Indium-111-Diethylenetriaminepentaacetic Acid-Octreotide Is Delivered in Vivo to Pancreatic, Tumor Cell, Renal, and Hepatocyte Lysosomes

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ABSTRACT

To better understand the factors that govern the target-to-background ratios of 111In-diethylenetriaminepentaacetic acid (DTPA) polypeptides, we studied 111In-DTPA-octreotide and a model nontargeted compound, 111In-DTPA-poly(D)lysine-biotin. We evaluated the fate of 111In-DTPA-octreotide after it localizes in somatostatin receptor-positive tissues and sought to determine why such a large fraction of these and other 111In-DTPA-polypeptides accumulate in the liver and kidneys.

Biodistribution studies in rats with an implanted pancreatic adenocarcinoma demonstrated rapid accumulation of 111In-DTPA-octreotide in the pancreas and tumor. Indium-111 also accumulated in the liver and kidneys. Subcellular fractionation of the liver, kidneys, tumor, and pancreas showed that the majority of the radioactivity copurified with lysosomal enzymes. Even at 1 h, little radioactivity was found in the fractions containing a cell surface enzyme. This suggests that in each tissue, the 111In-DTPA-octreotide was rapidly shuttled from the cell surface to lysosomes. In the liver, hepatocyte lysosomes were separated from sinusoidal and Kupffer cell lysosomes by administering chloroquine prior to sacrifice. This density shift experiment indicated that 111In-DTPA-octreotide accumulated predominantly in hepatocyte lysosomes.

A low molecular weight 111In-DTPA-poly(D)lysine-biotin compound was synthesized, and biodistribution studies showed substantial renal accumulation. The poly(D)lysine backbone conferred resistance to degradation, and this fact allowed determination of the net accumulation of this compound at the cellular level using an antibiotin antibody and immunohistochemical techniques. These experiments, as well as subcellular fractionation studies, demonstrated that the 111In-DTPA-poly(D)lysine-biotin compound accumulated in the lysosomes of proximal renal tubular cells.

These results indicate that lysosomes play a critical role in the cellular physiology of radiolabeled polypeptides. Using these data, we propose a comprehensive model that summarizes the factors that govern the target to background ratios of radiolabeled polypeptides.

INTRODUCTION

There are substantial differences between normal and diseased tissues at the molecular level, and it has long been hoped that these differences could be systematically exploited for diagnostic imaging. Radiopharmaceuticals are the most direct means of achieving this goal because the relevant biochemical pathways are often first elucidated and easily quantified. However, the in vivo results with nearly all radiolabeled polypeptides have been conducted with 111In-DTPA-octreotide, two important questions remain. First, what happens to the 111In-DTPA-octreotide molecule after it binds to the somatostatin receptors? Second, why does a sizable fraction of the 111In dose accumulate in the liver and kidneys? The answers to both questions could be applicable to many, if not all, polypeptide radiopharmaceuticals.

The data concerning the fate of somatostatin and its analogues following binding to cell surface receptors is conflicting. Experiments with somatostatin have demonstrated receptor-mediated endocytosis in several (4–8) but not all (9, 10) cell types. The fate of somatostatin analogues following internalization is also unclear. Autoradiographic studies with 125I-somatostatin analogues have found radioactivity in lysosomes (4–6, 8), but biochemical studies have failed to show significant intracellular metabolism (7, 9, 10). However, it remains possible that some of the iodinated somatostatin analogues are rapidly degraded and the resulting moniodotyrosine is rapidly released. Somatostatin analogues like octreotide were designed to resist proteolysis; thus, it is not surprising that octreotide is degraded slowly. Still, 24 h after injection the majority of the urinary radioactivity was not the intact compound but behaved much like 111In-DTPA (11). The site of this metabolism and the enzymes involved are unknown.

We and others have recently shown that a series of 111In-DTPA-polypeptides targeted to hepatocellular receptors were degraded in lysosomes (12, 13). The bonds linking the 111In to DTPA and the chelate to the amino acid backbone were stable (12–14). Thus, lysosomal metabolism of most 111In-DTPA-polypeptides should yield 111In-DTPA-lysine. We also proposed that 111In-DTPA-lysine accumulates within lysosomes because its rate of production greatly exceeds its rate of release (14). In this model, release is slow because 111In-DTPA-lysine cannot use any carrier-mediated transport system to cross the lysosomal membrane. We thus expected that following binding to somatostatin receptors, 111In-DTPA-octreotide is delivered to lysosomes and slowly degraded there. Following degradation, the resulting 111In-labeled metabolites could remain within lysosomes because they also could not use carrier-mediated transport systems for rapid egress.

The fate of molecules that fail to bind to somatostatin receptors is another important question. In vivo experiments have shown that nontargeted molecules like dextran, PVP, sucrose, and proteins accumulate in renal and hepatic lysosomes (15–20). Straus (17–19) injected horseradish peroxidase into rats and by cytochemical staining found that it rapidly accumulated in lysosomes of hepatocytes, sinusoidal cells, Kupffer cells, and proximal renal tubular cells. The uptake mechanisms have subsequently been extensively studied, and the predominant mechanism varies with the cell type. Renal tubular cells recapture proteins from the glomerular filtrate mainly by absorptive endocytosis, and the uptake efficiency correlates with the isoelectric point [see Maack et al. (21) for a review]. Positively charged proteins are bound, internalized, and degraded more efficiently than negatively charged proteins. Renal tubular cells can also accumulate molecules by fluid phase endocytosis (16, 22). Fluid phase endocytosis is a nonspecific process whereby any molecule can be delivered to lysosomes [see Silverstein et al. (23) for a review]. Hepatic cells accumulate horseradish peroxidase (18, 24), PVP (20, 25), sucrose (15, 26), and inulin (27) by fluid phase endocytosis. Scharschmidt et al.
(27) estimated that a hepatocyte internalizes 20% of its volume every hour by fluid phase endocytosis. The internalized molecules can be delivered to lysosomes and are then slowly released.

