SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

THE INVESTIGATION OF ANTI-TUMOR EFFECT OF FUNCTIONALIZED HYBRID AEROGEL MICROPARTICLES AGAINST LYMPH SPREADING TUMOR CELLS

BY GÁBOR KIRÁLY

SUPERVISORS: PROF. DR. ISTVÁN JUHÁSZ

DR. GÁBOR SZEMÁN-NAGY

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF DENTAL SCIENCES

DEBRECEN, 2019
THE INVESTIGATION OF ANTI-TUMOR EFFECT OF FUNCTIONALIZED HYBRID AEROGEL MICROPARTICLES AGAINST LYMPH SPREADING TUMOR CELLS

BY GÁBOR KIRÁLY

MSc in Biotechnology

SUPERVISORS: PROF. DR. ISTVÁN JUHÁSZ, MD, PhD

DR. GÁBOR SZEMÁN-NAGY, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF DENTAL SCIENCES

Head of the Examination Committee: PROF. DR. ILDIKÓ MÁRTON, MD, PhD, DSC

Members of the Examination Committee: DR. ESZTER BALTÁS, MD, PhD

PROF. DR. CSABA HEGEDŰS, MD, PhD

The Examination takes place at Department of Restorative Dentistry, Faculty of Dentistry, University of Debrecen, at 11:00 a.m. on July 11th, 2019.

Head of the Defense Committee: PROF. DR. ILDIKÓ MÁRTON, MD, PhD, DSC

Members of the Defense Committee: PROF. DR. GYÖNGYVÉR SOÓS, PhD

PROF. DR. ISTVÁN BÁNYAI, PhD, DSC

PROF. DR. CSABA HEGEDŰS, MD, PhD

DR. ESZTER BALTÁS, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1 p.m. on July 11th, 2019.
1. INTRODUCTION

Sentinel lymph nodes of the peritoneal tumors

Nowadays, despite the advanced diagnostic and therapeutic procedures, metastases represent a high proportion of cancer-related deaths and this complex process is the least understood aspect of tumor biology. To establish metastasis the tumor cell must be escaped from the primary tumor and enter the blood or lymph circulation. Subsequently by extravasation the tumor cell exits from the capillaries to the distal organ and begins to proliferate. The “seed and soil” theory proved that metastasis is a controlled process, which is controlled by the interaction between the tumor cell and the microenvironment of distant organ. In the case of lymphatic metastasis the tumor cells from the primary tumor reach the nearest lymph node and forms metastasis. The entry of tumor cells into the lymph nodes via lymphatic drainage created a sentinel lymph node theory. According to the sentinel lymph node theory the first lymph node get a lymphatic drainage from the site of the tumor, which will contain tumor cells. The state-of-the-art imaging techniques (MRI – magnetic resonance imaging, CT – computer tomography) are essentinals to detect a metastatic lymph nodes. In the case of peritoneal tumors, the relevant sentinel lymph nodes are associated to the major abdominal organs, but the tumor cells not only near the primary tumor, but also in the distal parts of the body can develop sentinel lymph node. Mostly, in the case of gastric cancer it could be observed, that the tumor cells from abdominal cavity enter the lymph nodes in the mediastinal region and then formed metastasis.
Animal models for the investigation of abdominal lymphatic drainage and metastasis

In many animal models have been described the lymphatic drainage pathways from the abdominal cavity, but it remains debated whether the abdominal cavity has a predetermined lymph flow pattern. As a result of the peritoneal injection of Evans blue dye or radioactively labeled albumin the particles appeared in the mediastinal and abdominal lymph nodes. Specific peritoneal lymphatic drainage in rat models, using different sized lymph tracers and infrared imaging, besides regional sentinel lymph nodes, metastasis was also seen in the upper mediastinal lymph nodes. In the lamb animal model, 3 major lymph nodes from the abdominal cavity were described, in which, like the different colloid and dye particles in the abdominal cavity, the tumor cells also passed through the diaphragm to the mediastinal direction, and passed through the parasternal lymph nodes to the parathymic lymph nodes.

