Evaluation of Selective Arterial Embolization Effect by Chitosan Micro-Hydrogels in Hindlimb Sarcoma Rodent Models Using Various Imaging Modalities

Tai Kyoung Lee · JeongIl Kwon · Kyung Sook Na · Hwan-Seok Jeong ·
Hyosook Hwang · Phil-Sun Oh · Dong Hyun Kim · Kyu Yun Jang ·
Seok Tae Lim · Myung-Hee Sohn · Hwan-Jeong Jeong

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Abstract

Purpose Embolization is mainly used to reduce the size of locally advanced tumors. In this study, selective arterial catheterization with chitosan micro-hydrogels (CMH) into the femoral artery was performed and the therapeutic effect was validated using different imaging methods.

Methods Male SD rats ($n=18$, 6 weeks old) were randomly assigned into three groups: Group 1 as control, Group 2 without any ligation of distal femoral artery, and Group 3 with temporary ligation of the distal femoral artery. RR1022 sarcoma cell lines were inoculated into thigh muscle. After 1 week, CMH was injected into the proximal femoral artery. Different

imaging modalities were performed during a 3-week follow-up.

Results The tumor size was significantly ($P<0.001$) decreased in both Group 2 and Group 3 ($P<0.001$) after selective arterial embolization therapy. ^{18}F -FDG-PET/CT revealed decreased intensity of ^{18}F -FDG uptake in tumors. The accumulation status of ^{125}I -CMH near the tumor was verified by gamma camera.

Conclusions Appropriate selective arterial embolization therapy with CMH was.

Keywords Selective arterial embolization · Chitosan micro-hydrogel · Molecular imaging · Ultrasonography · Positron emission tomography (PET) · Gamma camera · RR1022 · Sarcoma · Rodent

T. K. Lee · K. S. Na · H.-S. Jeong · H. Hwang · P.-S. Oh ·
S. T. Lim · M.-H. Sohn · H.-J. Jeong

Department of Nuclear Medicine, Research Institute of Clinical Medicine, Cyclotron Research Center, Institute for Medical Sciences, Molecular Imaging & Therapeutic Medicine Research Center Chonbuk National University Medical School and Hospital, Geumam-ro, Dukjin-gu, Jeonju, Republic of Korea

J. Kwon · D. H. Kim

Kai Bio Tech, Department of Nuclear Medicine, Research Institute of Clinical Medicine, Institute for Medical Sciences, Molecular Imaging & Therapeutic Medicine Research Center Chonbuk National University Medical School and Hospital, Geumam-ro, Dukjin-gu, Jeonju, Republic of Korea

K. Y. Jang

Department of Pathology, Chonbuk National University Medical School and Hospital, Geumam-ro, Dukjin-gu, Jeonju, Republic of Korea

H.-J. Jeong (✉)

Department of Nuclear Medicine, Chonbuk National University Medical School and Hospital, Geonji-ro, Dukjin-gu, Jeonju, Republic of Korea
e-mail: jayjeong@jbnu.ac.kr

Introduction

The main goal of tumor treatment is to remove tumors or to reduce subsidiarily the growth of tumor size. Radical therapy such as a surgical operation is one of the main approaches to remove tumors [1]. Although surgical operation helps remove a tumor from the body, it usually needs long admission with complications [2]. Neoadjuvant therapy, a pre-treatment before therapeutic agents are administrated to reduce tumor growth, makes further procedures of treatment easier and likely to succeed [3]. Various therapeutic techniques including immunotherapy, hormone therapy, chemotherapy, radiation therapy, and embolization therapy are commonly used as neoadjuvant therapy in clinical circumstances [4–6]. Embolization therapy is especially often used to reduce growth of locally advanced tumors in ischemic change induced by occlusion of blood vessels. Compared with other