It is very likely that radiolabeled antibodies and peptides are delivered to renal and hepatic lysosomes by the above mechanisms. Biodistribution studies with radiolabeled antibodies and peptides routinely show substantial hepatic and renal background. However, the concentration of the radiotracer in the liver and kidneys is much lower for iodinated proteins than those labeled using a radiometal and bifunctional chelate (28–37). Possible explanations have included dehalogenation and radiometal transchelation (30, 38, 39), but based on the available data concerning the metabolism of radiolabeled polypeptides and the specificity of lysosomal transporters, we proposed that the disparity results from differences in the rates various metabolites leave lysosomes (12, 14, 40).

To answer these questions, we studied the fate of \(^{111}\text{In}\)-DTPA-octreotide in several tissues including the pancreas, a pancreatic adenocarcinoma known to possess somatostatin receptors, the liver, and the kidneys. To better analyze the fate of nontargeted compounds in the kidney, we also synthesized a low molecular weight \(^{111}\text{In}\)-DTPA-poly(lysine)-biotin compound as a model for the nontargeted fractions of antibody fragments and other small polypeptides. The tissue, cellular, and subcellular distributions of these molecules were analyzed using a series of independent assays. The results affirm the important role lysosomes play in biodistribution of radiolabeled polypeptides, and we propose a model to explain the \textit{in vivo} behavior of polypeptide-based radiopharmaceuticals.

**MATERIALS AND METHODS**

**Materials.** Indium-\(^{111}\text{In}\) (3.8 × 10^6 Ci/mmol) and DTPA-Octreotide were obtained from Mallinckrodt Medical Inc. (St. Louis, MO). The Superose 12 HR 5/30 gel filtration column, PD-10 columns, low molecular weight gel filtration calibration kit, and NAP-5 columns were from Pharmacia (Almeda, CA). Mannose-BSA and galactose-BSA were from E-Y Labs (San Mateo, CA). Immunopure NHS-Biotin was from Pierce (Rockford, IL). Chelex-100, Bio-Rad DC Protein Assay reagents, and BioSpin-6 columns were from Bio-Rad (Richmond, CA). Centricon-10 and Centricon-30 ultrafiltration units were from Amicon (Beverly, MA). Ultrafree-MC 0.22 μm filter units were from Millipore (Bedford, MA). Hematoxylin, antibodies against fluorescein and biotin, and the peroxidase immunohistochemistry staining kit were from Vector Laboratories (Burlingame, CA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Mature female Sprague Dawley rats were purchased from Sasco Co. (Omaha, NE), and 6–8 week old Lewis rats were purchased from Charles Rivers Laboratories (Boston, MA). The rat pancreatic tumor CA20948 was obtained from the tumor bank at Biomeasure, Inc. (Hopkinton, MA).

**Octreotide Biodistribution Studies.** All animal experiments were performed in compliance with the guidelines established by the Washington University Animal Studies Committee. Indium-\(^{111}\text{In}\)-DTPA-d-Phe\(^6\)-octreotide was prepared using a commercial kit, and the radiochemical purity was assessed as described previously (41). Biodistribution studies were performed in male Lewis rats (220–260 g) bearing the rat pancreatic adenocarcinoma CA20948 as described previously (41). Animals were routinely given 10–50 μCi of \(^{111}\text{In}\)-DTPA-octreotide. In one experiment, 1.0 μCi of \(^{111}\text{In}\)-DTPA-octreotide and 250 μg of unlabeled octreotide were coincubated into a Sprague Dawley rat to evaluate whether somatostatin receptors contributed to renal uptake.

**Tissue Section Autoradiography and Pathology.** As part of the biodistribution experiments, tissue samples were removed, weighed, and placed in vials containing 30% (w/v) sucrose in 10% neutral buffered formalin. Samples were routinely fixed for at least 72 h before further processing. Frozen sections (200–400 μm thick) were then prepared using a Microm HM400 microtome equipped with a freezing stage. The sections were mounted on glass slides, covered with clear nail polish to prevent sample dehydration, and exposed to a Molecular Dynamics Storage Phosphor Plate for 5–7 days. The phosphor plate was then scanned with a Molecular Dynamics PhosphorImager SI to obtain digital autoradiographs with either 50- or 200-μm pixel sizes. Reflection images of the sections were obtained using a PowerMacintosh 7100 equipped with a Microtek IHR flatbed scanner and Adobe Photoshop 3.0. These digital images were superimposed on the autoradiographs to best assess which portions of the tissue contained the radiotracer. In some cases, the nail polish was removed using a xylene bath and the section stained with hematoxylin according to the manufacturer’s instructions. Transmission images of the stained sections were recorded on Ektachrome 200 film and digitized using a Polaroid Sprint 35 slide scanner.

After allowing the \(^{111}\text{In}\) to decay to background, specimens were submitted to the Washington University Research Histology laboratory, where 4-μm-thick paraffin sections were prepared. Some of these sections were stained with H&E, whereas others were processed for immunohistochemistry (see below). Paraffin sections were viewed using a Olympus BH-2 microscope equipped with a photomount. Images were recorded on Ektachrome 64 film and digitized as above.

The digital images were color corrected, cropped, labeled, and composed using Adobe Photoshop 3.0 software. The Figs. were printed at 300 dpi using a Kodak ColorEase dye sublimation printer.

**Subcellular Fractionation.** The \(^{111}\text{In}\)-labeled compounds (5–20 μCi/animal) were injected into the animals, and at the indicated time points, the tissues were removed, minced with razor blades, and homogenized in 4 volumes of ice-cold 0.25 m sucrose, 1 mM EDTA. To shift hepatocyte lysosomes, some animals were given chloroquine (two doses of 15 mg each injected into the peritoneal cavity at 100 and 50 min prior to sacrifice). The cells were then disrupted by 5 up-and-down strokes in a Potter-Elvehjem homogenizer. Nuclei and unbroken cells were sedimented by centrifugation at 400 × g for 5 min, and the postnuclear supernatant was transferred to a fresh tube. Calcium chloride was added (1 mM final) to swell mitochondria, and 6 ml of the supernatant were layered onto 30 ml of 37.5% Percoll (v/v), 0.25 m sucrose, 10 mM Hepes, pH 7.4. The gradients were formed by centrifugation for 30 min at 60,000 × g in a Ti70 rotor. Fractions (1.8 ml each) were collected from the bottom of the gradients using a peristaltic pump connected to a fraction collector.

