Chapter 7

Radioactive holmium loaded poly(L-lactic acid) microspheres for treatment of hepatic malignancies: efficacy in rabbits

in preparation

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Abstract
In this chapter the therapeutic effect of $^{166}$Ho loaded poly(L-lactic acid) microspheres in rabbits with liver tumours was investigated. New Zealand White rabbits with an implanted VX2 tumour were divided in three groups: sham-treated (n=3), cold microspheres (n=3) and microspheres loaded with $^{166}$Ho (0.9-1.0 GBq; n=2). After administration of the microspheres into the hepatic artery the biodistribution was studied with a gamma camera and tumour growth was followed in time with ultrasound. The radioactive microspheres were heterogeneously distributed over the liver and accumulated preferentially in the tumour area, which was confirmed by histological analysis. A transient increase of hepatic enzyme activity was observed after (radio)embolization. Sham-treated rabbits and rabbits treated with “cold” microspheres showed an exponential tumour growth. Therapeutic doses (100 Gy to the whole liver) arrested growth and resulted in necrosis of the tumour. No histological signs of pathological radiation effects, such as hepatic fibrosis or necrosis of the normal liver, were detected. This feasibility study demonstrates that $^{166}$Ho loaded poly(L-lactic acid) microspheres are promising systems for the treatment of patients with liver tumours.
7.1 Introduction
Malignant neoplasms of the liver, both primary and metastatic, are among the most common tumours worldwide. In western countries the majority of liver metastases originates from colorectal cancer. Its prognosis is poor, with a median survival of approximately 6 to 12 months [1]. Currently, chemotherapy, either systemic or by hepatic arterial infusion, is the main approach of treatment. Nevertheless, side effects of chemotherapy are commonly reported while life extension is often marginal [1-3]. Long-term survivors are frequently documented after partial liver resection, but this is only possible in 10% of the patients [2,4].

In the last decade a new therapy, viz. selective internal administration of radioactive yttrium loaded glass or resin based microspheres, resulted in a useful treatment with minor side effects [5]. The microspheres are administered into the hepatic artery and will lodge in the end arterioles in the liver. Tumours are usually rich in vasculature and they derive most of their blood supply from the arterial side. Thus accumulation of spheres can be found in and around the tumour when administered by the hepatic artery [6]. Estimated tumour doses exceed 300 Gy while the absorbed doses to the liver range from 50 to 150 Gy [5,7]. Although increased longevity and long term survivors are reported, this treatment modality is certainly far from optimal. Especially the high density of glass microspheres, the absence of a gamma component for imaging and the non-biodegradability are major drawbacks of both the resin- and glass-based 90Y loaded microspheres [5,8].

These disadvantages are solved to a large extent by the use of a new type of microsphere and isotope, as first described by Mumper et al. [8]. Neutron activated Ho-PLLA microspheres have more favourable properties. A disadvantage of these organic types of spheres is their high susceptibility to degradation induced by neutron irradiation needed for the activation of the holmium [9,10]. By carefully defining the irradiation conditions and increasing the holmium content we succeeded in the preparation of useful microspheres loaded with a therapeutic amount of radioactive holmium [11]. Experiments in a rat tumour model showed an average tumour/liver ratio of six for these PLLA microspheres [12].

In order to use these microspheres for therapy in humans, distribution in the liver should be inhomogeneous with preference for the tumour area and the delivered radiation dose should be able to induce necrosis of the tumour with acceptable toxicity to the normal liver. The objective of the present study was to investigate the effects of PLLA microspheres, containing therapeutic amounts of 166Ho, in the treatment of liver tumours in rabbits. Rabbits were either sham-treated or received a diagnostic or therapeutic (100 Gy) dose of microspheres. The dose of 100 Gy to the whole liver was chosen since it is well tolerated and results in effective tumour kill or reduction [5,13,14]. Tumour growth was investigated by ultrasound.
7.2 Materials and methods

7.2.1 Animals
All experiments were performed in agreement with The Netherlands Experiments on Animals Act (1977) and the European Convention guidelines (86/609/EC). Approval was obtained from the University Animal Experiments Committee (FDC/DEC-GNK nr. 99042). Ten adult female or male specific pathogen free New Zealand White rabbits of 2000 to 3500 g were used (Harlan, Horst, The Netherlands). The rabbits were housed individually in steel or plastic cages and provided daily with approximately 100 g “complete diet” pellets for rabbits (LKK-20, Hope Farms BV, Woerden, The Netherlands). Water was provided ad libitum. Rabbits were sacrificed after three or four weeks or earlier, when clear inconvenience for the animal was observed.

