every 2 days.

B16 melanoma homografts

The B16 melanoma homografts were produced by subcutaneous injection of 5×10^5 4-day-old cultured cells into the leg of 5-week-old female CD57 Bl/6J mice (IFFA CREDO, France). This cell line was maintained in RPMI medium supplemented with 10% FCS and containing the same antibiotics as the Capan-1 cells. The 25 cm^3 flasks were seeded with 10^5 cells and the media were changed every 2 days.

Culture checks

The cultures of Capan-1 and B16 melanoma cells were checked every month for contamination with mycoplasma using cultures grown in a suitable medium for three passages and polymerase chain reaction methods (Mycoplasma PCR kits, Stratagene, CA).

Tumor growth

Tumor growth was followed for 40 days for the Capan-1 xenografts and for 14 days for the B16 melanoma homografts by measuring tumor volumes in groups of five mice. The length l, width w, and height h of each tumor were measured with calipers. The volume was calculated from the ellipsoid volume

$$V = \frac{4}{3}\pi \frac{l}{2}\frac{w}{2}\frac{h}{2}$$

Tumors were obtained within 1 week after inoculation. The radiotracer was injected when the tumors reached a volume of 600 mm^3, i.e. approximately 40 days after inoculation of the Capan-1 cells and 12 days after inoculation of the B16 melanoma cells.

Cytology

In order to observe the features of cells maintained *in vivo*, samples of each tumor used for scintigraphic imaging

were removed for cytological analysis. They were fixed in Bouin's solution, dehydrated and embedded in paraffin. The morphological appearance of the tumor and the state of vascularization were examined after staining with hemalum–eosin and Mallory's trichrom.

Preparation of radiotracer ^{111}In–L–LDL

Human LDLs were isolated from fresh plasma from healthy volunteers by preparative ultracentrifugation in a KBr gradient using a vertical rotor (VTI 50, Beckman, Palo Alto, CA), according to the method of Paumay and Ronveaux-Dupal.[20] The LDLs were stored at 4 °C for no longer than 3 weeks. Before experimentation, LDL samples were filtered through a 0.45 μm pore size membrane (Millipore). The LDL were assayed as proteins by the method of Bradford[21] using BSA as standard. For reasons of simplicity, LDL concentration was expressed as milligrams of LDL per milliliter instead of milligrams of protein per milliliter. The preparation of L has been described elsewhere.[22] The standard LDL solutions were diluted with PBS. The L–LDL particles were prepared by adding solution L (1.5 mM) dropwise to a 2 ml solution of LDL (2 mg ml^{-1}) under stirring at room temperature for 1 h and filtration. The volume of L added to the LDL solution was varied to obtain different L/LDL ratios: 3/1, 30/1, 3/100. To $^{111}InCl_3$ (74 MBq, 2.0 mCi, 152 Ci per milligram of indium in 0.05 M HCl) cold $InCl_3$ (1.04×10^{-5} M in 0.05 M HCl) was added to produce a final solution of 50 μl. Sodium citrate solution (200 μl, 1.36×10^{-5} M) was added to 50 μl of $^{111}InCl_3$, and the pH of the final solution was adjusted to 7.4. This solution of ^{111}-indium citrate (pH 7.4) was added to L–LDL samples in appropriate volumes to give ^{111}In –L/LDL ratios of 3/1, 30/1 and 3/100 (^{111}In-to-L ratio, 1 : 1). The control solution used PBS instead of LDL, with a 3/1 ratio of 111-indium citrate/PBS.