A slightly different protocol was used to analyze the subcellular distribution of \(^{111}\text{In}\)-DTPA-poly(l)-lysine-biotin in the rat kidney. Six ml of the postnuclear supernatant were carefully added to tubes containing 24.5 ml of 27% Percoll (v/v), 0.25 m sucrose, 10 mM Hepes, pH 7.4, which had been layered over 5 ml of 2.5 m sucrose. After centrifugation as above, fractions were collected from the bottom of the gradient.

Unless otherwise specified, all assays were performed in 96-well plates, and results were measured using a Bio-Tek EL-311 microplate reader connected to a Macintosh SE computer equipped with Deltasoft 3.0 software. The lysosomal enzyme β-hexosaminidase was assayed by adding 100 μl of 10 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide dissolved in 100 mM NaOAc, 0.2% Triton X-100, pH 4.1, to wells containing 5 μl of the sample. After incubation at 37°C, the reactions were stopped by adding 100 μl of 1 M NaCO₃, pH 11, and the absorbance difference (405–750 nm) was measured. Control reactions were stopped immediately after the substrate was added. The plasma membrane enzyme alkaline phosphodiesterase was assayed by adding 100 μl of 5 mM thymidine 5'-monophosphate p-nitrophenylester dissolved in 100 mM Tris, pH 8.9, 0.2% Triton X-100 to wells containing 5 μl of the sample. Enzyme activity was measured using the microplate reader in a kinetic mode at 405 nm. The cytosolic enzyme, lactate dehydrogenase, was assayed as described previously (12). Total protein was assayed using the Bio-Rad DC method according to the manufacturer’s instructions. The relative amount of enzyme activity, radioactivity, or protein in each fraction was calculated by summing throughout each gradient and using that as the denominator.

**Preparation of \(^{111}\text{In}\)-DTPA-poly(l)-lysine-biotin.** A small nontargeted polypeptide was prepared by linking a series of reporter molecules to a poly-l-lysine backbone. Briefly, 60 mg of \(M\), 25,900 poly-l-lysine was dissolved in 1 ml Hepes, pH 7.4, to make a 2.5 mg/ml solution. Biotin residues were attached to this backbone by adding 24.4 mg of NHS-biotin dissolved in 1.8 ml of DMSO (an approximately 20-fold molar excess). The reaction was held at room temperature in the dark for 30 min before the addition of 1 ml of a 2 mg/ml solution of DTPA cyclic dihydroxydissolved in DMSO. After a 15-min incubation at room temperature, 500 μl of a 10 mg/ml solution of FITC in DMSO were added, and the mixture was held at room temperature for...
another 15 min. Finally, 2 ml of a 200 mg/ml succinic dihydride solution in DMSO were added and allowed to react for 30 min.

Following the addition of side groups to the poly(D)lysine backbone, the conjugate was concentrated using a Centriprep-10 diafiltration unit according to the manufacturer’s instructions. Following this, the conjugate was centrifuged at 11,000 rpm in a microcentrifuge for 5 min, and no precipitate was observed. A PD-10 gel filtration column was next used to remove any free DTPA, FITC, succinate, or biotin. The column was equilibrated and eluted with 20 mM Hepes, 150 mM NaCl, pH 7.4 (HBS), according to the manufacturer’s instructions. The conjugate was again concentrated using a Centricron-10 ultrafiltration device. The concentrated sample was passed through a 0.22-μm filter to remove any aggregates that formed during concentration.

The DTPA-poly(D)lysine-biotin conjugate was next labeled with 111In using our previously published method (12). Briefly, a 10-μl aliquot (approximately 400 μg) was diluted into 80 μl of 0.1 M NaOAc, 150 mM NaCl, pH 6.0. Between 200 and 350 μCi 111InCl3 in 0.1 N HCl was added, and the mixture was held at room temperature for 40 min. The 111In-DTPA-poly(D)lysine-biotin was separated from the free metal by gel filtration using Biospin-6 columns equilibrated with HBS (20 mM Hepes, 150 mM NaCl, pH 7.4). The efficiency of labeling ranged from 95—97%. The 111In-DTPA-poly(D)lysine-biotin was sized by gel filtration as described previously (12).

The efficiency of biotinylation was assessed by streptavidin-agarose affinity chromatography. The labeled sample was diluted into 200 ml of HBS containing 1.5 mg of BSA as carrier. The sample was applied to a 0.2-ml column of streptavidin-agarose. Binding was determined after washing the column with 1 ml of HBS. Typically, more than 98% of the radioactivity bound to the column.

**RESULTS**

**111In-DTPA-Octreotide Is Delivered to Lysosomes in Target Tissues.** The distribution of 111In-DTPA-octreotide in normal and tumor-bearing rats was first studied at the organ/tissue level. As shown in Fig. 1, the radioactivity rapidly cleared from the blood and accumulated in tumor, pancreas, liver, and kidney. Other studies have indicated that pancreatic and tumor accumulation is mediated by somatostatin receptors because the accumulation was much lower in animals coinjected with a large excess of unlabeled octreotide (41). The results agree well with biochemical and autoradiographic studies that have shown somatostatin receptors in rat pancreas and this tumor line (8, 42–44). In contrast, the hepatic and renal accumulation was also seen in animals coinjected with the large excess of unlabeled octreotide (41, 45), and, thus, uptake was likely independent of somatostatin receptors. We will refer to the pancreas and the pancreatic adenocarcinoma as target tissues. The liver and kidneys are termed nontarget tissues.