approaches, embolization therapy has several advantages, including minimal invasions, no scars, rare risk of infection, and high success rate of treatment with minimal complication [7]. One of the representative approach of embolization therapy is transcatheter arterial chemoembolization (TACE) on hepatocellular carcinoma (HCC). TACE has been used to treat cancer or to reduce the size of tumor in HCC by administration of anticancer drug and lipiodol, one of the embolic agents, into the selected hepatic artery in the liver before main treatment such as brachytherapy [8]. The embolic agents include liquid embolic agents (*n*-butyl-2-cyanoacrylate and ethiodol), sclerosing agents (ethanol, ethanolamine oleate, sotradecol), mechanical occlusion devices (coils, detachable balloon), and particulate embolic agents (gelfoam hemostasis, polyvinyl alcohol, acrylic gelatin microspheres) [9]. Chitosan, a natural carbohydrate polymer modified by partial *N*-deacetylation of chitin, is commonly used in biomedical fields recently due to its biological safety, non-toxicity, biocompatibility, biodegradability, and easy preparation method [10]. We have established a synthetic method for the preparation of chitosan micro-hydrogel (CMH) that is uniform and stable. In this study, we tried to use these particles as an embolic agent. The purpose of this study was to evaluate the efficacy of selective arterial embolization therapy for cancer treatment using molecular imaging modality to trace the infused molecules and to evaluate cancer metabolism. To validate the therapeutic effect of the embolic agents, ultrasonography was used to measure the diameter size, and also gamma camera and positron emission tomography with computed tomography (PET/CT) using ^{18}F -fluorodeoxyglucose (^{18}F -FDG) were utilized [11–15].

Materials and Methods

Materials

Chitosan (molecular weight, 300 kDa; deacetylation, 98 %) was purchased from Youngchipharm (Paju, Korea). Sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (SHPP) was bought from Thermo Fisher (Waltham, MA, USA). Sodium triphosphate pentabasic (TPP) and boric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Poly-mesh was purchased from TEXTOMA (Daegu, Korea). Isoflurane was obtained from Hana Pharmaceutical (Hwasung, Korea). Na^{125}I was obtained from Perkin Elmer Life Science (Boston, MA, USA). Surgical sutures were purchased from ETHICON (Somerville, NJ, USA). Human serum and Cyclosporin A were provided by Chonbuk National University Hospital (Jeonju, Korea).

Synthesis of Chitosan-SHPP

Chitosan (1,000 mg) was dissolved in HCl (0.1 N, 100 mL) at 4 °C for 5 h followed by the addition of borate buffer (pH 7.3, 200 mL). Sulfo SHPP (10 mg) diluted in DMSO (0.2 mL) was added into chitosan solution and reacted under N_2 purge for 15 h at −4 °C. This chitosan-SHPP solution was dialyzed for 3 days and then lyophilized for 1 week. The morphology of CMH was identified by optical microscope (DP72; OLYMPUS, Shinjuku, Tokyo, Japan).

Preparation of ^{125}I -Labeled Chitosan-SHPP

Chitosan-SHPP solution (20 mg) was dissolved with 1 % acetic acid (0.7 mL). ^{125}I (37 MBq, 100 μL) was then added into the chitosan-SHPP solution. Chloramine T (1 mg/0.1 mL PBS) and sodium bisulfite (1 mg/0.1 mL PBS) were successively added and reacted for 30 min. To measure the labeling efficiency, ^{125}I -CMH was spotted by thin layer chromatography (TLC, B-AR-2000-1; BIOSCAN, Washington, DC, USA) using normal saline solution.

Chitosan Micro-Hydrogel (CMH) Formulation by Electro Spinning System

^{125}I -labeled chitosan-SHPP solution (1 mL) was dropped into sodium triphosphate pentabasic (TPP) solution (12 g/100 mL) at 12 kV with flow rate of 0.1 mL/min and working distance of 10 cm using an electro spinning system. The ^{125}I -labeled chitosan micro-hydrogels (^{125}I -CMH) filtered by polymer mesh (mesh opening, 100 μm) were stored in normal saline.

Animal

All animal experiments were performed in compliance with policy and procedure of the Institutional Animal Care and Use Committee in Chonbuk National University of South Korea. Male Sprague–Dawley rats ($n=18$, 6 weeks old) were purchased from Orient-Bio (Seoul, Korea). Rats were randomly assigned into three groups: Group 1 as control, Group 2 without any ligation of femoral artery, and Group 3 with permanent ligation at the distal part which faces down from inoculation site in femoral artery.