Imaging procedure and biodistribution

^{111}In–L–LDL 9.3 MBq (250 μCi) and ^{125}I-albumin 37.5 kBq (1 μCi) per mouse was injected intravenously through the tail vein. Mice were anesthetized before experimentation with a sterile solution of Immenoctal (Houdé, Paris, France) (300 μl intraperitoneal per mouse). Scintigraphic imaging of the mice ($n = 5$) was performed at different times (24 h, 48 h) after injection of various ^{111}In–L/LDL ratios (3/1, 30/1, 3/100) and ^{125}I-albumin using a gamma camera (Sophycamera) equipped with a pinhole collimator set at 70 mm from the animal. Approximately 200 000 counts were obtained during the 15 min acquisition. After image acquisition, the animals were killed and organs of interest (stomach, intestine, pancreas) and tissues (muscle, tumor) were dissected out, washed three times in cold PBS (4 °C), blotted dry, weighed, and counted for radioactivity in a Packard counter (Packard Auto Gamma 5780). The radioactivity (counts per minute: cpm) found in each of the tissues studied for different ^{111}-In/lipoprotein ratios and for the ^{111}-In alone is represented in histograms. The results are expressed by the ratio cpm per gram organ/cpm per milliliter for the different tissues. In the absence of biomarker tissue uptake, activity is only from the blood in the organ.[23] A ratio above unity is indicative of biomarker-specific uptake.

RESULTS

Visualization of the tumor by injection of the ^{111}In–L–LDL radiotracer

Scintigraphic imaging in the nude mouse transplanted with a human pancreatic tumor (Capan-1) and in the black mouse implanted with a murine tumor (B16 melanoma) showed that radiolabeled LDL led to detectable uptake of tracer by the Capan-1 (Plate 1a, arrow) and B16 tumors (Plate 1c, arrow) 48 h after intravenous injection of the tracer. For both nude (Plate 1b) and black mice the tumor was not visible after injection of indium citrate. The arrow in Plate 1b indicates the tumor site for the nude mouse. It is noteworthy that the tumor was slightly visible for the nude mouse xenografted with the human pancreatic tumor Capan-1 (Plate 1a, arrow), whereas it was clearly visualized for the mouse homografted with the B16 melanoma cells (Plate 1c, arrow).

Biodistribution of radioactivity within the tumor

Biodistribution studies performed 48 h after injection indicated that the radioactivity in both Capan-1 and B16 tumors was significantly higher than in the other organs after administration of the 3/100 ^{111}In–L/LDL radiotracer (Fig. 1a and b). No relative increase in the radioactivity in the tumors was observed after injection of the 3/1 and 30/1 ^{111}In–L/LDL radiotracers or indium citrate (Fig. 1a and b).

For the nude mouse with the Capan-1 tumor, the radioactivity in the tumor was double that found in stomach, intestine, pancreas or muscle (Fig. 1a). However, it should be noted that for the mouse with the B16 melanoma, the level of radioactivity in the tumor was three fold that of the stomach, 11-fold that of the intestine and 34-fold greater than that found in muscle (Fig. 1b). Similar results were obtained 24 h after injection.

In order to demonstrate the *in vivo* specificity of radiolabeled LDL binding, ^{125}I-albumin was injected intravenously to mice bearing B16 tumor. The B16 tumor was chosen as the amounts of intratumoral radioactivity were higher than those in the Capan-1 tumor. The amounts of radioactivity in organs 48 h after injection of ^{125}I-albumin or ^{111}In–L–LDL were compared (Fig. 2). The level of radioactivity in the B16 tumor was comparable to that in the other organs after injection of radiolabeled-albumin (Fig. 2a), whereas four fold more radioactivity was noted in the tumor than in the other organs after injection of radiolabeled-LDL (Fig. 2b). When ^{125}I-albumin was injected into the CD57Bl/l6J mouse, more than 80% of the radioactivity remained in the blood (ratio cpm organ/cpm blood between 0.2 and 0.4). For the stomach, the ratio was about unity (Fig. 2a) and was due to 125-I released after degradation of the albumin. It was noteworthy that the