This distribution of radioactivity within the target tissues was further evaluated by tissue section autoradiography (Fig. 2). The distribution of the radiotracer was correlated with images of the same

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![Fig. 1. Octreotide biodistribution. Tumor-bearing Lewis rats were injected with 111In-DTPA-octreotide, and the tissue distribution was determined at 1 (○) and 20 (■) h as described in “Materials and Methods.” Three animals were used for each time point, and results are expressed as % initial dose per gram (A) and % initial dose per organ (B).](image-url)
tissue section before and after each was stained. The stained sections (Fig. 2C) more clearly define the boundary between the tumor and the surrounding connective tissue, but the staining protocol also introduced dehydration artifacts. By comparing these three sets of images, it was clear that the majority of the radiolabel lies in the tumor. Within the tumor, the slight heterogeneity results from numerous small vascular spaces (data not shown).

The autoradiographs of the pancreas showed a uniform distribution of the radiotracer (data not shown). The subcellular distribution was assessed by subcellular fractionation. These studies found a substantial fraction of the radiotracer in the dense portion of the gradients at both 1 and 20 h (Fig. 3). The same fractions contained a nearly equivalent fraction of total lysosomal enzyme activity. The remainder of the radioactivity and lysosomal enzyme activity were distributed throughout the gradient in a roughly parallel fashion. The broad lysosomal enzyme peak likely reflects the heterogeneity of lysosomes in the various cells of the pancreas. The presence of both radioactivity and lysosomal enzymes in the uppermost portion of the gradient likely results from breakage of lysosomes during the homogenization procedure. The slight difference in the distribution of the lysosomal enzyme between 1 and 20 h could reflect small differences in homogenization, which lysed more of the lysosomes in the 1-h sample. It is also possible that some of the radioactivity in the upper portion of the gradient represents molecules bound to somatostatin receptors on the cell surface and in the endocytic apparatus. However, the finding that the majority of the radioactivity did not copurify with the plasma membrane marker suggests that the 111In-DTPA-octreotide does not remain at the cell surface after binding to somatostatin receptors. The lack of a major peak at intermediate densities typical of endosomes indicates that the radioactivity does not accumulate in the endocytic apparatus, although the slight discrepancy between the distribution of the 111In and lysosomal marker at 1 h could result from 111In-DTPA-octreotide in both dense endosomes and lysosomes. The better correlation seen at 20 h supports this possibility.

A similar pattern was seen with the pancreatic adenocarcinoma (Fig. 4). Again, the bulk of the radiotracer was found in the dense portion of the gradient. The same fractions also contained the majority of the lysosomal enzyme. The lack of a major peak copurifying with the plasma membrane marker suggests that the neoplastic cells rapidly internalized 111In-DTPA-octreotide and delivered it to lysosomes.

111In-DTPA-Octreotide Is Delivered to Renal and Hepatic Lysosomes. In the kidney, tissue section autoradiography demonstrated that the 111In was concentrated in the renal cortex (Fig. 5). The same pattern was observed at both the 1- and the 20-h time points (data not shown). The cortical uptake was not blocked in animals in which an excess of unlabeled octreotide was coinjected (Fig. 5, C and D). This result suggests that the cortical accumulation was independent of somatostatin receptors. Bakker et al. (46) also found that octreotide accumulated in the renal cortex of Wistar rats. Ligand binding studies with tissue sections failed to detect somatostatin receptors, and they suggested that the cortical radioactivity resulted from either fixation in the proximal tubules during reabsorption or tubular excretion.

The distribution was next assessed by subcellular fractionation. As shown in Fig. 6, the radiotracer was found concentrated in the dense portion of the gradient. The same fractions contained a nearly identical fraction of a lysosomal enzyme but only a minority of the total protein. The major radioactivity peak was also well separated from the plasma membrane marker. The radiotracer copurified with lysosomes and this pattern did not change significantly between 1 and 20 h. A second peak containing both radioactivity and the lysosomal enzyme was found near the top of the gradient. This likely results from some breakage of lysosomes during the homogenization procedure because it is difficult to prepare renal homogenates without breaking some lysosomes (47). These results suggest that the observed cortical activity results from tubular reabsorption of octreotide from the glomerular filtrate and retention of the radiolabel in lysosomes.

In the liver, the radiotracer was also found concentrated in the dense portion of the gradient (Fig. 7). The same fractions contained the bulk of the lysosomal enzyme. The radioactivity peak was well separated from a plasma membrane enzyme and the majority of cellular proteins. At 1 h, a small amount of 111In (22%) was found near the top of the gradient, and this broad peak decreased by 20 h. This radioactivity likely represents residual blood pool activity because the liver-to-blood ratio was only 1.6 at 1 h. These results suggest that the majority of the hepatic 111In activity lies within lysosomes.

The mechanism of 111In octreotide accumulation by the liver has been unclear. It is possible that the observed accumulation in hepatic lysosomes could simply result from fluid phase endocytosis. Because fluid phase endocytosis is nonspecific, lysosomal 111In concentrations should lag behind plasma 111In concentrations at early time points.
Fig. 3. Subcellular fractionation of the pancreas. Tumor-bearing rats were sacrificed at 1 (A and C) or 20 (B and D) h after the injection of \(^{\text{111}}\text{In-DTPA-octreotide. The pancreas was removed, homogenized, and fractionated on 37.5% Percoll gradients as described in "Materials and Methods." Fractions were collected from the bottom of the gradient and assayed for radioactivity (•); a lysosomal enzyme, \(\beta\)-hexosaminidase (○); a plasma membrane enzyme, alkaline phosphodiesterase (□); and total protein (■). The distribution of the lysosomal enzyme and radioactivity are shown separate (A and B) from the distribution of the total protein and plasma membrane marker (C and D) to improve clarity.