Drug delivery systems for targeting cytostatic agents

In the treatment of cancer diseases different toxic drugs are used which have side effects, limited efficacy and the major drawback is the lack of target specificity. High dosage ranges and low penetration distance (only 2-3 mm) are further disadvantages of cytostatic drugs, which are used in the case of peritoneal tumors. In conventional tumor therapy, methotrexate (MTX) is an effective antineoplastic agent, but it has many side effects. MTX absorption at increasing concentrations follows saturation kinetics in the gastrointestinal tract, bioavailability of the drug to the tumor is dose-dependent and the kidneys are quickly filter out most of the MTX at high concentrations. This results short plasma half-life (5-8 hours) and low drug concentration in the target tissue. Several methods have been used to
target MTX release: packing of the drug into lipids, the binding to hydrophilic gelatin microspheres and the application in emulsion similar to kilomicrons was also useful in the enhancement of target specificity and bioavailability of MTX.

**Aerogels and hybrid aerogels as drug delivery systems**

Aerogels as porous systems, because of their large internal surface, surface / volume ratio, large pore volume and uniform pore size, are ideal candidates for usage as drug carrier. Because silica aerogels are not biodegradable, and because of severe side effects (silicosis) due to their usage, researches has turned to an organic part containing hybrid aerogels. In the case of hybrid aerogels, the combination of the inorganic component, which has a large surface area with the biodegradable organic part produces a new material that makes them suitable as a carrier material. In many publications, the release of drug molecules from aerogels was based on the different properties of the target tissue, such as the altered pH of the target tissue, increased enzyme function, redox changes, or competitive inhibition. In tumor treatment studies, different intracellular enzyme activities, which are different from normal cells, have been used to release drug from hybrid aerogels. Mesoporous silica nanoparticles functionalized with doxorubicin (DOX) were injected into the tail vein of a mouse with hepatocellular carcinoma. The particles accumulated in the liver tumor, and the increased matrix metalloprotease (MMP-9) activity of hepatocellular carcinoma cells was responsible for drug release.
2. RESEARCH OBJECTIVES

Our previous studies showed that intraperitoneally administered colloidal ink particles and cancer cells traversed the diaphragm and appeared in the upper mediastinal and parathymic lymph nodes in rat model. In the first part of our work the main objective was to investigate the appearance of intraperitoneally administered colloidal particles and metastatic SCC VII tumor cells in the parathymic lymph nodes in a mouse model. The following experiments were performed for these tests:

1) We wanted to investigate whether mouse cell carcinoma (SCC VII) tumor cell suspension in mice i.p. administered, then the tumor cells from the peritoneum through the lymphatic circulation enter the parathymic lymph nodes and form metastasis. We wanted to investigate the tumor metastasis pathway macroscopically (examining the metastatic pathway by i.p. administered colloidal Ink particles) and microscopically (histological staining and immunohistochemical labeling).

2) Methotrexate (MTX) as a model cytostatic was used to treat SCC VII cells, which were used in the experimental mouse model. In the in vitro studies, we wanted to demonstrate the genotoxic, micronucleus forming effect of MTX on tumor cells by cytotoxic assays and by examining the chromatin condensation intermediates of the SCC VII cells and the chromatin structures formed.

The hybrid, biodegradable material containing aerogels are suitable candidates as drug delivery systems due to their many advantageous structural properties. Hybrid aerogel microparticles were designed to perform the following experiments:

3) We wanted to prove that the newly synthesized, hybrid silica-gelatin aerogel microparticles (H-AG) are biocompatible with the cells in vitro, long-term time-lapse video microscopy was used for these observations.

4) The release of drug from MTX-functionalized H-AG microparticles (MFH-AG) was observed in various cell-free and cell-containing media. Finally, the cytotoxicity of MFH-AG microparticles were tested on mouse SCC VII and human HL-60 and HaCaT cell lines using MTT viability assay.
3. MATERIALS AND METHODS

Cell cultures

The experiments were performed with mouse carcinoma (SCC VII) and human (HaCaT, HL-60) cell cultures. SCC VII cells were grown in Dulbecco’s Modified Eagle’s Medium Nutrient Mixture (DMEM-HAM’S F12) (Sigma-Aldrich) supplemented with 2mM L-glutamine, 23 mM NaHCO₃, 100 U/mL penicillin, 100 U/mL streptomycin, 1% non-essential amino acids and 10% fetal bovine serum (FBS). The HaCaT cells, which are immortalized, but not transformed epithelial cells were propagated in DMEM supplemented with 2mM L-glutamine, 23 mM NaHCO₃, 100 U/mL penicillin, 100 U/mL streptomycin, 1% non-essential amino acids and 10% fetal bovine serum. The promyelocytic human leukemia cell line was cultured in RPMI 1640 (Roswell Park Memorial Institute) (Sigma-Aldrich) supplemented with 2mM L-glutamine, 23 mM NaHCO₃, 100 U/mL penicillin, 100 U/mL streptomycin, 1% non-essential amino acids and 10% FBS.

