In Vivo Cellular Distribution and Endocytosis of the Somatostatin Receptor–Ligand Complex

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Radioactive tumor targeting agents are highly interesting and for treatment of neuroendocrine tumors expressing somatostatin receptors, radiolabeled somatostatin analogues (including [^{111}In-DTPA-D-Phe^1]-octreotide) has been tried in a small number of patients with encouraging results. To increase our knowledge about the in vivo processing of administered [^{111}In-DTPA-D-Phe^1]-octreotide we have examined tumor and normal tissue material from a patient with a midgut carcinoid tumor. By ultrastructural autoradiography, silver grains indicating the presence of [^{111}In-DTPA-D-Phe^1]-octreotide could be identified within tumor cells, both in the primary tumor and in the mesenteric metastases. Silver grains were also found in leukocytes and in blood vessels. However, normal enterocytes did not show any specific radioligand uptake. This study indicates that the binding and endocytosis of [^{111}In-DTPA-D-Phe^1]-octreotide is a specific process that takes place in cells expressing somatostatin receptors. However, the importance of the number of somatostatin receptors and subtypes expressed will have to be further studied.

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Somatostatin is a peptide hormone which exerts its action on many different tissues and cells including the central and peripheral nervous system, endocrine cells and immune cells (1, 2). The substance acts as an inhibitor of a wide variety of functions such as endocrine and exocrine secretion, intestinal movements and cell proliferation (1, 3). Somatostatin also functions as a neurotransmitter (4) and binds with high affinity to specific receptors present in the cell membrane. These somatostatin receptors (sst) belong to the seven-transmembrane receptor family and, up until now, five different subtypes have been cloned (5–7).

Somatostatin analogues are used to treat patients with hormone-producing neuroendocrine tumors. Subcutaneous treatment induces a decrease in hormone levels and a concomitant relief of symptoms (8). The effect of administered analogues seems mainly to be mediated via binding to sst_2 through the blocking of Ca^{2+}-channels (3, 9). Other functions designated to somatostatin are probably mediated through other receptor subtypes, and consequently, involve other second messengers.

Somatostatin analogues have been used to visualize sst-expressing tumors by using the radioactive compound [^{111}In-DTPA-D-Phe^1]-octreotide (10, 11). The octreotide molecule binds with high affinity to sst subtypes 2, 3 and 5 and tumors with expression of these receptor subtypes are easily demonstrated at scintigraphy. However, about 20% of patients with a positive sst scintigraphy do not respond to somatostatin analogue treatment. This might be due to the fact that these patients do not express sst_2 but one of the other sst subtypes that bind the analogue but do not transmit the message to inhibit hormone secretion (9, 11).

Recently, it has been suggested that [^{111}In -DTPA-D-Phe^1]-octreotide can be used to treat patients with sst-expressing tumors. The initial results are promising with objective responses in several of the patients treated (12). However, in order for [^{111}In-DTPA-D-Phe^1]-octreotide treatment to be successful, the tumor targeting agent has to be internalized into the cell interior. ^{111}In emits gamma rays which have too high an energy to cause tissue damage. Instead, the therapeutic effect has been designated to Auger electrons also emitted from the ^{111}In radionuclide. The Auger electrons have a very short range of about 100 nm but are very effective and one single decay may cause several DNA double-strand breaks (13).

The process of internalization is probably a matter of receptor-ligand endocytosis. It has been shown that in transfected CHO-K1 cells the receptor is internalized dif-

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ferently according to the sst subtype of the receptor (14). It was shown that, after ligand binding, sst_3 and sst_5 were internalized by more than 50% while sst_2 internalizes to only about 20%. Therefore the amount of internalized ligands is dependent on the subtype and number of sst expressed on the surface of the cells composing each individual tumor.

Most endocytotic studies are carried out as in vitro experiments on cell cultures. However, the in vivo processes are much more complex. We therefore developed a method to study in vivo ligand binding and internalization. The method made it possible to evaluate to what degree the receptor-ligand complex is internalized. The distribution of [^{111}In-DTPA-D-Phe^1]-octreotide in cells apart from the neuroendocrine tumor cells can also be revealed. In this report we concentrate on the process of in vivo endocytosis and the presence of [^{111}In-DTPA-D-Phe^1]-octreotide in non-tumor cells.