This agrees well with the biodistribution data shown in Fig. 1. However, the biodistribution data indicates that at later time points, hepatic \(^{\text{111}}\text{In concentration far exceeds plasma }^{\text{111}}\text{In concentration. Such a disparity could result from large differences in the rates of }^{\text{111}}\text{In egress from the blood and lysosomes. Indium-111-DTPA-octreotide is rapidly cleared from the blood by glomerular filtration. The }^{\text{111}}\text{In can leave hepatic lysosomes by any of three possible routes: carrier-mediated transport, diffusion, or fluid phase vesicular transport. Our recent results with }^{\text{111}}\text{In-DTPA-poly(D)lysine-galactose, which is targeted to hepatocyte lysosomes, indicates that fluid phase vesicular transport is the dominant mechanism for egress from hepatic lysosomes.}

Fig. 4. Subcellular fractionation of the tumor. Tumor-bearing rats were sacrificed at 1 (A and C) or 20 (B and D) h after the injection of \(^{\text{111}}\text{In-DTPA-octreotide. The tumor was removed, homogenized, and fractionated on 37.5% Percoll gradients as described in "Materials and Methods." Fractions were collected from the bottom of the gradient and assayed for radioactivity (•); a lysosomal enzyme, \(\beta\)-hexosaminidase (○); a plasma membrane enzyme, alkaline phosphodiesterase (□); and total protein (■). The distribution of the lysosomal enzyme and radioactivity are shown separate (A and B) from the distribution of the total protein and plasma membrane marker (C and D) to improve clarity.
transport from lysosomes to the cell surface is quite slow.4 Our prior studies have also suggested that 111In-labeled metabolites are poor substrates for lysosomal egress by carrier-mediated transport and transmembrane diffusion (12, 14). Because water and other solutes can rapidly leave lysosomes, the trapped 111In becomes more concentrated. Thus, at later time points, the blood to liver 111In concentration difference results from 111In retention in hepatic lysosomes. Munnikma et al. (20) studied the biodistribution of a known fluid phase endocytosis tracer and found the same dynamic relationship between blood and liver concentrations.

If the liver does accumulate octreotide by fluid phase endocytosis, we expected that it should accumulate predominantly in hepatocyte lysosomes because hepatocytes are the most common cell type in the liver, and this factor far outweighs any cell-to-cell differences in the rates of fluid phase endocytosis (20, 25, 48, 49). Hepatocyte lysosomes were separated from Kupffer and sinusoidal cell lysosomes by a chloroquine density shift technique (50). A hepatocyte-specific marker, 111In-DTPA-albumin-galactose, showed a substantial shift with chloroquine treatment (Fig. 8). In contrast, a Kupffer and sinusoidal cell marker, 111In-DTPA-albumin-mannose, showed a lesser shift. With chloroquine treatment, 111In-DTPA-octreotide shifted substantially, and the pattern was nearly identical to that seen with the hepatocyte-specific marker. This indicates that the radiolabel from 111In-DTPA-octreotide resides predominantly in hepatocyte lysosomes. This result supports our hypothesis that octreotide accumulates in the liver by fluid phase endocytosis.

**Synthesis and Characterization of a Model 111In-DTPA-Polypeptide.** To determine which cells types were responsible for the renal accumulation of 111In-DTPA-polypeptides, a model compound was prepared and characterized. The model compound was based on a poly(ol)lysine backbone to prevent lysosomal proteolysis. Reporter molecules were attached to this backbone via the ε-amines. Indium-111 was linked to this backbone using DTPA cyclic dihydride as a bifunctional chelate. The 111In served as a reporter group for the biodistribution, stability, and subcellular fractionation studies. Biotin was added to the ε-amines and used as a reporter group for the stability and immunohistochemical studies. Fluorescein was used as a reporter group to determine yields during synthesis and purification. Because positively charged compounds such as poly(D)Lysine can bind nonspecifically to tissue culture cells (51, 52), the remaining amines were succinylated. This step effectively converted most of the remaining positively charged amines to negatively charged carboxylic acids. The resulting compound showed essentially no nonspecific binding to a human hepatoma cell line in vitro (data not shown).

The length of the poly-D-lysine backbone was chosen so that following attachment of the various reporter molecules, the resulting compound would be similar in size to F(ab)2 fragments and genetically engineered single-chain antigen-binding proteins (53) and so that like those proteins, it would undergo glomerular filtration. Gel filtration of the 111In-labeled conjugate demonstrated a peak centered just after the BSA peak (data not shown). Albumin served as a convenient standard because its size and charge exclude it from passing through the glomerular pores. Because gel filtration separates compounds on the basis of their hydrodynamic (Stokes) radius, compounds that elute after albumin have a smaller Stokes radius than albumin. Although glomerular filtration is affected by factors other than size, we expected that this 111In-DTPA-poly(D)Lysine-biotin compound, which had a Stokes radius equivalent to a Mr 55,000 protein would readily undergo glomerular filtration.

The Model Compound Accumulates in Renal Lysosomes. Biodistribution studies indicated that the model compound was rapidly cleared from the blood and accumulated predominantly in the kidney (Fig. 9A). This result is similar to that seen with other low molecular weight polypeptides labeled with metal chelates (29, 32). To ascertain whether the renal uptake was mediated by absorptive or fluid phase endocytosis, a blocking experiment was performed (Fig. 9, B and C). Renal 111In content decreased when 111In-DTPA-poly(D)Lysine-biotin was coinjected with a large excess of the unlabeled compound. This decrease was accompanied by a large increase in urinary 111In excretion. The inability to completely block the renal uptake likely reflects the high capacity of the uptake system and nonspecific uptake by fluid phase endocytosis. This result agrees well with the work of Behr et al. (54, 55), who recently showed that the renal uptake of 111In-DTPA-polypeptides can be partially inhibited by coadministering large doses of polylysine or lysine. Other groups have shown that the renal uptake of other antibodies can be partially inhibited by coinjecting large amounts of lysine (56, 57).