Mouse experiments

Male and female (5-5) C3H mice were kept in a conventional laboratory environment and fed on a semi-synthetic diet (Charles River Mo, Kft, Godollo, Hungary) and tap water ad libitum. Ink particles (0.2 ml) was injected i.p. into the peritoneum of the 8-10 weeks old C3H mice to mimick metastatic spread, the experiment lasted for 48 h, before animals were sacrificed and the necropsy performed. C3H mice were infected with SCC VII cells, SCC cells (1.5 × 10⁵) suspended in 0.2 ml saline were injected i.p. into mice, and after 30 days the mice were sacrificed by cervical dislocation. The abdominal cavity was opened, the primary tumor and the thymus were isolated. During mouse autopsy the images were taken with a camera with 5 megapixel resolution.
Histology, immunohistochemistry

After necropsy, the thymus including parathymic lymph nodes were fixed in 4% buffered formaldehyde. The specimens were embedded in paraffin, sectioned, stained with hematoxylin-eosin (H&E) and examined under the microscope. Histological studies of the primary tumors grown on the abdominal wall and the tumor-bearing lymph nodes of the thymus were performed at the Department of Pathology, Kenézy Hospital of Debrecen. For the identification of SCC VII cells in formalin fixed and paraffin-embedded mouse lymph nodes, immunohistochemical analysis of cytokeratin 14 (clone: LL002, Biocare Medical, Pacheco, CA, USA) positive cells was performed. Images from the histological samples were taken with a LEICA DM 2000 microscope.

Isolation of chromatin structures, fluorescent microscopy

Firstly, the control and methotrexate (MTX) treated cells (0.1-1-10-100 μM concentrations) were reversibly permeabilized, which ensures temporary penetrability of cellular membranes. For this experiment we used a T-150 dextran containing hypotonic buffer, then the cells were regenerated in a 10% FBS containing cell culture medium. Thereafter colcemid were added (0.2 ng/ml) to the medium, which was followed by 2 hours incubation, depending on the number of the cells. The cells were washed with PBS 2 times. Then, swelling buffer was used for osmotic swelling of the cells, incubating them at 37 °C for 10 minutes. The swelling buffer was removed by centrifugation (5 minutes, 500g). Nuclei were isolated by the slow addition of 1 ml Fixative (methanol:glacial acetic acid, 3:1) washed twice in Fixative and then resuspended in 1 ml of Fixative. Cells taken in the Fixative solution were dropped using a Pasteur pipette on a microscope slide from a height of 30 cm, then the slides were dried at room temperature overnight. Slides were rinsed with PBS
and dehydrated using increasing concentrations of ethanol (70, 90, 95 and 100%). Dehydrated slides containing isolated chromatin structures were mounted in 35 µl Antifade Medium under 24x50 mm coverslips. Blue fluorescence of DAPI was monitored with an Olympus AX70 fluorescence microscope.

**Micronucleus formation tested by Giemsa staining**

This experiment served only to test that MTX treatment indeed generates micronuclei. Nuclei were isolated from intact cells and stained with Giemsa. Nuclei isolated from SCC cells after 10 µM and 100 µM MTX treatment.

**Preparation of hybrid silica-gelatin aerogel (H-AG) and methotrexate functionalized hybrid silica-gelatin aerogel (MFH-AG) microparticles**

**Preparation of gelatin-methotrexate conjugate**

0.50 g gelatin was dissolved in 30 mL 0.05 M NaHCO₃ solution. This was mixed with a solution of 150 mg MTX in 15 mL 0.05 M NaHCO₃. The pH of the mixture was set to 6.0 with HCl solution, and it was stirred at 300 rpm at room temperature for 2 h. 750 mg 1-ethyl-3-(3-diaminopropyl)-carbodiimide hydrochlorid (EDC) was introduced into the previous solution and the reaction mixture was stirred overnight. The conjugate was purified using 7000 MWCO SnakeSkin Dialysis Tubing submerged in phosphate buffer (0.05 M, pH = 7.4), and later in water. The aqueous medium was replaced after 24 h soaking, and 5 dialysis cycles were performed. The final, dry G-MTX conjugate was obtained by lyophilization.