MATERIAL AND METHODS

Patient

A patient with a malignant midgut carcinoid tumor was scheduled for abdominal surgery. Two days before surgery the patient was injected with [^{111}In-DTPA-D-Phe^1]-octreotide and one day before surgery an sst scintigraphy was performed showing pathological radionuclide accumulation in the central part of the abdomen indicating the primary tumor and the mesenteric metastasis (Fig. 1).

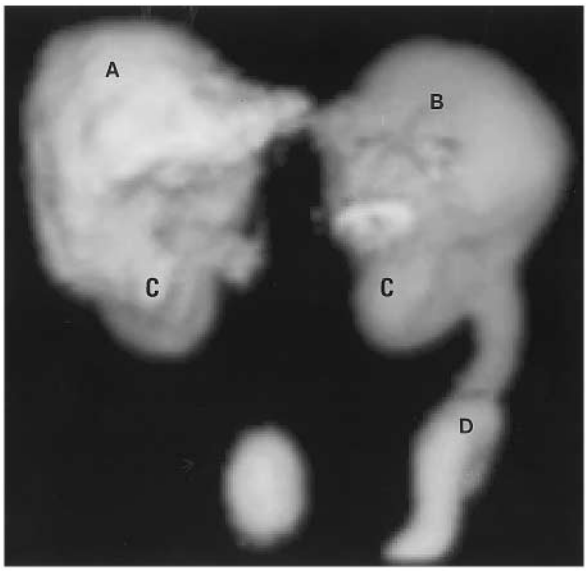

Fig. 1. A three-dimensional rendered SPECT somatostatin receptor scintigraphy image collected 24 h post injection. Pathological accumulation of [^{111}In-DTPA-O-Phe^1]-octreotide is shown in the central part of the abdomen, indicating the presence of the primary midgut carcinoid tumor and the mesenteric metastasis. A: liver; B: spleen; C: kidney; D: activity in the left colon.

Surgery

The patient was operated on and a primary carcinoid tumor and a mesenteric metastasis were removed. The histopathological examination showed a classical midgut carcinoid tumor and immunohistochemical staining for chromogranin A was positive. Adjacent normal intestinal tissue was also collected. Biopsies from the removed tumor and metastasis (150 mg each) were collected together with 200–300 mg adjacent normal intestinal and mesenteric tissue. All tissue specimens were blotted dry and weighed. These samples, together with peripheral blood (1.2 ml), were analyzed in a scintillation wellcounter (LKB Wallac, 1282 CompuGamma, Turku, Finland) calibrated for ^{111}In. Attempts were made to standardize specimen geometry. The tumor to blood and normal tissue to blood radioactivity ratios were calculated with standardization for weight.

Somatostatin receptor scintigraphy

The sst scintigraphy was performed as described earlier (15). Briefly, the patient was injected intravenously with 160 MBq [^{111}In-DTPA-D-Phe^1]-octreotide (Mallinckrodt Medical of Petten, The Netherlands). Anterior–posterior wholebody images were taken the day after injection, and a SPECT (single photon emission computed tomography) was performed over the abdomen using a gamma scintillation SPECT-camera equipped with a medium-energy general purpose collimator (Nuclear Diagnostics, Hägersten, Sweden and London, UK). The collection of original data for the SPECT images was performed using a 64-step rotation of 360° in a 64×64 word matrix. Data were processed in the HERMES® system (Nuclear Diagnostics). Energy windows of 173 and 247 keV ($\pm$ 10%) were used. The collection time for each angle was 40 s, giving a total of about 30000 counts/angle. A Wiener filter was applied to the original data for the reconstruction of SPECT images.