The distribution of the radiotracer within the kidney was determined by autoradiography, biochemical studies, and subcellular fractionation. Autoradiography demonstrated cortical accumulation of the radiotracer (data not shown). Analysis of renal lysates showed that over 84% of the 111In activity was still bound to a streptavidin-agarose affinity column. This result indicated that the bonds linking 111In to the biotin residues were resistant to degradation, and this fact allowed evaluation of the distribution of the agent at the microscopic level with immunohistochemical techniques. These studies found that the compound accumulated within the proximal renal tubular cells (Fig. 10).

The reaction product of the immunohistochemical reaction was found predominantly in cortical tubules (Fig. 10B). Little or no staining was observed in glomeruli or blood vessels. Also, little or no staining was observed in control sections (Fig. 10, C and D). The cortical staining agrees well with the autoradiography results of Bakker et al. (46), who studied 111In-DTPA-octreotide, and Yokota et al. (58), who studied the distribution of antibody fragments in the kidney. At higher magnifications (Fig. 10, E and F), the intensely stained tubules could be identified as proximal convoluted tubules based on morphological criteria. These criteria include columnar cell shape,

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Fig. 6. Subcellular fractionation of the kidney. Tumor-bearing rats were sacrificed at 1 (A and C) or 20 (B and D) h after injecting $^{111}$In-DTPA-octreotide. The kidney was removed, homogenized, and fractionated on 37.5% Percoll gradients as described in “Materials and Methods.” Fractions were collected from the bottom of the gradient and assayed for radioactivity (●); a lysosomal enzyme, β-hexosaminidase (□); a plasma membrane enzyme, alkaline phosphodiesterase (■); and total protein (●). The distribution of the lysosomal enzyme and radioactivity are shown separate (A and B) from the distribution of the total protein and plasma membrane marker (C and D) to improve clarity.

Fig. 7. Subcellular fractionation of the liver. Tumor-bearing rats were sacrificed at 1 (A and C) or 20 (B and D) h after the injection of $^{111}$In-DTPA-octreotide. The liver was removed, homogenized, and fractionated on 37.5% Percoll gradients as described in “Materials and Methods.” Fractions were collected from the bottom of the gradient and assayed for radioactivity (●); a lysosomal enzyme, β-hexosaminidase (□); a plasma membrane enzyme, alkaline phosphodiesterase (■); and total protein (●). The distribution of the lysosomal enzyme and radioactivity are shown separate (A and B) from the distribution of the total protein and plasma membrane marker (C and D) to improve clarity.

abundant cytoplasm, relative frequency, well-developed microvilli, tubular, and luminal diameters (59). At the highest magnifications, the staining was found to be heterogeneous within each cell. Such a pattern suggests that the antigen is localized within subcellular organelles. For the 1 h time point, a small amount of apical staining was occasionally observed (Fig. 10E). This included staining of the microvilli, as well as a line of staining near the base of the microvilli. Such a pattern agrees well with the earlier work of Straus (17) and
Maunsbach (60), who found tracers initially in the apical portions of proximal convoluted tubular cells. The apical staining was not seen at the 20-h time point (data not shown). At both the 1- and the 20-h time points, the staining was predominantly in the basolateral portion of the cells, consistent with the location of secondary lysosomes.

The subcellular localization of the $^{111}$In-DTPA-poly(d)Lys-biotin was next determined by subcellular fractionation. As shown in Fig. 11, the distribution of $^{111}$In paralleled the distribution of the lysosomal enzyme. Both the lysosomal enzyme and the $^{111}$In radiolabel were recovered in the dense portion of the gradient, and these were separated from cytosolic and plasma membrane proteins. A second peak was found at the top of the gradient, and it likely resulted from disruption of lysosomes during the homogenization procedure. Together with the immunohistochemical results, these data indicate that the $^{111}$In-DTPA-poly(d)Lys-biotin was rapidly transported to lysosomes in the proximal convoluted tubule.

**DISCUSSION**

To better understand the factors that govern the target-to-background ratios of radiolabeled polypeptides, we have studied the fate of both the targeted and nontargeted fractions of $^{111}$In-DTPA-octreotide and a model nontargeted compound. Our results clearly demonstrate that lysosomes play an important role in determining this balance.

The subcellular fractionation experiments indicate that in tissues bearing somatostatin receptors (pancreas and a pancreatic adenocarcinoma), the $^{111}$In-DTPA-octreotide does not remain at the cell surface. Rather, it is rapidly internalized and delivered to lysosomes. Breeman et al. (45) concluded that $^{111}$In-DTPA-octreotide is rapidly internalized after conducting a series of biodistribution studies in normal Wistar rats. They found that the distribution of $^{111}$In-DTPA-octreotide was not altered by injecting a 4–20-fold excess of octreotide or pentetreotide 20 min later. If the $^{111}$In-DTPA-octreotide remained bound to cell surface receptors, one would have expected to see some displacement by the unlabeled somatostatin analogues. In humans, two groups have now reported that $^{111}$In-DTPA-octreotide imaging is not compromised but rather is improved by concurrent therapy with unlabeled octreotide (61, 62). The likely explanation is that receptors that bind octreotide are rapidly replaced by receptors, the binding sites of which are unoccupied. The finding by Hofland et al. (7) that octreotide is rapidly internalized and can up-regulate somatostatin receptors suggests that receptor up-regulation could explain the increased target-to-background ratios seen in these patients.

On the basis of our results, we propose that $^{111}$In-DTPA-octreotide is delivered to lysosomes by receptor-mediated endocytosis. At present, it is unclear whether the somatostatin receptors recycle to the cell surface or are degraded in lysosomes along with their ligands. Many hormone receptors do not recycle (63), but the up-regulation of receptors by octreotide indicates that either the receptors recycle or cells rapidly synthesize new receptors to replace those that are degraded in lysosomes. One could potentially distinguish between these
The results of Bakker et al. (46), who found the possibilities by measuring uptake after inhibiting protein synthesis, as was done for the asialoglycoprotein receptor (64).

