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RESEARCH ARTICLE

Experimental Peritoneal Metastasis Model: Which Type of Rodents Should we Choose, and which Method Should we Perform for the Intraperitoneal Inoculation of Tumor Cells?

Berke Manoğlu[1], Tayfun Bişgin[1], Aras Emre Canda[2], Safiye Aktaş[2], Zekiye Sultan Altun[2], Osman Yılmaz[3]

[1]Dokuz Eylül University Faculty of Medicine, Department of General Surgery, İzmir, Turkey
[2]Dokuz Eylül University Institute of Oncology, Department of Basic Oncology, İzmir, Turkey
[3]Dokuz Eylül University Faculty of Medicine, Department of Laboratory Animal Science, İzmir, Turkey

ABSTRACT

Aim: This study aimed to create a peritoneal metastasis (PM) model in Wistar albino rats and nude mice and compare PM models and different tumor cell inoculation methods in the two experimental animal types.

Method: There were two main groups: group 1 comprised Wistar albino rats (n=16), and group 2 comprised nude mice (n=16). The group comprising rats was divided into two subgroups (1A and 1B), to which different tumor inoculation methods were applied. Group 2, comprising nude mice, was divided into two subgroups (2A and 2B), to which different tumor inoculation methods were applied. Euthanization was performed on the 7th and 14th days after tumor inoculation. The obtained samples were evaluated macroscopically, microscopically, and biochemically.

Results: Although no PM model was formed in group 1, a PM model occurred in the subjects in group 2 who were euthanized on the 14th day. There was no statistically significant difference between the mean peritoneal carcinomatosis index scores, tumor diameters, and the amount of intra-abdominal ascites in the subgroups (2A vs. 2B), in which the PM model was created by two different methods.

Conclusion: The inoculation of tumor cells with the peritoneal injection method enabled the creation of a PM model that can be used in experimental studies. Although a PM model could not be established in rats, a complete PM model was established in nude mice. In future studies, we plan to evaluate the efficacies of different drugs in the PM models we have created.

Keywords: Peritoneal metastasis model, colorectal cancer, tumor inoculation

Introduction

Colorectal cancer (CRC) is the second most common cause of cancer-related deaths worldwide. Peritoneal metastasis (PM) has the worst prognosis among all CRC metastases.[1] While an average survival of 1 year can be achieved with systemic chemotherapy in patients with PM due to CRC, 5-year survival rates can reach up to 40-58% with cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC).[2] Therefore, as standard treatment in selected patients, the use of CRS + HIPEC is recommended.[3] Although it is more successful against systemic chemotherapy, there is not enough scientific evidence on CRS + HIPEC. While only hyperthermia has a cytotoxic effect on cancer cells, CRS and HIPEC potentiate each other's effects when combined with chemotherapy.[4] When compared with systemic chemotherapy, intraperitoneal chemotherapy provides a more intense concentration of chemotherapeutic agents on tumor cells, with lower systemic toxicity.[5] Thanks to all these favorable effects, when HIPEC is applied, a 20-50 times more intense tumoricidal effect is achieved compared with using systemic chemotherapy.[6]

Address for Correspondence: Berke Manoğlu, MD,
Dokuz Eylül University Faculty of Medicine, Department of General Surgery, İzmir, Turkey
E-mail: berkemanoglu@hotmail.com **ORCID ID:** orcid.org/0000-0003-4755-2200
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In our study, we created a PM model in Wistar albino rats and nude mice using CC-531 (rat-origin colon adenocarcinoma cell line). We also compared the PM models created in the two different experimental animal types using two different tumor cell inoculation methods. Using the results, we determined the most suitable type of rodent and the most appropriate technique to be used.

Materials and Methods

Our study was conducted at The Experimental Animals Laboratory between January and May 2022 with the approval of the Dokuz Eylül University University Local Animal Ethics Committee (approval number: 53/2019, date: 25.12.2019). In the process of establishing the PM model, 16 male 10-to-12-week- old Wistar albino rats and sixteen 7-to-8-week-old nude mice (athymic mice) bred in the Experimental Animals Laboratory were used. Nude mice caged in groups of four under laboratory conditions in air-filtered laminar flow cabinets were monitored. The mice were fed irradiated food and autoclaved reverse-osmosis-treated water. All treatments were carried out under sterile conditions in a laminar flow hood. The Wistar albino rats were caged in groups of eight.