**Preparation of methotrexate functionalized silica-gelatin aerogel**

0.55 g gelatine-MTX conjugate and 70 mg (NH₄)₂CO₃ were dissolved in 20 mL hot distilled water. After cooling the aqueous solution near to room temperature, a second solution of 3.0 mL tetramethyl ortosilicate (TMOS) dissolved in 7.0 mL
methanol was mixed into it. The reaction mixture was vigorously stirred and poured into a cylindrical plastic mold for gelation. After 24 h, the alcogel was removed from the mold and placed into a perforated aluminum container for multiple step solvent exchange. First, the alcogel was soaked in methanol for 24, next the methanol was replaced by acetone in four 24 h soaking steps, and acetone was replaced 2 more times after 24 h soaking. The acetone was replaced 3 more times to remove any trace amounts of water from the porous system. No MTX leach was observed during the multiple steps of solvent exchange, confirmed by fluorimetry. Finally the gel was dried with supercritical CO₂ at 14 MPa and 80 °C in a pumpless drying system. The composition of the hybrid aerogel (MFH-AG) was estimated by thermal analysis to be ca. 65 wt.% silica and 35 wt.% G-MTX.

**Aerogel characterization**

**Electronmicroscopy and porosity investigations of hybrid aerogel particles**

Scanning electron microscopic (SEM) images were recorded on a Hitachi S-4300 instrument (Hitachi Ltd., Tokyo, Japan). Aerogel shards, freshly split from the monoliths were immobilized on carbon tapes and covered by 5 – 6 atomic layer thick sputtered gold conductive layers. Typically, 10 – 30 kV accelerating voltage was used.

Specific surface areas, pore size distributions and total pore volumes of aerogel samples were measured by N₂ adsorption-desorption porosimetry (Quantachrome Nova 2200e) after degassing at 40 °C for 24 h. Specific surface area was calculated using the multipoint BET method. Pore size distribution was calculated from the desorption curves using the BJH method.
Sterilization and conditioning of the functionalised silica-gelatin aerogel

Silica-gelatin aerogel slabs and particles were sterilized with UV light for 48 hours before the in vitro experiments. 1–2 mm thick slabs were used for the adherence experiments, while ground and sieved (d < 125 μm) particles were used for the migration studies. After sterilization, the aerogel samples were placed into cell free DMEM-F12 medium for conditioning for 3 h, then the cells were added to the cell culture flasks.

Time-lapse videomicroscopy and digital image analysis

For the biocompatibility studies time-lapse video microscope system was used, which is suitable for long-term observation of the cells: the applied 4 microscope system with near infrared illumination (960 nm) provided the long-term observations of the cell cultures in the cell culture incubator. The image sequences, which are generated by the video microscopy system, were used to obtain qualitative and quantitative informations from the cells with high temporal resolution for dynamic analysis of toxic processes.

Measuring the size distribution of cells and MFH-AG microparticles

The size distribution of micronized MFH-AG particles and SCC VII cells were characterized by using a hemocytometer and image analysis. Images were taken with a 1.3 MP USB microscope camera (MicroQ Industrial Digital Camera), and the ImageJ program was used for measuring the size distribution of the cells and the particles. The results were evaluated with Microsoft Excel.

Additionally, the size distribution of aerogel microparticles was measured by laser diffraction size measurement (Mastersizer 2000, MALVERN Instruments). A
series of measurements were conducted in 10 min intervals over 2 h in order to monitor the spontaneous degradation of the aerogel particles.

**Testing the release of MTX from functionalized (MFH-AG) particles**

The MTX release from MFH-AG particles firstly was studied in cell free buffers and cell culturing medium. The micronized aerogel particles were soaked for 72 h in different aqueous media, in addition the concentration of the particles was always 1.00 mg/mL. 3 different buffer solutions were used: 0.01 M HCl solution, pH = 5.0 acetate buffer and pH = 7.4 PBS. Besides these, dissolution was also tested in Dulbecco's Modified Eagle's Medium Nutrient Mixture (DMEM-HAM’S F12) supplemented with 2 mM L-glutamine, 23 mM NaHCO$_3$, 100 U/mL penicillin, 100 U/mL streptomycin and 1% non-essential amino acids. The DMEM medium was tested both in the absence and in the presence of 10% fetal bovine serum (FBS).