Preparation of Rat Models Inoculated With Sarcoma Cells

During experiment days, all rats were treated with the immunosuppressant cyclosporin A (CsA, 20 mg/kg) at −1, 0, +1, +2, +4 days of inoculation of the RR1022 sarcoma cell lines. Before surgery for inoculation, rats were anesthetized with isoflurane (2 %) through inhalation. Surgery started with a small incision in the proximal site of the right thigh. The RR1022 sarcoma cells

Fig. 1 A conceptual diagram for preparation of ^{125}I -CMH. ^{125}I -CMH for the treatment is prepared by electro-spinning method and administered by selective arterial injection into the proximal femoral artery

(1×10^7 /rat), combined with normal saline, were inoculated into the space between the femoral muscle and fascia near the femoral artery using insulin syringes (31-gauge). After the inoculation, the small incision was sutured using 3–0 surgical black silk.

One week after the inoculation, in Group 2, CMH (300 μL) was injected into the proximal femoral artery via intravenous catheter (24-gauge); in Group 3, the distal site of the femoral artery was ligated by 3–0 surgical black silk and ^{125}I -labeled CMH (300 μL) was injected into the proximal femoral artery.

Fig. 2 Optical microscopic image showing the size of CMH between 100 and 150 μm . Morphology of CMH was shown with smooth surfaces and spherical shapes

Fig. 3 Radio TLC image showing ^{125}I labeling efficiency of CMH measured over 90 % in the saline and PBS. Labeling stability of CMH with ^{125}I was highly stable

Fig. 4 Ultrasonography providing anatomical information and size measurement of tumors in real time. The follow-ups of ultrasonography were performed at every week from the beginning of experiment. All

images shown in this figure represented transverse sections. The tumor size change, as well as the tumor development, has been monitored with difference of echogenicity

Fig. 5 Glucose metabolic activity of tumor in each group. The yellow-colored circle indicates a tumor as expected. The red-colored ellipse in the 3rd week of Group 2 indicates an inflammatory cellular uptake induced by ischemia due to CMH distribution into arterial system of right hindlimb

The injection site punctured by catheterization was repaired with a small piece of polyethylene catheter tube.

Assessment of Tumor Status Using Imaging Modalities

All rats during imaging acquisition were anesthetized with isoflurane (2 %). After 1 week of tumor inoculation, ^{18}F -FDG (18 MBq) was injected into all rats through tail vein injection. Rats were lied down at prone position on the bed. ^{18}F -FDG PET/CT images were acquired for 30 min to identify glucose metabolic status of tumor by using the Animal PET/CT system (FLEX Pre-clinical Platform; Gamma Medica-Ideas, Salem, USA) at the 1st and the 3rd week. Volume of interest (VOI) for tumor was drawn by visualized three-dimensional analysis software from Life Science (Amira; FEI, MA, USA). To estimate the intensity of ^{18}F -FDG, the point source of ^{18}F -FDG (370 KBq) was used as the standard source and the intensity in the tumor was calculated by proportional expression ($\text{ACTIVITY}_{\text{standard}}:\text{COUNTS}_{\text{standard}}=\text{ACTIVITY}_{^{18}\text{F}\text{-FDG}}:\text{COUNTS}_{^{18}\text{F}\text{-FDG}}$). To monitor size change of tumor growth, ultrasonography was obtained by ultrasound system

(Accuvix V10; Samsung Medison, Suwon, Korea) using a linear probe with 8.0-MHz frequency (L5-13IS; Samsung Medison, Suwon, Korea) in transverse and longitudinal sectional images of the tumor at the 1st, 2nd, and 3rd week. Using the three-axis information estimated by ultrasonography, the volume (V) of tumor was calculated by the volume formula for a three-dimensional sphere:

$V = \frac{3}{4}\pi \times \left\{\frac{1}{2} \times (\text{width} \times \text{height} \times \text{depth})\right\}$. For Group 3, scintigraphic images were also acquired to check the accumulation of ^{125}I -labeled CMH near the tumor by using an Animal single photon emission computed tomography with X-ray computed tomography (SPECT/CT system, FLEX Pre-clinical Platform; Gamma Medica-Ideas, Salem, USA). A total of 35 keV of gamma energies emitted from ^{125}I were detected by a gamma camera and presented to planar imaging. Photographic images of body (prone position) were also obtained to verify the location where ^{125}I -labeled CMH was in the body. In Groups 2 and 3, photographic images were taken during experiments to verify hindlimb ischemia induced by CMH.

Assessment of Hematoxylin and Eosin (H & E) Stain

To analyze histology of femoral muscle and tumor mass, all rats were euthanized in a N_2 chamber for subsequent histological analysis. Axial sections sampled across the center of the tumor were fixed in 10 % buffered formaldehyde solution and paraffin embedded. H & E staining of every section was performed. After tumor removal for H & E staining, each rat was euthanized in a nitric oxide (NO) chamber.