The group of rats was divided into two separate subgroups (1A and 1B), in which two different tumor inoculation methods were applied separately. Group 2, consisting of nude mice, was divided into two subgroups (2A and 2B), each of which received a different tumor inoculation method. In the animals in groups 1A and 2A, tumor cell inoculation was performed by intraperitoneal injection, while in the animals in groups 1B and 2B, it was performed after peritoneal irritation via a laparotomy incision (Table 1). The mean weights of the Wistar albino rats and nude mice were 300 (±50) g and 32 (±2) g, respectively.

Intraperitoneal inoculation of tumor cells: Cancer cells from the CC531 colon adenocarcinoma cell line were harvested during the logarithmic growth stage by incubation at 37 °C under a humidified 5% CO_2 atmosphere. Cells were then resuspended in phosphate-buffered saline (PBS) for intraperitoneal injection. The suspended cells were administered to the animals in groups 1A and 2A by intraperitoneal injection using 16 mm long and 0.45 mm diameter needles. After sterilization of the abdominal area, the abdominal wall was passed by entering the abdominal midline from the right lateral at a 90° angle using a 16 mm needle. Then, a 1-2 mm needle was advanced at an angle of 45°. It was confirmed by aspiration that there was no intestinal content or bleeding. After these steps, tumor cells were injected into the abdominal cavity.

Table 1. Groups and tumor cell inoculation method

Groups	Subgroups (tumor cell inoculation method)
Group 1 (n=16) Wistar albino rat	Group 1A (inoculation by intraperitoneal injection)
	Group 1B (inoculation with laparotomy)
Group 2 (n=16) Nude mouse	Group 2A (inoculation by intraperitoneal injection)
	Group 2B (inoculation with laparotomy)

Diethyl ether inhalation anesthesia was applied to the animals in groups 1B and 2B, and the abdominal skin was cleansed with povidone-iodine solution. The necessary sterilization conditions were provided by covering the mice or rats with sterile surgical drapes. A midline abdominal incision of approximately 5 mm in length was made, and the abdominal cavity was entered (Figure 1). Peritoneal irritation was performed with the help of sterile fine-tipped forceps, and peritoneal cells were inoculated into the abdomen. The midline incision was closed primarily with 4/0 prolene sutures. Intraperitoneal inoculation of $5x10^6$ cells (0.3 cc in 200 µL PBS) was performed in all groups in line with referenced studies.[4]

Follow-up, euthanization, and evaluation of subjects: The animals were followed up daily. Laparotomies were performed under diethyl ether inhalation anesthesia in four animals from each group on the 7th and 14th days to evaluate the results obtained. Those with macroscopic PM findings were scored according to the peritoneal carcinomatosis index (PCI). The PCI was calculated according to the largest tumor diameter obtained from the experimental model, the number of organs involved, and the presence of intra-abdominal acid, with scores from two adjusted points (small bowel, peritoneum, diaphragm, ascites, and other organs).[4] Peritoneal cancer indices were determined, and scoring totaling 8 points was performed with consideration of the organ involved and the tumor diameter.

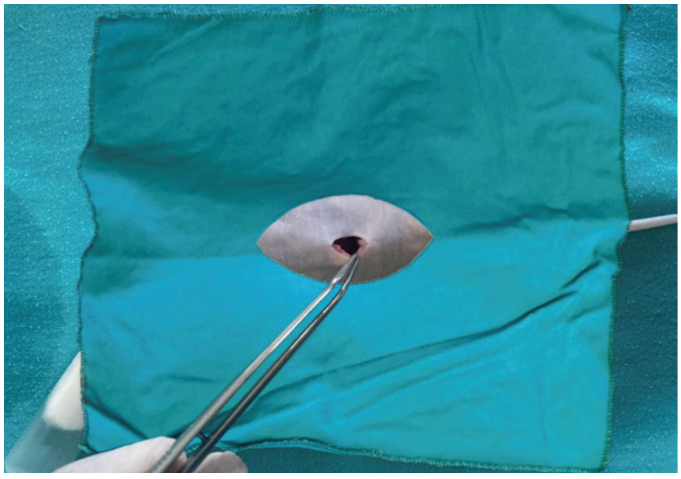

Figure 1. Tumor cells were inoculated into the intraperitoneal area by making an incision of approximately 5 mm