7.2.2 Tumour cells
The VX2 cell line was obtained from the Department of Oral and Maxillofacial Surgery of the University Medical Center, Utrecht, The Netherlands [15]. The original tumour cells were obtained from a virus induced papilloma rabbit carcinoma [16]. The VX2 tumour was propagated by subcutaneous passage in the hip region of the rabbit or was derived from a part of the implanted tumour in the liver of a freshly killed rabbit. In order to facilitate implantation, the tumour tissue was dissected and small parts (2 mm in diameter) were chosen for implantation.

7.2.3 Tumour implantation
Prior to inhalation-anaestheticum, premedication of 0.5 ml methadon (10 mg/ml; Veterinary Pharmacy, University of Utrecht, The Netherlands) and 0.5 ml Vetranquil® (acepromazine, 10 mg/ml; Sanofi Sante Animale Benelux BV, Maassluis, The Netherlands) was given. Subsequently, the rabbits were anaesthetized by an intravenous injection of Hypnomidate® (2 mg/ml; B. Braun Melsungen AG, Melsungen, Germany) and N₂O and halothane (Albic BV, Maassluis, The Netherlands) as inhalation anaestheticum. A laparotomy was performed by ventral mid-line incision in order to expose the lobes of the liver. Tumour tissue was implanted in the lobus sinister lateralis by injection with an Abbocath-T® 18G (Abbott Ltd., Ireland), together with a piece of titanium to serve as a localization marker for ultrasound. After approximately 12 days the first ultrasound investigation (HDI 3000 ATL, Entos™ CL10-5 transducer) was performed to check tumour growth.

7.2.4 Preparation of microspheres
Radioactive holmium loaded microspheres were prepared as previously described [11]. Briefly, holmium acetylacetonate (HoAcAc) is incorporated into PLLA by
solvent evaporation, resulting in microspheres of 20-50 µm after sieving. Neutron activation of the microspheres was performed by irradiation for 1h in the PRS facility of the high-flux nuclear reactor in Petten, The Netherlands. Neutron activated microspheres were used the next day for therapeutic purpose (900-1000 MBq in 35-40 mg) or as diagnostic, “cold” microspheres after decay for four to five days (40-80 MBq in 35-40 mg). Microspheres were sonicated for 10 min in an ultrasonic cleaner and suspended in Gelofusine® (Vifor Medical SA, Switzerland) prior to administration.

7.2.5 Administration of microspheres
When the tumour had reached a diameter of ≥20 mm, a second laparatomy was performed in order to administer the holmium loaded microspheres (or Gelofusine® in case of sham-treated rabbits). Administration of microspheres was similar as described for rats in chapter 6. The gastroduodenal artery was cannulated with an Abbocath-T® 24G (Abbott Ltd., Ireland). Back flow was checked with 0.1% methylene blue in 5% glucose. A pre-flushed administration system similar as described by Herba et al. [17] was connected to the Abbocath®. The suspended microspheres were administered and the administration system was measured for activity pre- and post injection, in order to calculate the injected dosage. The gastroduodenal artery was sealed with tissue glue (Histoacryl, B. Braun Melsungen AG, Melsungen, Germany) and wires were removed to restore the arterial hepatic circulation.

7.2.6 Biodistribution and assessment of tumour growth
Rabbits were divided in three treatment groups which received either Gelofusine® (control group, n=3), “cold” microspheres containing 40-100 MBq ¹⁶⁶Ho for diagnostic imaging (n=3) or microspheres containing therapeutic amounts of activity (900-1000 MBq; n=2) resulting in approximately 100 Gy to the whole liver. The rabbits were monitored by planar and SPECT imaging, using a triple-head gamma camera (Prism 3000, Cleveland, USA). A whole body scintigram was made 5 days after administration of therapeutic and 1 day after diagnostic microspheres, in order to determine gross distribution.

Ultrasound studies were performed at approximately 4, 10, and 18 days after administration of Gelofusine® or microspheres and before sacrificing, and were evaluated by the same observer. Examination of the liver, stomach and part of the intestines was established by sagittal and transversal scanning of the abdominal region. The tumour was measured in three directions and its volume was calculated by applying the equation for the volume of an ellipsoid [18]. Ultrasound measurements before treatment and sacrificing were compared with calliper measurements at time of treatment and autopsy of the rabbit.
7.2.7 Toxicity evaluation
Blood samples were taken three or four times: prior to tumour implantation, before administration of microspheres, after administration and before sacrifice. Blood was centrifuged at 2500 g for 10 min and the plasma immediately frozen. The plasma samples were analysed for alkaline phosphatase as an indicator of biliary toxicity and for alanine aminotransferase (ALAT), gamma glutamyltransferase (γ-GT) and bilirubine as indicators of hepatocellular toxicity. At autopsy the thoracic and abdominal organs were inspected and fixed in phosphate-buffered 4% formaldehyde. In order to verify presence of microspheres, tissue samples from the tumour site, liver, lungs, spleen, and stomach were embedded in paraffin and histologically evaluated after staining with haematoxylin-eosin.