Statistical Analysis

All data sets were calculated and represented as mean $\pm$ SD. Statistical differences between three groups were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc tests for multiple comparisons (Group 1 versus Group 2, Group 1 versus Group 3 and Group 2 versus Group 3) on SPSS 12.0 statistics software (SPSS, Chicago, IL, USA). Statistically significant difference was considered when the *P* value was less than 0.05.

Results

Characterization of ^{125}I -Labeled Chitosan Micro-Hydrogels (CMH)

The conceptual diagram for the preparation of ^{125}I -CMH is described in Fig. 1 to understand the experimental procedure. The size of CMH was between 100 and 150 μm as measured under an optical microscope (Fig. 2). CMH had smooth surfaces and spherical shapes. The efficiency of CMH measured for around 140 min in

normal saline solution was over 90 %, suggesting that the labeling condition between CMH and ^{125}I was highly stable (Fig. 3).

Assessment of Imaging Modalities

From the results of the ultrasonography, we calculated the change of tumor size at follow-up time point. Tumor size change as well as tumor development were easily monitored with difference in echogenicity (Fig. 4). Transverse images of the right thigh represented heterogeneous and decreased echogenicity in the region of the tumors in Groups 2 and 3 with time. ^{18}F -FDG-PET/CT imaging revealed that the glucose metabolic uptake of tumors had higher intensity than other normal sites (Fig. 5). The intensity (I) of ^{18}F -FDG uptake in tumors was reflected as similar to change of tumor growth; Group 1 ($I_{1w}=4.4$ MBq/ 1.54 cm^3 , $I_{3w}=16.72$ MBq/ 5.73 cm^3), Group 2 ($I_{1w}=4.5$ MBq/ 1.32 cm^3 , $I_{3w}=3.8$ MBq/ 1.16 cm^3) and Group 3 ($I_{1w}=3.6$ MBq/ 1.44 cm^3 , $I_{3w}=2.5$ MBq/ 0.91 cm^3). ^{125}I planar images using gamma camera revealed that ^{125}I -CMH was distributed all over the right hindlimb in Group 2 and accumulated near the tumor as expected in Group 3 (Fig. 6).

Pathologic Findings

In visual photographic imaging, serial change of the right limb was observed in Group 2, suggesting hindlimb ischemia induced by CMH which became worse during follow-ups. However, no significant change was detected in Group 3 (Fig. 7). H & E stains showed that tumor cells in Group 1 were intact without dominant inflammatory infiltration or tumor necrosis. In contrast, tumor cells in Group 2 were pyknotic with inflammatory infiltration. In Group 3, there were significant ischemic necrosis and inflammatory infiltration (Fig. 8).

Analysis of Tumor Volume Changes

In the result of Group 1, the tumor volume ($V_{1w}=1.54\text{ cm}^3$, $V_{2w}=6.47\text{ cm}^3$, $V_{3w}=5.73\text{ cm}^3$) was highly increased initially and then slightly decreased for 3 weeks. However, in Group 2 ($V_{1w}=1.32\text{ cm}^3$, $V_{2w}=2.71\text{ cm}^3$, $V_{3w}=1.16\text{ cm}^3$) and Group 3 ($V_{1w}=1.44\text{ cm}^3$, $V_{2w}=2.84\text{ cm}^3$, $V_{3w}=0.91\text{ cm}^3$), the tumor volumes were very slowly increased initially and then decreased to the small size at the beginning of follow-up periods (Fig. 9).

Fig. 6 ^{125}I -labeled chitosan micro-hydrogels (CMH) were detected by gamma imaging system after 1 day of ^{125}I -CMH injection via proximal femoral artery. The tumor site was identified in Group 3 (yellow-colored circle). The red-colored circle in Group 2 indicates the distributed ^{125}I -CMH over the tumor and the limb. The white-colored solid lines indicate body contours of SD rats

Fig. 7 Visual photography was taken to monitor hindlimb ischemia induced by CMH in Group 2 as compared with Group 1 and Group 3 at the 3rd week of experiment