The results were evaluated as follows: small bowel and/or mesenteric involvement: 1 point; peritoneal involvement: 1 point; diaphragmatic involvement: 1 point; ascites (+): 1 point; involvement of other organs: 1 point. Tumor diameters were measured and scored as follows: no tumor growth: 0 points; nodule diameter ≤2 mm: 1 point; nodule diameter 2-5 mm or >5 tumor nodules: 2 points; nodule diameter ≥5 mm or >10 tumor nodules: 3 points (Figure 2). Ascites fluid was aspirated and quantified. Intra-abdominal lavage was performed using saline solution, and the examination of the samples retrieved did not reveal the presence of macroscopic tumors.

After the retrieval of samples from the small intestine, peritoneum, intra-abdominal fluid, and blood, the animals were euthanized, and the tissue and intra-abdominal fluid samples were evaluated histopathologically and biochemically. The tissue samples were fixed in 10% formaldehyde, cassetted, and embedded in paraffin blocks after tissue follow-up. Frozen sections of 5 µm in thickness were prepared from the optimal surface area of the sections. The sections were then stained with hematoxylin and eosin and examined under an Olympus x50 light microscope.

Tissue samples were evaluated for the presence of tumors, tumoral pattern, differentiation, apoptosis, mitosis, and necrosis. An evaluation was made by calculating the total number of mitoses in 10 different tumor areas by magnifying the field of vision 400 times under a light microscope with a 40x objective. The number of apoptotic cells was calculated by evaluating 5,000 cells and determining their percentage in 1,000 cells. The tissue samples were evaluated for evidence of tumor necrosis. Supernatants remaining after the centrifugation of the mice's intra-abdominal fluid samples were studied using lysyl oxidase-like protein 1 (LOXL1) and TWIST transcription factor (TWIST) mouse-compatible ELISA kits. Vascular endothelial growth factor (VEGF) levels in the diluted fluid samples were calculated, taking into account the mouse-compatible ELISA kit application steps. According to the absorbance values obtained from the standards, standard graphs of each test were created. Concentrations were expressed by calculating the absorbance values of the samples. The measuring range and the measurement sensitivity of the LOXL1 ELISA kit were 78-5,000 and 29 pg/mL, respectively. The measuring range and the measurement sensitivity of the TWIST test kit were 0.156-10 and 0.056 ng/mL, respectively. The measuring range and the measurement sensitivity of the VEGF test kit were 15-1,000 and 9,375 pg/mL, respectively.

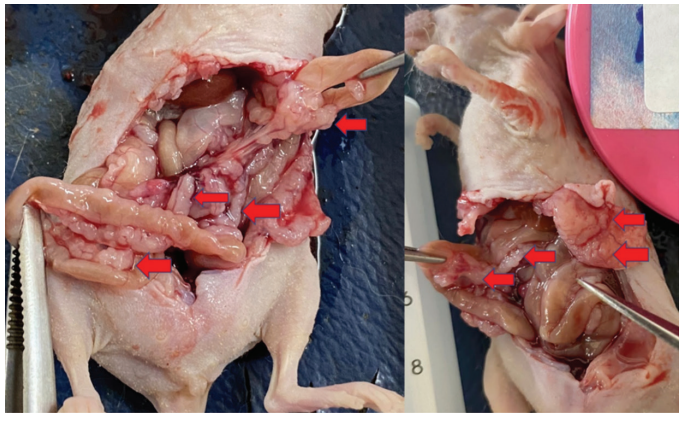

Figure 2. Metastatic nodules (shown with red arrows). The peritoneal metastasis model was scored macroscopically using the peritoneal cancer index

Statistical Analysis

Before the study, the number of experimental animals was determined by a power analysis. The maximum number of animals allowed by the animal experimentation ethics committee was used to ensure statistically significant results. Statistical analyses were performed using IBM's SPSS 24.0 statistics software program. The significance of differences was assessed using the Kruskal-Wallis test. Continuous variables were compared using an independent-samples t-test. Descriptive statistics were presented in the median (25^{th}-75^{th} percentile) format. Fisher's exact test, the chi-squared test, and t-tests were used for the analysis of qualitative data, and descriptive statistics were shown in the form of frequencies. A value of $p<0.05$ was defined as statistically significant.