Ultrastructural autoradiography

Immediately after tumor dissection, small, randomly collected, tissue samples were taken and processed for morphological electron microscopy. The tissue samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, supplemented with 0.1 M sucrose for 6 h, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded series of ethanol, immersed in propylene oxide and finally infiltrated with epoxy resin (Agar 100, Agar Aids Scientific, Stansted, UK) followed by polymerization at 60°C. Ultrathin sections (60–80 nm) were placed on Formvar coated gilded grids and contrasted with 4% uranyl acetate for 30 min and Reynolds lead citrate for 3 min. Some grids were left for morphological examination, the rest were used for autoradiographic examination. Ilford L4 nuclear research emulsion (Ilford Anitec, Gothenburg; Sweden) for autoradiography was used after dilution 1:5 in

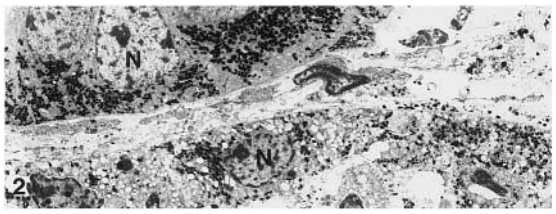

Fig. 2. A morphologic survey of neuroendocrine tumor cells growing in the typical nests. Cells in the upper part of the micrograph show gathering of secretory granules to the basal parts of the cells and round, big nuclei (N) with the spotty pattern of the chromatin. In the lower part, the cells reveal dilated vacuolar structures and nuclei with condensed chromatin. × 8400

distilled water. The emulsion gel was melted and diluted at 43°C. The grids were coated with film using the wire loop technique (16). After drying for 4 h, the grids were placed in a light tight box containing silical gel and placed at 4°C. Exposure time was 4 and 7 days. Development was performed in Kodak D19 for 3 min and fixation in Kodak Unifix for 6 min (Kodak AB, Järfälla, Sweden).

RESULTS

The radioactivity ratios were analyzed in a wellcounter. The tumor to blood ratio was 86.5 for the primary tumor and varied between 13.8 and 173.1 for the metastasis. Measurements for normal tissues were 7.2 for the intestine and 1.8 for mesentery.

The tumor cells grow in nests surrounded by connective tissue, which is characteristic of carcinoids (Fig. 2). The cell/cell contacts were normal and showed membrane interdigitations, desmosomes and tight junctions. A high endocytotic activity was revealed by the high number of clathrine-coated and uncoated vesicles close to the cell membrane. Hormone granules were numerous in cells at the periphery of the nests, and less numerous in the central parts of the nests. The granules were heteromorphous in shape with an amorphous matrix. Many of the tumor cells showed early signs of necrosis, i.e. swollen organelles and irregularly shaped nucleus. The normal intestine tissue was composed of enterocytes of normal appearance.

Binding and internalization of radioactive labeled octreotide was easily revealed by the highly electondense silver precipitations (Ag grains) in the film emulsion. The number of Ag grains slightly increased with exposure duration (4 and 7 days). Very few background or unspecific Ag grains were seen in the material. Control exposure of sections without radioactivity was negative. The morphology of sections covered with emulsion was not as distinct as naked sections, but ultrastructural details were still detectable.

In the primary tumor, Ag grains were located on the plasma membrane and throughout the cytoplasm in almost all cells examined (Figs. 3 and 4). Most Ag grains were located among the granules, many in close proximity

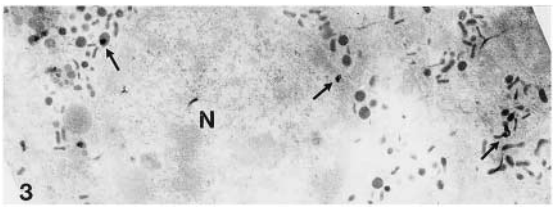

Fig. 3. Micrograph of an autoradiographic section after 4 days of exposure. Ag grains (arrows) representing location of [^{111}In-DTPA-D-Phe^{1}]-octreotide can be seen among the heteromorphous granules in the cytoplasma and in close proximity to the tumor cell nucleus. × 12000.

to the nucleus (Figs. 3 and 5). Solitary cells, or groups of cells, demonstrated a remarkably high number of Ag grains compared with the number in most other cells (Fig. 6).