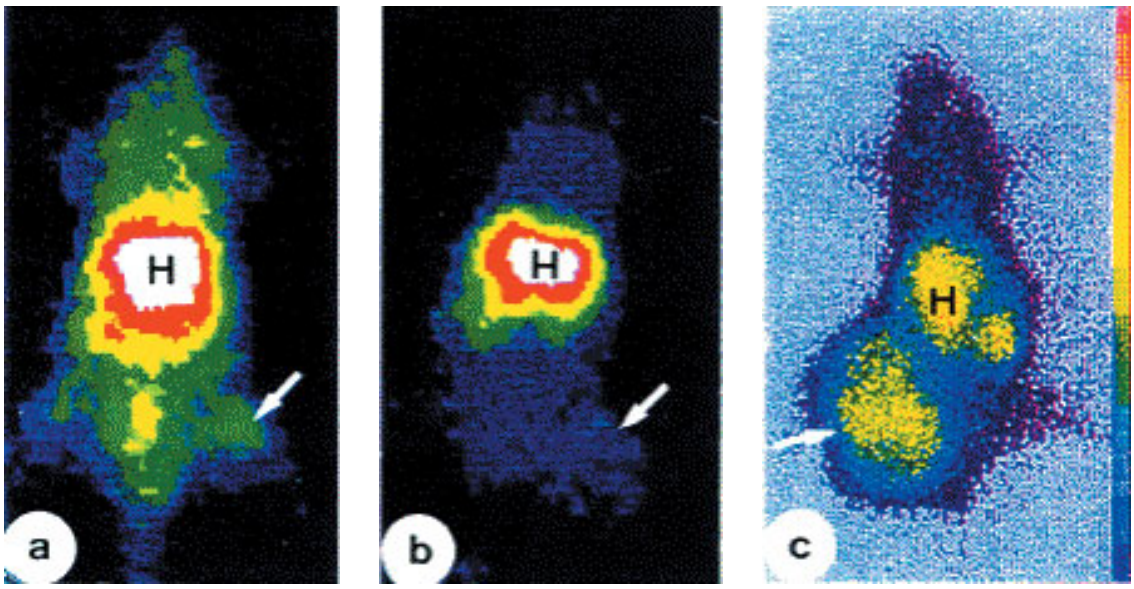

Plate 1. Scintigraphic images of nude mice with xenografted human pancreatic Capan-1 tumors after (a) intravenous injection of the 3/100 ^{111}In–L/LDL radiotracer or (b) 111-indium citrate alone. (c) Scintigraphic images of black mice (CD 57 Bl/6J) with homografted murine melanoma B16 tumors after intravenous injection of the 3/100 ^{111}In–L/LDL radiotracer. The images were taken 48 h after the intravenous injection. Note the lack of radioactivity (b, arrow), the weak radioactivity of a xenografted Capan-1 tumor (a, arrow) and the strong radioactivity of a homografted B16 tumor (c, arrow). Note the strong reactivity in the hepatic area (H). The color intensity scale for scintigraphic imaging goes from blue for weak radioactivity to white for strong radioactivity.