During the co-incubation of SCC VII cells and MFH-AG particles we used 5 test groups: 1) The free MTX content of the supernatants of cell cultures was measured after incubating the cultures with MFH-AG particles. 2) Additionally, after growing cells in DMEM-HAM’S F12, the supernatant of SCC VII cells was also used as a dissolution medium to test MTX release from MFH-AG. A complementary test was also conducted where 1% gelatin coating was applied to induce the growth of the SCC VII cells for 72 h. Cells were then harvested and sonicated. 3) The lysate and the 4) supernatant of the intact culture were incubated separately with MFH-AG particles to test the release of MTX. 5) The enzymatic cleavage of MFH-AG was tested by incubating the particles for 24 h with 1 unit of Cat-B in PBS.
All experiments were run in triplicates. The amount of released MTX was quantified in every experiment by UV-Vis spectroscopy using the method of standard addition for each sample.

**Determination of cell viability after treatment of MFH-AG microparticles**

The viability investigations of different tumorous (SCC VII and HL-60) and nontumorous cell lines were tested on a 96-well plate. During the experiments after the incubation times (24, 48 and 72 hours) the cells were washed twice with PBS (100 µl), 100 µl MTT reagent (0.5 mg/ml) was added to each well, then the plates were placed back into the cell culturing incubator for 3 hours. Next, the MTT solution was removed from each well and the resulting formazan crystals were dissolved in dimethyl-sulfoxide (200 µl each well). The viability of treated cells was determined as a percentage (%) viability relative to the control cells.

**Statistical analysis**

The presented results show data from at least three independent experiments conducted on different days. The results are determined as mean ± standard deviation. Statistical analysis was performed with a *t-test* or, in the case of multiple comparisons, by ANOVA. Statistical analysis was performed using GraphPadPrism 6 Windows software (Graphpad software, San Diego California, USA). The results of *p < 0.05, **p < 0.01 were considered to be significantly different.
4. RESULTS AND DISCUSSION

The investigation of the peritoneum-parathymic lymph node complex in metastatic mouse model *in vivo*

4.1. The Ink particles appeared in the thymus and in the parathymic lymph nodes after intraperitoneal injection

In the first mouse experiments Pelikan ink colloid particles was administered i.p. into 2 male and 2 female C3H mice and we investigated the appearance of colloid particles in the mouse parathymic lymph nodes. After 48 hours the colloid particles accumulated in the parathymic lymph nodes on the 2 sides of the thymus. The ink particles were enriched in the 2 upper and below in the 2 smaller symmetrically located parathymic lymph nodes in the thymus. Hematoxylin-eosin staining also showed the presence of ink particles in the cortex region of the thymus.

4.2. Mouse SCC VII tumor cells traversed the diaphragm and appeared in the parathymic lymph nodes by metastasis after i.p. administration

C3H mice were administered i.p. SCC VII cells (1.5 × 10⁵) and after 30 days the resulting primary tumor was isolated from the peritoneum, tumors of the abdominal organs and the metastatic cells containing thymus were removed from the mice. During autopsy the enlargement of the inflamed and reddish thymus could be observed. Heamatoxylin-eosin staining was able to distinguish well the basophil stained cells of healthy thymic tissue from eosinophil stained cancer cells. Tumor tissue was characterized by high cell density and large number of dividing nuclei. The presence of tumor cells in the lymphatic tissue was also demonstrated by immunohistochemical labeling (cytokeratin 14), during which the tumor cells were stained dark brown.
The investigation of the intermediates of chromatin condensation of SCC VII cells after MTX treatment

4.3. We have demonstrated the genotoxicity of MTX in the nuclei of SCC VII cells after reversal permeabilization

Four major types of chromatin changes were seen in nuclei of SCC VII cells after the 1 µM MTX treatment: a) micronucleus-like forms; b) extrusion of fibrillar chromatin at one or more locations of the nucleus, pointing to the membrane damage, rather than single micronucleus formation; c) appearance of one or more small wholes in the nuclear membrane confirming the notion of membrane damage by MTX; d) extensive membrane damage represented by large wholes. After MTX treatment chromosome condensation did not reach metaphase and was inhibited after ribbon formation probably due to insufficient supercoiling.