Our results also indicate that a sizable fraction of the $^{111}$In-DTPA-octreotide that failed to bind to somatostatin receptors was delivered to hepatic and renal lysosomes. In the kidney, the results suggest the following scheme: the $^{111}$In-DTPA-octreotide passes through the glomerulus and binds, albeit inefficiently, to the brush border in the proximal renal tubule; it is then rapidly internalized and delivered to lysosomes. Studies that indicate that $^{111}$In-DTPA-octreotide is degraded in the kidney and other tissues and that lysosomal enzymes can degrade $^{111}$In-DTPA-octreotide (65) are consistent with this model. The results of Bakker et al. (46), who found the $^{111}$In concentrated in renal cortical tubules, are also consistent with this scheme. The stability and immunohistochemical studies with $^{111}$In-DTPA-poly(lysine)-biotin also support this scenario.

Hepatic uptake and delivery to lysosomes likely results from fluid phase endocytosis. Receptor-mediated endocytosis is unlikely because hepatic uptake is not blocked by excess octreotide. Absorptive endocytosis is possible, but aside from the polyligand hepatic receptors like the scavenger receptor found on Kupffer and sinusoidal cells (66), we do not know of any broad specificity uptake mechanism that parallels that found in the kidney. Measurements of hepatic fluid phase endocytosis in vivo (20) indicate that it could easily account for the observed 1.8 and 0.9% of the initial dose that we found in hepatocyte lysosomes 1 and 20 h after injection.

The finding that $^{111}$In-DTPA-octreotide is rapidly internalized and delivered to lysosomes in target and nontarget tissues has several important implications. First, it illustrates that cellular physiology must be considered when evaluating biodistribution results. Radiolabel concentration in any tissue depicts the flux through multistep/multicompartment pathways where the rates of delivery to and egress from various compartments, including subcellular organelles, and reaction rates in the various environments all contribute to the observed results. Thus, the radiolabel concentration of a tissue will not be governed by simple binding constants, as predicted by some (39, 67), but rather by rate-determining steps (68, 69). Although receptor binding may be a rate-determining step, other possible rate-determining steps include the rates of receptor recruitment from intracellular pools, intracellular metabolism, and radiolabel release. Second, based on the lysosomal metabolism of other $^{111}$In-DTPA-polypeptides (12–14, 40), we predict that the $^{111}$In-DTPA and DTPA-(D)Phe bonds are stable. Because $^{111}$In-DTPA-octreotide is degraded to the same metabolite in a variety of tissues and because lysosomal enzymes can degrade $^{111}$In-DTPA-octreotide (65), we expect that the likely metab-

![Graph](image1)

**Fig. 9.** Biodistribution of $^{111}$In-DTPA-poly(lysine)-biotin. In A, Sprague Dawley rats were injected with a mixture of $^{111}$In-DTPA-poly(lysine)-biotin and unlabeled In-DTPA-poly(lysine)-biotin, and the tissue distribution was determined at 1, 4, and 20 h (C, E, and F, respectively) as described in "Materials and Methods." In B and C, Sprague Dawley rats were injected with $^{111}$In-DTPA-poly(lysine) alone (tracer) or with a mixture of $^{111}$In-DTPA-poly(lysine)-biotin and unlabeled In-DTPA-poly(lysine)-biotin (+cold). The kidneys (B) were removed at 4 h, and the urine (C) was collected during the entire 4 h experiment. Three animals were used for each time point.

![Graph](image2)

**Fig. 10.** Renal immunohistochemistry. Sprague Dawley rats were injected with a mixture of $^{111}$In-DTPA-poly(lysine)-biotin/FITC, and the kidneys were removed 1 h later. After the $^{111}$In was allowed to decay to background, paraffin sections were prepared and probed for In-DTPA-poly(lysine)-biotin/FITC using antibody and antifluorescein antibodies and counterstained with hematoxylin as described in "Materials and Methods." The peroxidase/diaminobenzidine reaction product is seen as a brown precipitate, and the nuclei were stained blue. A, the brown precipitate is seen in the renal cortex (RC) but not the medullary ray (MR), outer medulla (OM), or inner medulla (IM). B and F, staining is seen in the proximal convoluted tubule (PCT) but not the glomeruli (GL). E and F, the staining within tubular cells is predominantly within basolateral structures (vertical arrows), but occasional apical staining was seen (horizontal arrows). C and D, an excess of free biotin was added during the incubation with avidin-peroxidase complexes. Approximate magnifications are shown in the upper right corner of each panel.
olites are $^{111}$In-DTPA-(D)Phe or $^{111}$In-DTPA-(D)Phe-Cys. We expect that these metabolites leave lysosomes slowly because they are not substrates for carrier-mediated transport. The combination of receptor-mediated endocytosis and radiolabel retention in lysosomes suggests that tumors with somatostatin receptors could accumulate large amounts of radiolabeled metabolites in their lysosomes. Although internalization and lysosomal retention of the radiolabel make targeted radiotherapy with somatostatin analogues more attractive (70), therapies will also have to address radiolabel accumulation in normal and nontarget tissues. Radiotherapy will also be limited by the short range of the Auger electrons that are emitted in the decay of $^{111}$In. Due to their short range, the Auger electrons will be most effective when $^{111}$In reaches the nucleus. Although we did not determine the amount of $^{111}$In in the nucleus, our results indicate that lysosomes contain the majority of the activity in somatostatin receptor positive tissues. This suggests that $^{111}$In may not be the ideal isotope for radiotherapy.