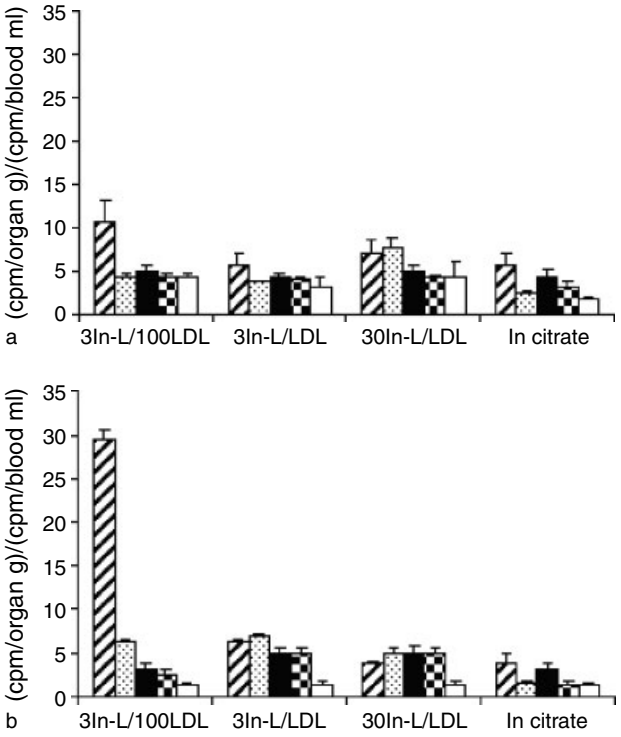

Figure 1. Biodistribution of the radioactivity in a xenografted human pancreatic Capan-1 tumor (a) and a murine B16 tumor (b). Note the strong radioactivity in tumors (▨) compared with that in stomach (▩), intestine (■), pancreas (▩) and muscle (□). Three ^{111}In–L/LDL radiotracer formulations were injected intravenously into the mice 3/100, 3/1, 30/1) and the 3/1 control solution of 111-indium citrate/PBS. The results shown in this figure are expressed as the ratio cpm per gram organ/cpm per milliliter blood plus/minus the standard error of the mean (SEM) of five values.

ratio of radioactivity was above unity for all the organs after injection of ^{111}In–LDL. Similar results were obtained 24 h after injection.

Tumor cytology

Cytological studies of the Capan-1 and B16 tumor samples *in vivo* showed features of the pattern of vascularization (Fig. 3).

Capan-1 xenografts

From the tenth day after injection of cells, growth of the tumor became exponential and the volume reached 600 mm^3 on day 40. Cytological analysis showed that, at this stage, the tumor was surrounded by abundant connective tissue and it was composed of many small cancerous nodules presenting numerous mitoses (Fig. 3a, arrows). These proliferating nodules were surrounded by thick reactive connective tissue. Adenoid structures were rather rare, with few areas of lysis. The tumoral tissue was not irrigated, because no blood capillaries were observed. Only the peritumoral connective tissue contained numerous blood vessels.

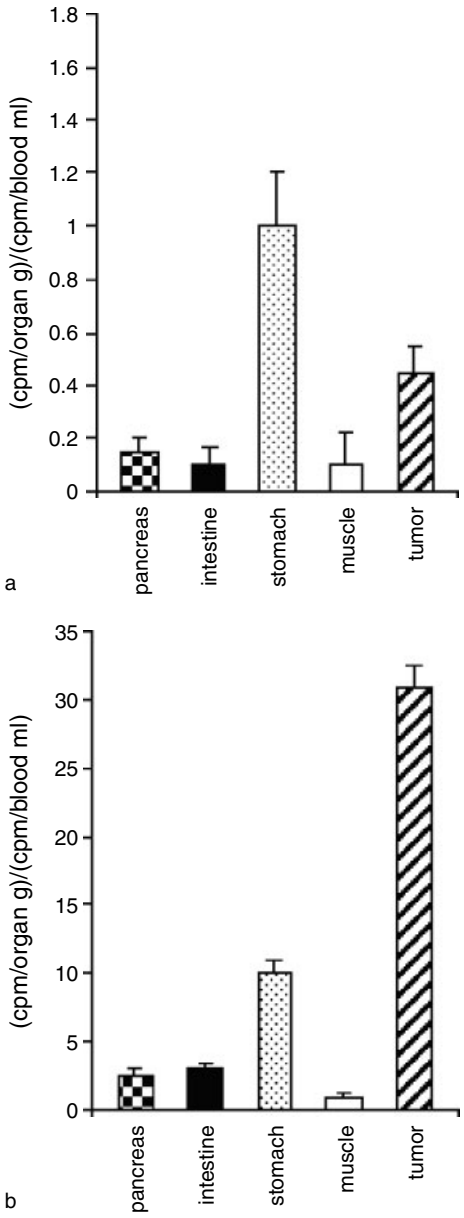

Figure 2. Biodistribution of the radioactivity in a murine B16 tumor after injection of ^{125}I-albumin (a) or ^{111}In–L–LDL (b) to the mice. Note the strong radioactivity in tumors (▨) when ^{111}In–L–LDL was injected instead of ^{125}I-albumin, compared with that observed in stomach (▩) intestine (■), pancreas (▩) and muscle (□). The 3/100 ^{111}In–L/LDL radiotracer formulation was injected intravenously into the mice. The results shown in this figure are expressed as the ratio cpm per gram organ/cpm per milliliter blood plus/minus SEM of five values.