Discussion

We used selective arterial embolization to reduce tumor growths to reveal that our CMH would be useful as an embolic agent. We also used molecular imaging to evaluate the procedure and tumor status. Embolization of peripheral tumor using CMH showed successful diminishment of tumor size and metabolism. Appropriate utilization of molecular imaging modalities informed us of the proper approach for therapeutic substances to target lesion and predict therapeutic effect or complications. In this study, it was important to know whether

Fig. 8 Pathologic examinations ($\times 200$) with hematoxylin and eosin (H & E) stain of RR1022 tumor mass inoculated to SD rats. H & E stains showed Group 2 with pyknotic tumor cells and inflammatory infiltration and Group 3 with significant ischemic necrosis and inflammation

adequate part of artery was ligated before the administration of CMH. Results from Group 2 showed an excellent effect of reducing tumor size as from Group 3. However, hindlimb ischemia was induced because the descending part of the femoral artery was not ligated, so that CMH flowed into the arterial system of lower limb over all. On the other hand, the administrated CMH accumulated in the arteries of Group 3, providing blood supplies to the tumor which induced the embolization effect because the descending part of femoral artery was ligated to guide CMH into the branched artery heading to the tumor.

To prepare rat models with sarcoma, we inoculated the RR1022 sarcoma cells into the space between the thigh

Fig. 9 The tumor size in Group 2 and 3 was changed significantly compared with Group 1. The groups treated by embolization showed greater inhibition rate in tumor growth. * $P < 0.05$, ** $P < 0.001$

muscle and fascia near the femoral artery and selected superficial femoral artery for easy access and lower complication [16]. So, the proximal and superficial femoral artery was catheterized by 24-gauge needle of IV catheters during the selective arterial catheterization. In Group 3, we raised the probability that injected CMH was guided more into the artery heading to the tumor by occluding the distal part of the superficial femoral artery selected by catheterization.

A number of imaging modalities in radiology and nuclear medicine can provide anatomical and functional information through the whole body to diagnose pathological interpretation in pre-clinical and clinical circumstances. A radiopharmaceutical such as ^{18}F -FDG offers functional information on the glycolic metabolism of a tumor to verify tumor status with quantitative information as well as localization. We assessed the change of ^{18}F -FDG uptake as similar to the change of tumor volume. Thus, tumor status could be verified by comparing the change of ^{18}F -FDG uptake and tumor size. In addition, the reason that Group 3 showed lower ^{18}F -FDG uptake than Group 2 is assumed to be because the tumor necrosis induced by the embolization effect of CMH into tumor in Group 3 had progressed more than in Group 2. Ultrasonography has been well known to provide anatomical information to diagnose diseases or tumor in real time. The tumor size was easily measured by ultrasound during experiments. Even though the operator needs to be skilled at handling the ultrasound device, ultrasonography is very helpful to diagnose several targets. Accordingly, nuclear medicine and radiology based imaging technique could be useful with pre-clinical and clinical follow-ups [17].

Specific iodine radioisotopes have been commonly used to diagnose or treat diseases of clinical importance. ^{125}I used in the current study is most commonly used to produce radiolabeled compounds for in vitro experiments. ^{125}I has very

low gamma energy (27–35 keV) of photons, which preclinical gamma camera can acquire in vivo study image. Radioisotope ^{131}I has been used to irradiate mainly cancer cells therapeutically. ^{131}I also emits gamma and beta emission simultaneously. This characteristic permits this radioisotope to be used in the theranostics field as well [18, 19]. Therefore, if CMH labeled with radioisotope ^{131}I instead of ^{125}I is used, more highly effective selective arterial radioembolization as main treatment is expected in future studies.

Conclusion

Selective arterial embolization therapy with CMH was significantly effective for tumor treatment in a rodent sarcoma model involving implantation in thigh muscle. Molecular imaging modalities including ultrasonography are feasible to be utilized as anatomical imaging tools to provide valuable information on the location and amount of embolic agent and glycolic metabolism of a tumor. The information will help researchers predict the possibilities of therapeutic success, complication development, and tumor metabolic status.

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Ethical Statement This study was approved by the animal ethics committee in our hospital and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Conflict of Interest Tai Kyoung Lee, JeongIl Kwon, Kyung Sook Na, Hwan-Seok Jeong, Hyosook Hwang, Phil-Sun Oh, Dong Hyun Kim, Kyu Yun Jang, Seok Tae Lim, Myung-Hee Sohn, Hwan-Jeong Jeong declare that they have no conflict of interest.

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