4.4. Micronucleus formation tested by Giemsa-staining

Exponentially growing SCC VII cells were treated with MTX at various concentrations (10 and 100 µM) for 9 hours followed by isolation of the nuclei and staining. Compared to the control nuclei the apoptotic nuclei of 10 µM MTX treated SCC VII cells, and the 100 µM MTX treated nuclei with large macronuclei were clearly visible.

4.5. We proved MTX induced micronucleus formation in non-permeabilized cells

Although, in SCC cells without permeabilization the interphase chromatin structures were not visible, but after MTX treatment generated micronuclei between 0.1 and 100 µM concentrations. MTX generated relatively small micronuclei at low (0.1-1.0 µM) concentrations, reduced the size of macronuclei at apoptotic (1-10 µM) concentrations, increased the size of macronuclei and caused the disrup-
tion of nuclei at necrotic (100 µM) MTX concentration. The frequency of micro-nucleus formation depended on the MTX concentration. This observation indicated that micronucleus formation in early S phase was crucial not only in chromatin folding but also a sensible stage for their appearance upon membrane damage caused by the genotoxic agent.

**Characterization of hybrid silica-gelatin aerogel particles**

4.6. Scanning electron microscopy and N$_2$ porosimetry

Electron microscopic images of methotrexate functionalized hybrid silica gelatin aerogel microparticles (MFH-AG) were taken at 20.000x magnification. No significant structural differences are present between H-AG and MFH-AG analogous silica-gelatin hybrid aerogels. The MFH-AG aerogel backbone consists of spherical blocks with diameters between 40 and 100 nm. As a summary, the results of the porosimetry measurement do not reveal any structural changes that could be attributed to the incorporation of MTX into the hybrid aerogel.

4.7. Size distribution of aerogel particles and cells

Determining size of the SCC VII cells and the MFH-AG microparticles, it was found that when the 2-3 mm pieces obtained from the aerogel monolith were placed in an aqueous medium for almost 2 hours, the particles have fallen to near cell size ca. 15-20 µm. After treatment with the tissue homogenizer and sonication the particles (12,13 ± 1,5 µm) have fallen to approximately the same size as the SCC VII cells (14,5 ± 1,6 µm).
The investigation of the interaction of H-AG and MFH-AG particles

4.8. We proved that the hybrid silica-gelatin aerogel (H-AG) microparticles are biocompatible with the cells

Biocompatibility tests were performed with the custom-built *in vitro* long-term video-microscopy system. The adhesion time was approximately two-fold longer on the aerogel surface than in the case of the control cells. The SCC VII cells took a longer pathway and moved faster than the control cells in the presence of H-AG microparticles, in addition the cells migrated toward the aerogel particle. The directional movement of SCC cells is attributable to the chemoattractant property of gelatin content in H-AG particle. Neither apoptosis, nor necrosis were observed in adhesion and migration experiments, so the results showed the biocompatibility of H-AG microparticles.

4.9. The presence of the cells was required for the MTX release from the MFH-AG particles

The release of drug from methotrexate functionalized H-AG particles was firstly measured in buffers with different pH (0.01 M HCl solution, in pH = 5.0 acetate buffer and in pH = 7.4 PBS), and in cell culture medium. After 72 hours of incubation in both buffers and medium, the amount of released MTX was below detection limit. MFH-AG particles were incubated with cells and cell supernatant, which resulted 8.6% and 12% MTX release. The MFH-AG particles were incubated with the supernatant of cells grown on 1% gelatin coating and incubated with cell lysate obtained after sonication of the cells provided 8.7% and 11.6% MTX release. From the MFH-AG particles 4.6% MTX release was measured by the effect of Cathepsin-B enzyme. These results showed that *in situ* synthesized collagenase enzyme by tumor cells was responsible for drug release.
4.10. HaCat, SCC VII and HL-60 growth inhibition