B16 melanoma homografts

After 5 days latency, the tumor grew exponentially until the 14th day. Macroscopically, it appeared as a spongy nodule. Cytological analysis of the B16 melanoma cells in the exponential phase showed them to be round or oval with

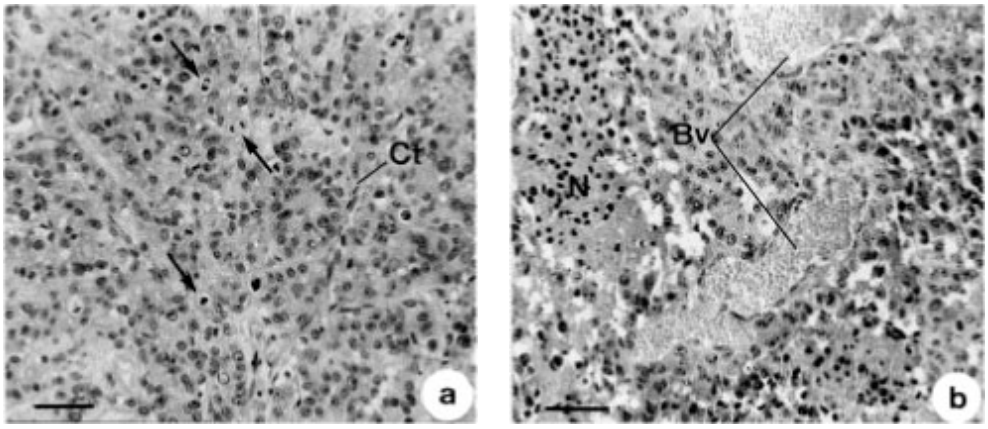

Figure 3. (a) General appearance of a xenograft of Capan-1 cells in the nude mouse. The cells form in large clusters separated by thin connective tissue (ct). Note the absence of blood vessels and the abundant mitoses (arrows). First heterotransplantation tumor; aged 40 days; stained with hemalum–eosin. (b) General appearance of a B16 melanoma homograft transplanted into a CD57 Bl/6J mouse. Note the presence of numerous blood vessels (Bv) and necrotic zones (N). Tumor explanted for the first time; aged 12 days; stained with hemalum–eosin (bar: 50 μm).

a voluminous nucleus. The cytoplasm was filled with black granules, thought to be mostly melanin, indicating that the tumor was differentiated. Areas of necrosis were rare. On the other hand, abundant blood capillaries were observed in the tumoral tissue (Fig. 3b). At the latest stages of growth, around the 15th day, the tumor became hemorrhagic.

DISCUSSION

The present study highlights the difficulty in detecting pancreatic xenografts in the nude mouse after injection of the radiotracer ^{111}In–L–LDL.

In a previous study, we showed that the density of LDL receptor sites in Capan-1 pancreatic cells was tenfold that of normal cells.[18] This property can be exploited to bind radioactive isotopes, such as ^{111}-In using LDL as vehicle, enabling gamma-scintigraphic imaging. We established that radioelements can be bound to LDL via a lipophilic complexing agent (L) to generate metal–L–LDL radiotracers.[16] Moreover, in Capan-1 pancreatic cells in culture, the LDLs with radiotracer did not lose the ability to bind to their receptor sites: electron microscopy clearly showed their internalization.[18] As the LDL pathway did not seem altered by indium radiolabeling,[18] both Capan-1 and B16 melanoma tumors should be clearly detected by scintigraphic imaging.