7.3 Results
7.3.1 Tumour growth and survival
Implantation of the tumour resulted in a 100% “take-rate”. In tumours transplanted from subcutaneous site to liver a slower tumour growth was observed (approximately 21 days before reaching a diameter >20 mm) compared with tumour tissue that was transplanted from liver to liver (approximately 16 days before reaching >20 mm). Tumours >2 mm in diameter were visible with ultrasound and were well vascularized as illustrated in Fig. 1.

Fig. 1a-b. Doppler ultrasound (square) of the vasculature of the VX2 tumour (1.2 x 1.3 cm) before treatment. The high blood flow in and around the tumour is indicated in the ultrasound graph in the white/light grey regions (a). In the schematic drawing the blood flow is indicated as grey regions (b). *Branch of the portal vein.
Tumour growth was exponential in both the control group and the rabbits treated with “cold” microspheres (Fig. 2 and Table 1). No primary tumour growth was seen in the rabbits treated with 900 MBq of activity.

After the second operation most rabbits showed a decline in physical condition mainly manifested by a decrease in appetite.

**Fig. 2.** Tumour growth in the liver of rabbits after treatment (day 0). Measurements of tumour sizes in time for individual rabbits of control (n=3), cold microspheres (n=3) and therapeutic group (n=2) are shown by grey square, light grey triangle and black rhomb.
Table 1. Tumour size at treatment and sacrifice.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>(^{166})Ho (MBq)</th>
<th>Tumour size at treatment (cm(^3))</th>
<th>Tumour size at sacrifice (cm(^3))</th>
<th>Day of sacrifice</th>
<th>Increase of tumour size (%)</th>
</tr>
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<tr>
<td>Control 1</td>
<td>---</td>
<td>4.8</td>
<td>132.8</td>
<td>34</td>
<td>2767</td>
</tr>
<tr>
<td>Control 2</td>
<td>---</td>
<td>13.6</td>
<td>201.1</td>
<td>29</td>
<td>1479</td>
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<tr>
<td>Control 3</td>
<td>---</td>
<td>0.1</td>
<td>25.7</td>
<td>30</td>
<td>25700</td>
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<tr>
<td>Cold 1</td>
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<td>0.2</td>
<td>11.7</td>
<td>23</td>
<td>5850</td>
</tr>
<tr>
<td>Cold 2</td>
<td>123</td>
<td>3.2</td>
<td>254.9</td>
<td>28</td>
<td>7966</td>
</tr>
<tr>
<td>Cold 3</td>
<td>46</td>
<td>12.9</td>
<td>37.7</td>
<td>24</td>
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<td>14.9</td>
<td>11.8</td>
<td>21</td>
<td>-79</td>
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</tbody>
</table>

7.3.2 Metastatic disease

Ultrasound appeared to be a useful modality in predicting the size of the tumours (Fig. 3). In all treatment groups several massive lung and liver metastases were found with ultrasound or subsequently at autopsy. Histology showed high proliferation grade and necrotic centre indicative for non-differentiated and aggressive tumours. Also metastases on the exterior of the stomach and diaphragm were present in all three groups. Metastases on the incision wound were sometimes present and often visible before treatment. If possible this tumour tissue was resected during the second laparotomy.

![Fig. 3. Correlation between tumour volume measured with ultrasound and calliper measurements during operation and autopsy of the rabbits.](image)

7.3.3 Biodistribution and histological analysis

Cannulation of the liver artery resulted in deposition of 80-90% of the initial activity of microspheres. Based on SPECT and planar scintigraphic images radioactivity remained in the liver and tumour with preferential accumulation of activity in the tumour (Fig. 4). Activity in the liver was heterogeneously distributed which was
confirms microscopic analysis showing that microspheres were distributed unequally over liver and tumor. Although single spheres were found in similar amounts in tumor and liver tissue, the greater part of clustered microspheres had accumulated around the (primary) tumor and in the larger vessels (Figs. 5 and 6). No microspheres were found in lungs, stomach, or spleen. Particularly in the rabbits that were embolized with (radioactive) microspheres, cholestasis was noted in the liver and this was sometimes visualized as small ridges in the ultrasound image.

Fig. 4a-b. Whole body scintigraphic image of a tumor-bearing rabbit 3 days after injection of approximately 1GBq $^{166}$Ho poly(L-lactic acid) microspheres into the hepatic artery. Contour of the rabbit was obtained by using a “flood source”.

In the control group and the rabbits embolized with “cold” microspheres, histology showed compression of liver parenchyma caused by the rapid expansion of the tumor. The tumor tissue appeared to be viable and was unaffected by the treatment. In contrast, in the therapeutic treated rabbits the primary tumor and the larger metastases were entirely necrotic. Also necrotic liver tissue was seen close to large blood vessels containing clusters of microspheres. Compression of normal liver tissue was scarcely observed.