Based on the results of MTX release experiments, the MFH-AG particles were incubated with tumorous and non-tumorous cell cultures with different collagenase activity. The 0.2 mg/ml MTX and 2 mg/ml MFH-AG concentrations contained a weight equivalent agent in the experiments. After 24 hours of incubation growth inhibition was observed for all 3 types of cell. Only the viability of HL-60 cells showed significant difference between the 0.2 mg/ml MTX and 2 mg/ml MFH-AG concentrations. 48 hours of treatment showed significant cytotoxicity at concentrations of 0.2 mg/ml MTX and 2 mg/ml MFH-AG for HaCaT and HL-60 cells. The toxicity of HL-60 cells was dose-dependent. At the end of 72 hour treatment the mortality of HL-60 cells at all concentrations was higher than 80%. The SCC VII and HaCaT cell lines showed a near 30% decrease in viability at the highest MFH-AG concentration. After 72 hours there was no difference between 0.2 mg/ml MTX and 2 mg/ml MFH-AG for any cell line.
5. **SUMMARY**

In the first part of our work we examined the development of metastasis from the abdominal cavity through the lymphatic drainage to the upper *mediastinal* lymph nodes in mouse model. We have shown that, similarly to the India ink colloidal particles, the mouse cancer cells (SCC VII) traversed the diaphragm and appeared in the parathymical lymph nodes after intraperitoneal injection. Hematoxylin-eosin and immunohistochemical stainings have shown that parathymic lymph nodes contained metastatic cells of abdominal tumor. In the used mouse model the parathymic lymph nodes are sentinel lymph nodes, which have a significant role in medical diagnostics. The described mouse metastatic model may be suitable for testing various antitumor agents and/or drug carriers *in vivo*, that are potentially suitable for the treatment of lymphatic metastasis.

In the second part of the research we investigated the effect of methotrexate (MTX), as a model cytostatic agent, on the chromatin condensation processes of murine SCC VII tumor cells. We found that micronucleus was formed at low (0.1-1 µM) concentrations, at higher concentrations apoptotic (1-10 µM) and necrotic (100 µM) alterations could be observed. It should be noted, however, that micronucleus formation, which is an indicator of citotoxicity, is part of the normal intermediates of chromatin condensation and may be due to chromatin structures passing through the openings of the nuclear membrane.

The third part of our study was designed to investigate the biocompatibility of newly synthesized H-AG microparticles with cells. For these studies a time-lapse video microscope system was used, which enables a long-term observation on the cells, and MTT-assay was also used. Based on the biocompatibility results, it was visible that the H-AG microparticles stimulated cell divisions cells and the cells
migrated towards the particles. Based on these results it was desirable to create an anti-tumor agent containing aerogel-based system. Aerogel particles were functionalized with MTX (MFH-AG) and the cytotoxic effect of the particles were tested on various cell lines with different collagenase activity. The cytotoxicity of the near cell-sized MFH-AG particles (at 2 mg/ml concentration) was equivalent to the effect of the free MTX (at 0.2 mg/ml concentration) in the aqueous medium, furthermore the different collagenase activity of the cells was the requirement of the drug release from the aerogel particles.
List of publications related to the dissertation


DOI: http://dx.doi.org/10.1556/030.65.2018.015 
IF: 1.107 (2017)


IF: 3.338

DOI: http://dx.doi.org/10.1016/j.jtemb.2014.12.011 
IF: 2.55

DOI: http://dx.doi.org/10.1155/2014/976394 
IF: 3.516

10. Scharfenberger, L., Hennerici, T., Király, G., Kitzmüller, S., Vernooij, M., Zielinski, J. G.: 
Transgenic mouse technology in skin biology: generation of complete or tissue-specific knockout mice. 
DOI: http://dx.doi.org/10.1038/jid.2013.457 
IF: 7.216

DOI: http://dx.doi.org/10.1089/dna.2013.2147  
IF: 1.991

**Total IF of journals (all publications): 31.97**  
**Total IF of journals (publications related to the dissertation): 12.252**

The Candidate's publication data submitted to the IDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

29 May, 2019
LECTURES AND POSTERS RELATED TO THE THESIS

Király G, Szemán-Nagy G, Veres P, Kalmár J, Bánfalvi G, Juhász I. The application of functionalised aerogel particles against a lymph spreading tumor cell line. MEDPécs 2018, Medical Conference for PhD students and Experts of Clinical Sciences, 27th of October 2018


LECTURES AND POSTERS NOT RELATED TO THE THESIS