On the basis of our results, we propose the model shown in Fig. 12 to explain the biodistribution patterns observed with polypeptide-based radiopharmaceuticals. This model predicts that following injection, a small fraction of the molecules will accumulate in the target tissue. This accumulation will be limited by factors that include concentration of available target sites, agent concentration in this local environment, and the kinetics of target binding and dissociation. Agent concentration at the target site will in turn depend on blood flow, capillary permeability, interstitial pressure, and attrition from competing pathways or competing binding sites (e.g., the binding site barrier; Refs. 71–76). After binding to the target, the agent will be internalized and eventually delivered to lysosomes. The rate of internalization will vary depending on the cell surface target and the radiolabeled agent. Agents targeted to receptors that undergo receptor-mediated endocytosis will be rapidly internalized. Agents targeted to other cell surface molecules will be internalized at a lower rate.

The fate of molecules that fail to bind to the target depends primarily on glomerular filtration. Molecules that pass through the glomerular filter become candidates for delivery to renal lysosomes by absorptive endocytosis. The efficiency of renal tubular capture depends in part on the density of positive charges (21). Thus, whereas most polypeptides are readily delivered to lysosomes, small polypeptides, such as octreotide, that contain only a single positive charge may largely escape renal tubular recapture. Although some peptides, such as the enkephalins, are degraded to amino acids by proteases within the renal tubular brush border (77), polypeptides like octreotide are resistant to such degradation and are thus recovered intact from the urine (11). This model outlines a series of competing pathways, and the bulk of any radiolabeled polypeptide will follow the path of least resistance. Thus, if the radiolabeled polypeptide cannot pass through the glomerular filter, it will circulate for an extended time, and this will provide ample opportunity for hepatic uptake.

Hepatic uptake can occur by receptor-mediated endocytosis, absorptive endocytosis, and fluid phase endocytosis (23). Because fluid phase endocytosis is nonspecific, we predict that every compound will be cleared from the blood at a basal rate that represents hepatic fluid phase endocytosis. Using $^{125}$I-PVP to measure fluid phase endocytosis, Munnikinsma et al. (20) determined that the hepatic lysosomes contained nearly 8% of the initial dose at 24 h. This figure slightly underestimates hepatic fluid phase endocytosis because a fraction of the $^{125}$I-PVP was cleared by glomerular filtration. Still, this 8% figure is very similar to the hepatic accumulation of $^{111}$In-antibodies seen in normal and nontarget tissues. Radiotherapy will also be limited by the glomerular filter, which serves as a barrier to the delivery of radiolabeled polypeptides to the target tissue. This accumulation will be limited by factors that include concentration of available target sites, agent concentration in this local environment, and the kinetics of target binding and dissociation.
after blocking any receptor-mediated or absorptive uptake by coinjecting a large excess of the unlabeled antibody (78). Hepatic uptake would exceed 8% of the initial dose whenever other uptake pathways come into play.

The fate of the radiolabel after uptake and lysosomal delivery will be addressed in another report. Briefly, once delivered to lysosomes, radiolabel concentrations will depend on the rate of lysosomal degradation and egress of the low molecular weight metabolites. Some radiolabeled metabolites, such as moniodotyrosine, can rapidly leave lysosomes by carrier-mediated transport (79). Radiometal-chelate polypeptides can be degraded to radiometal-chelate amino acids(s), and the available data indicate that with a few exceptions, these metabolites are poor substrates for the carrier-mediated transport systems found in lysosomes. Although these metabolites can leave lysosomes by other routes, these alternate routes are slow. Thus, the metabolites containing the radiometal accumulate in lysosomes.

Because radiolabel concentration at any site reflects the balance between rates of radiolabel uptake and release, this model suggests that the target-to-background ratio could be increased by manipulating these rates. It is therefore important to delineate the rate-determining steps of uptake and release in target and nontarget tissues. For example, a radiometal or residualizing iodinated label would lower the rate of radiolabel release from the target tissue (80–84). Blocking absorptive endocytosis in the proximal renal tubule with large doses of positively charged amino acids or polymers (54, 55) would lower renal background. We also expect that blocking strategies might also lower hepatic uptake, but fluid phase endocytosis will always result in some hepatic background. Others have investigated “metabolizable chelates” as a means of facilitating release from nontarget tissues (85–89). These chelates have not succeeded because the rate-limiting step in release is not production of a low molecular weight metabolite but rather its passage through cell membranes. One could potentially use one of the numerous transmembrane transporters found in hepatocyte (90) and renal tubular (91) cells to lower renal and hepatic background. Rapid release from the target tissue could be avoided by designing compounds the radiolabeled metabolites of which would be substrates for the transporters that are found only in hepatocytes and renal tubular cells. Possible candidates are proteins labeled using EDTA-benzyl-isothiocyanate and DTPA-benzyl-isothiocyanate as bifunctional chelates. Arano et al. (13) have shown that 111In-EDTA-benzyl-lysine is rapidly released from hepatocytes but not Kupffer and sinusoidal cells. Esteban et al. (38) and Kimura et al. (92) have presented results that suggest that 111In-DTPA-benzyllysine should be cleared rapidly from hepatocytes. The next steps will be to determine whether these metabolites are rapidly released from renal tubular cells and to further characterize their transport.

It is obvious that a more detailed understanding of the radiotracer physiology at the molecular level is needed. It has always been tempting to bypass such studies and to instead continue searching for new agents and new radiolabeling methods. However, such approaches will likely continue to yield disappointing results. Although elucidating mechanisms is slow and exploiting them problematic, it is the best long-term strategy.

NOTE ADDED IN PROOF

Andersson et al. (J. Nucl. Med., 37: 2002–2006, 1996) recently reported that primary cultures of human neoplasms internalized 111In-DTPA-octreotide in vitro. A single image from their autoradiographic studies shows grains over cytoplasmic granules which could represent lysosomes. These results agree well with our data. They also reported that a small fraction of the grains were seen over the nucleus. Although we cannot exclude the possibility that a small fraction of the 111In accumulates in the nucleus, it is difficult to reconcile this observation with the data regarding the stability of the 111In labeled metabolites in the lysosome (12–14).

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Indium-111-Diethylenetriaminepentaacetic Acid-Octreotide Is Delivered in Vivo to Pancreatic, Tumor Cell, Renal, and Hepatocyte Lysosomes

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