Results

None of the rats in group 1 were lost during the experiment and follow-up. However, wound infection/dehiscence occurred in two animals in the group in which tumor inoculation was performed by laparotomy. No macroscopic or microscopic evidence of tumors was found in the evaluation performed after the euthanization of the experimental animals, and no PM developed in the Wistar albino rats.

In group 2, wound infection/dehiscence developed in three animals in the subgroup in which tumors were inoculated by laparotomy, one of whom exited on the 4^{th} postoperative day. On day 7, four subjects from both subgroups were euthanized. No macroscopic tumor or intra-abdominal ascites was detected. However, microscopic tumor cells were found in the intra-abdominal lavage fluid. On day 14, four experimental animals from each subgroup were euthanized. Macroscopic tumors and intra-abdominal ascites were detected in all subjects. Diffuse intra-abdominal ascites and widespread tumor implants were observed in the small intestines and peritoneum. In groups 2A and 2B, in which the PM model was created by two different methods, there was no statistically significant difference between the

mean PCI scores, tumor diameters, and the amount of intra-abdominal ascitic fluid [PCI: 7.50 (±0.57) vs. 7.25 (±0.50); tumor diameter: 3.75 (±1.70) mm vs. 3.50 (±1.29) mm; ascitic fluid: 3.50 (±1) mL vs. 3.37 (±0.85) mL] (Table 2).

Microscopic findings: When the tissue samples harvested from the intestinal system, peritoneum, and liver after euthanization were evaluated under a microscope, tumor cell infiltration was observed in all tissues. There were nodular and undifferentiated tumor samples. There was no statistically significant difference between groups 2A and 2B in terms of mitotic and apoptotic cell counts (Table 3).

Biochemical findings: There was no statistically significant difference between groups 2A and 2B in terms of the mean VEGF, LOX1, and TWIST values of intra-abdominal ascites (Table 4).

Table 2. There was no statistically significant difference between the mean PCI scores, tumor diameters, and the amount of intra-abdominal ascites in the subgroups (group 2A vs. group 2B), in which the PM model was created by two different methods

Mean ± SD	Group 2A	Group 2B	p-value
PCI	7.50±0.57	7.25±0.50	0.537
Tumor diameter (mm)	3.75±1.70	3.50±1.29	0.823
Ascites (mL)	3.50±1	3.37±0.85	0.855

Kruskal-Wallis test, t-test, PCI: Peritoneal carcinomatosis index, PM: Peritoneal metastasis, SD: Standard deviation

Table 3. The means of tumor tissues in the groups, mitosis counts, and apoptosis counts

	Mitosis count (40x)	Apoptosis count/1,000 cells
Group 2A Inoculation by intraperitoneal injection	12,250 (std 2.06)	6.75 (std 2.21)
Group 2B Inoculation with laparotomy	13,000 (std 1.41)	6.00 (std 2.70)
p-value	0.570	0.683

Kruskal-Wallis test, t-test, std: Standard deviation

Discussion

Experimental models created for the treatment of patients with PM will enable the realization of preclinical studies and new treatment options in the future. Studies based on mouse models allow researchers to learn about diseases with highly complex and dynamic pathophysiologies, such as cancer.[7,8] Clinical advances in cancer research in recent years have been associated with the efficient use of preclinical tumor models. They have also provided us with the opportunity to understand tumor growth, physiology, and interactions with the tumor microenvironment. Models created by grafting tumor cells into genetically engineered mouse models (nude/athymic mice) constitute useful and usable experimental tools in cancer research.[9,10] In our study, we used Wistar albino rats and genetically engineered nude mice, a species routinely used in experimental trials. We evaluated the relevant differences between both species. We also compared rats that were more suitable in terms of both size and endurance during surgical procedures and follow-up with much smaller and fragile immunosuppressive nude mice, in which the performance of surgical procedures could be more difficult. A suitable PM model could not be established using rats; however, we were able to create a suitable PM model in nude mice that could be used in experimental studies.