In the present study, we xenografted human pancreatic cancer cells (Capan-1) into the leg of nude mice, rather than into the dorsal region, to avoid confusing the image of the pancreatic tumor xenograft with that of the liver, which is also known to express LDL receptor sites.[24] The results of the biodistribution study showed that, 48 h after the intravenous injection of ^{111}In–L–LDL tracers, the radioactivity in the pancreatic tumor was twofold that found in the organs of the digestive tract (stomach, intestine, pancreas) or in muscle. Despite this enhanced radioactivity, which indicated that at least some of the radiolabeled LDL had penetrated the tumor, the levels of radioactivity were not sufficient to distinguish tumors from other organs by scintigraphy. Furthermore, in all experiments, we noted that the radioactivity in the pancreatic tumor was lower than that in the liver, an organ in which the LDL receptor density is similar to that in Capan-1 cells *in vitro* (liver-G2 $K_D = 15\ \mu g\ ml^{-1}$; Capan-1$K_D = 27.5\ \mu g\ ml^{-1}$).[18,25] We wished to know, in contrast to the B16 melanoma, why the xenografted Capan-1 pancreatic tumors were not visible by gamma-scintigraphic imaging despite an uptake of radioactivity twofold higher than in the other organs. The cytological investigations showed that one of the main differences between the Capan-1 and the murine B16 tumors was that the Capan-1 tumoral tissue was poorly vascularized. They were seen as an accumulation of small clusters of proliferating tumoral nodules or adenoid structures surrounded by a thick layer of connective tissue. It may be that the enshrouding reactive connective tissue is an obstacle to the entry of ^{111}In–L–LDL tracer from peripheral blood vessels to the tumoral cells. The rarity of blood capillaries in these tumors could explain their low levels of radioactivity and the difficulty in visualizing them scintigraphically. In the B16 melanoma, on the other hand, many blood vessels were observed between and inside the clusters of tumoral cells. The tumor had a spongy appearance and was clearly visualized with after injection of the radiotracer.

The high level of radioactivity in the blood after injection of ^{125}I-albumin can be explained by the volume of blood remaining in the organ.[23] The weak radioactivity observed in the B16 tumor (compared with the other organs) when

the non-specific tracer ^{125}I-albumin was injected strongly suggests that the high level of radioactivity observed in this tumor after injection of ^{111}In–LDL was receptor mediated. This is supported by the fact that B16 tumors are known to over-express LDL receptors.[13,26] The ratio of radioactivity above unity observed for all the organs after injection of ^{111}In–LDL is consistent with the presence of LDL receptors in non-cancerous cells.[24,27]

The B16 melanoma showed up clearly on scintigraphy and exhibited marked radioactivity after injection of the ^{111}In–L–L tracer: B16 cells express the LDL receptor abundantly and the tumor is well vascularized. The Capan-1 pancreatic xenografts appeared faint and exhibited low levels of radioactivity after injection of the ^{111}In–L–L biomarker: the tumor was poorly vascularized, although the Capan-1 cells over-express LDL receptors in culture.[18] These observations suggest that the difficulty in detecting the xenografted Capan-1 pancreatic tumor stemmed largely from poor vascularization. On the other hand, the question arises as to whether the LDL receptor sites are also expressed in lower quantities in tumors. We do not think so, since it has been shown in numerous tumors that LDL receptors remain expressed. Thus, melanocytes express LDL receptor sites that are able to internalize tracers.[15] Moreover, in older Capan-1 cell cultures, nodules resembling those found in solid tumors were observed containing LDL-immunofluorescent cells, indicating the expression of LDL receptor sites (data not shown). Internalization is possible, since it has been shown that tracers inserted into LDL do not denature the recognition between apolipoprotein B and the receptor sites of cancerous cells.[16] With Capan-1 cells, the LDL receptor sites are also over-expressed,[18] but, unlike in the melanoma, the Capan-1 xenografts were faint on gamma-scintigraphy. This was attributed to the low infiltration of blood vessels into the Capan-1 tumoral tissue compared with that formed into the melanoma B16 cells.

In summary, the present study shows that a major reason for the difficulty in visualizing Capan-1 pancreatic xenografts by scintigraphy could be a poor vascularization of the tumoral tissue. Nevertheless, the In–L–LDL conjugate remains a good radiotracer since it allows tumors visualization, especially those that are well vascularized.

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