Fig. 5a-b. Typical example of a liver tissue section after embolization with cold microspheres in the liver artery (a). A schematic overview of the micrograph shows A) tumor tissue B) liver tissue and C) connective tissue with bile ducts and blood vessels (b). The magnification of the white square of (a) shows five microspheres in small arteries close to the tumor (schematic drawing 5 black circles) (c). Since microspheres were cold no damage is seen in liver or tumor tissue.
Fig. 6a-b. Typical example of a liver tissue section after embolization with microspheres with therapeutic amounts of activity (a). A schematic overview of the micrograph shows A) tumour tissue and B) liver tissue (b). A cluster of microspheres is seen in a large blood vessel in the tumour. No cell nucleus is seen in cells of both liver tissue and tumour tissue indicating necrotic tissue (c).

7.3.4 Hepatic toxicity
At this stage of the study plasma data were too incomplete to allow for statistical analysis of hepatic enzyme levels. Therefore only the trends observed in the individual animals can be described. Total bilirubin levels remained unchanged in all rabbits. For alkaline phosphatase, ALAT and γ-GT a transient elevation in activity was observed which is illustrated in Fig. 7 for a rabbit that was treated therapeutically with 928 MBq of radioactive microspheres. This phenomenon was not observed in sham-treated rabbits, which showed no change in activity of liver enzymes during the follow up until sacrificing.

Fig. 7. Liver enzyme levels in a rabbit treated (at day 0) with 928 MBq $^{166}$Ho-microspheres.
7.4 Discussion
Treatment of liver malignancies in humans remains a challenge and prognosis of these patients is still poor. Conventional therapies such as systemic chemotherapy and external radiotherapy are insufficient [1,2]. New approaches such as regional chemotherapy result in increased survival and palliation [1,3,19]. Nevertheless, the side effects of regional chemotherapy are a considerable drawback.

A good alternative with less side effects are radioactive yttrium containing glass or resin based microspheres. Although these spheres showed some favourable clinical results, they have their own disadvantages such as a relatively high density, lack of \( \gamma \)-emission and non-biodegradability [5]. This is reflected in the risk that these microspheres are deposited into the gastroduodenal vasculature under gravity. The microspheres can not be tracked during and after administration and repeated administration of spheres is difficult. The use of neutron activated holmium loaded poly(L-lactic acid) microspheres may overcome these disadvantages.

In this study the proof of principle of this radioactive holmium loaded system was investigated in a rabbit tumour model. A major difference with human liver metastases and transplantable rodent tumours is the relatively slow doubling time in humans [20]. In the human situation microspheres can be administered via the femoral artery using standard radiological intervention techniques. The present rabbit model has the disadvantage of relatively rapid tumour growth, the concomitant spread of metastases and the need for two laparotomies.

The present study shows that holmium loaded microspheres infused into the hepatic artery do not give rise to backflow to the gastrointestinal vessels and that virtually all injected activity is deposited in the liver and tumour.

The high dose of activity in the liver and tumour was surprisingly well tolerated. This effect (external radiotherapy is limited to 30-35 Gy [5,21]) is similar as described for the radioactive yttrium loaded microspheres and is probably caused by the inhomogeneous distribution of activity in the liver [22,23]. Fox et al. [23] described for a patient that 86% of the normal liver parenchyma received less than the dose that would be expected for a uniform distribution, and 34% of the tissue received even less than one third of the dose [23]. Thus, approximately one third of the tissue receives less or equal to 30 Gy (total dose 90 Gy).

Histology as well as planar and SPECT imaging of the tumorous rabbit livers confirmed the high variability in microsphere and activity distribution for this embolization technique. In the rabbits embolized with microspheres at least two times more activity was found in and around the tumour compared with normal liver parenchyma based on SPECT imaging and histology. These values and variability are in accordance with results in other animal studies and clinical trials [13,24,25].
It was shown in this study that radioactive microspheres with therapeutic amounts of $^{166}$Ho, approximately 100 Gy to the whole liver, can arrest tumour growth and induce tumour necrosis. Studies, in other animal models, will be needed to further investigate the potential survival benefit of this therapy.

The administration of (radioactive) microspheres often gave rise to cholestasis in the liver and was also observed in one sham-treated rabbit. This phenomenon was probably caused by repeated halothane exposure during the two laparotomies. Impaired bile flow and cholestasis were reported as liver injury induced by halothane [26] and these effects may be enhanced by microsphere embolization.

In conclusion, this study has demonstrated that holmium loaded PLLA microspheres with a therapeutic dose of activity can arrest tumour growth and induce necrosis of tumour tissue while sparing normal liver tissue. These results warrant further studies in other models before this therapy can be tested in the human situation.

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