The amount of Ag grains in the mesenteric metastases was less than that in the primary tumor. Internalized [^{111}In-DTPA-D-Phe^{1}]-octreotide was demonstrated at the same sites as in the primary tumor cells.

The control tissue samples, the normal intestine specimen, also revealed some Ag grains. In these normal cells the [^{111}In-DTPA-D-Phe^{1}]-octreotide reaction product seemed to be unspecific with the few Ag grains found demonstrated in endocytotic vacuoles and at the microvilli striated border (Fig. 7). White blood cells also showed a small number of Ag grains on cell membranes and in their cytoplasm.

DISCUSSION

In the future, development of tumor-targeting agents will be of great importance for the introduction of possible new anti-cancer therapeutic drugs. One such compound is [^{111}In-DTPA-D-Phe^{1}]-octreotide which is used to perform scintigraphic investigations in patients with somatostatin

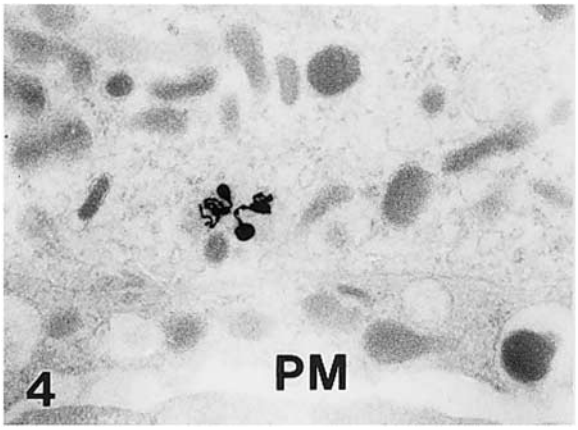

Fig. 4. Detail of Ag reaction products localized in the vicinity of the plasma membrane (PM) of a tumor cell. Note that close to the plasma membrane many vacuoles and tubular systems are seen among the granules. Exp. 4 days. × 36000.

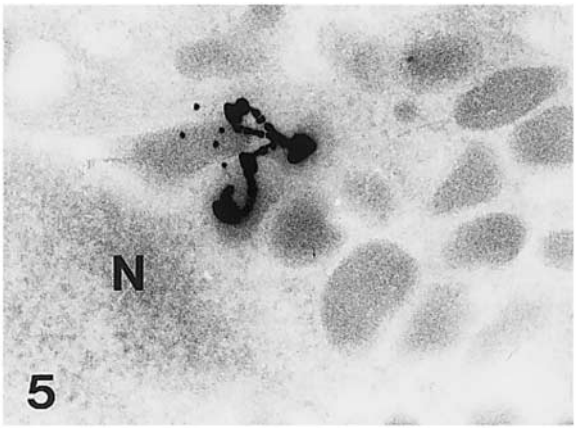

Fig. 5. Detail of Ag reaction products localized in the vicinity of the nucleus (N) where vacuoles are seldom seen. Exp. 7 days $\times$ 54000.

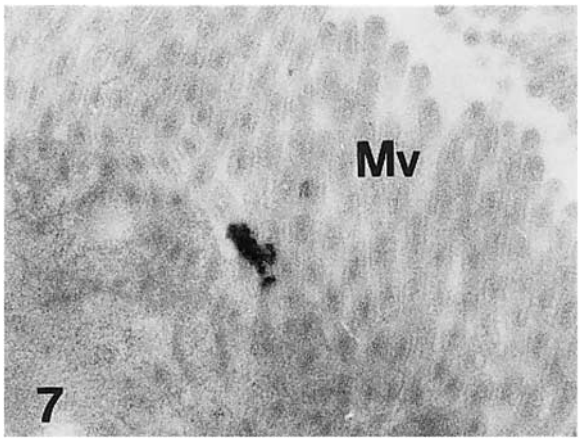

Fig. 7. Detail from an enterocyte in the normal intestine specimen. Occasionally Ag grains were seen on the microvilli (Mv)—striated border—facing the lumen. Exp. 4 days. $\times$ 24000.

receptor-expressing tumors (10, 11). It is known that the sst-ligand complex is internalized and that the amount of internalized ligand depends on the number and subtypes of sst expressed (14).

To be able to understand the biological background for the successful treatment with [^{111}In-DTPA-D-Phe^1]-octreotide, we have studied the radioligand uptake in tumor cells in vivo. We have examined tumor tissue from the primary tumor, mesenteric metastases and normal intestine from a patient with a midgut carcinoid tumor. The patient received a diagnostic dose of [^{111}In-DTPA-D-Phe^1]-octreotide and, after removal of the tumor, the amount of bound and internalized radionuclide was assessed.

Silver grains indicating the presence of [^{111}In-DTPA-D-Phe^1]-octreotide were found in both the tumor cells from the primary tumor and the metastases. In the tumor tissues examined there was a variability in ^{111}In uptake between different tumor cells. Reaction products were seen in all tumor cells, but in some of the cells a remarkably high number of Ag grains were detected. Cells with many Ag grains were located mainly in the primary tumor, while the metastases displayed less radionuclide binding. Possible explanations for this discrepancy could be differences in sst subtypes and/or numbers in different tumor cells, but the discrepancy could also be due to factors such as differences in vascularization of the tissues and development of fibrosis which might influence the accessability for the [^{111}In-DTPA-D-Phe^1]-octreotide to reach the tumor cell. These questions will have to be further addressed in forthcoming studies, but it is worth noting that cells in the center of the tumor nests displayed fewer Ag grains than cells in the border facing the surrounding capillaries.

There were very few Ag grains present in the fibrous stroma surrounding the tumor nests, although some Ag grains were demonstrated in the capillaries and also in leukocytes. This was expected, since resting monocytes and lymphocytes express low affinity sst expression and upon activation the expression changes towards high affinity sst expression.

In normal intestine we did not identify any specific uptake of ^{111}In giving rise to Ag grains within the enterocytes. Some grains were detected in vacuoles, which is a main feature in these absorptive cells. This uptake is unspecific, i.e. not receptor mediated. Some Ag grains were also found in the striated border of the enterocytes facing the intestinal lumen. This is also what might be expected, since the [^{111}In-DTPA-D-Phe^1]-octreotide is partly excreted through the intestine.

For the purpose of tumor-cell-targeting treatment it is important that Ag grains can be found throughout the cytoplasm and also in the vicinity of the tumor cell nucleus. Because the Auger electrons have a very short range, the presence of Ag grains close to the DNA is necessary for the Auger electron to cause DNA breakage and cellular death (13). It is shown in this study that ^{111}In-octreotide binds to somatostatin receptors and is internalized through receptor-mediated endocytoses to endosomes and further into the cell interior. However, the intracellular processing of somatostatin and somatostatin analogues is not yet understood and will have to be further investigated.

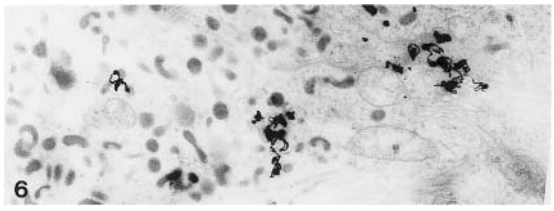

Fig. 6. Example of a cell with a remarkably high number of [^{111}In-DTPA-D-Phe^1]-octreotide uptake and the Ag reaction products located throughout the cytoplasm. Exp. 7 days. $\times$ 2400.

We conclude that this method reveals the in vivo cellular distribution of injected [^{111}In-DTPA-D-Phe^{1}]-octreotide. The knowledge gained from these studies may help us to interpret the radiobiological effects observed in patients treated with [^{111}In-DTPA-D-phe^{1}]-octreotide and, in the future, knowledge on the biological action of somatostatin.

ACKNOWLEDGEMENTS

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