The most common cell lines used to induce the development of PM in mouse models include MC38 and CT26 (colon adenocarcinoma cell lines).[11-16] We used the CC531 rat colon adenocarcinoma cell line in our study, which enabled us to create an effective model for use in experimental studies. Peritoneal inoculation can be performed by intraperitoneal injection or via a laparotomy approach directly into the peritoneal cavity. The desired number of cells for the peritoneal inoculation model is determined according to the tumor cell line used and the degree of aggression (i.e., MC38: 2-5x10^5 cells and ID8 (epithelial ovarian cell line): 5-10x10^6 cells). For this reason, the number of cells used for peritoneal inoculation and the volume of cells suspended both for peritoneal inoculation and the wider dissemination of cells throughout the peritoneal cavity are of critical importance.[11-13,17] In our study, 5x10^6 cells were resuspended

Table 4 VEGF, LOX1, and TWIST values in intra-abdominal fluid

	VEGF	LOX1	TWIST
Group 2A	377,382 (std ± 174,620)	496,250 (std ± 166,851)	1,241 (std ± 0.205)
Group 2B	335,535 (std ± 128,002)	510,000 (std ± 140,059)	1,165 (std ± 0.219)
p-value	0.712	0.904	0.629

Kruskal-Wallis test, t-test, VEGF: Vascular endothelial cell growth factor, LOX1: Lysyl oxidase-like protein 1, TWIST: Twist transcription factor, std: Standard deviation

(0.3 cc, 200 µL PBS) and injected into the intraperitoneal area of all groups. Two different methods were used to inoculate cells intraperitoneally: either intraperitoneal injection or peritoneal irritation through a laparotomy approach under anesthesia, with the cells released directly into the peritoneal area. In both groups of different species of rodents, post-procedural morbidities (wound infection/dehiscence) were observed in the groups that underwent laparotomy using these two different methods. One animal died in the nude mouse group. The morbidity rate was 25% (n=2) in group 1B (Wistar albino rats), and the morbidity and mortality rates in group 2B (nude mice) were 50% (n=4) and 12.5% (n=1), respectively.

In different studies, a PM model was created between the 7th and 40th days, depending on the type of cells given after intraperitoneal inoculation.[4,18,19] In our study, the experimental animals divided into groups after the inoculation of tumor cells were euthanized on the 7th and 14th days. No tumors were detected macroscopically or microscopically in the animals in group 1. In group 2, extensive peritoneal tumors were observed in the group that was euthanized on the 14th day. On the 7th day, tumor cells were observed in the cytological samples obtained by intra-abdominal lavage, but no macroscopic tumors were observed.

There are significant differences between the peritoneums of rodents and humans. The most important difference is related to the omentum, which is a highly vascularized organ critical to the development of PM in humans; however, the mouse omentum is hypovascular and does not play the same role in mice.[20-22] Due to the complexity of cancer pathophysiology, it is very difficult to establish an ideal PM model. Therefore, results in mice should always be carefully evaluated and interpreted. However, rodents belong to a species suitable for simulating PM in experimental studies. Considering all these facts, PM models are applicable and suitable models for testing different chemotherapeutic agents for application to the peritoneal cavity.

Study Limitations

In our study, two different species produced by us in the animal experiments laboratory were used. The diversity of rodent species can be increased.

Conclusion

In our study, a suitable PM model was developed that can be used in studies performed with nude mice. We determined that intraperitoneal injection is the most appropriate method for the intraperitoneal inoculation of tumor cells. Using this method, a PM model can be created with acceptable morbidity and mortality rates. In future studies, we plan to use this model for intraperitoneal therapeutic approaches.

Ethics

Ethics Committee Approval: Our study was conducted at The Experimental Animals Laboratory between January and May 2022 with the approval of the Dokuz Eylül University University Local Animal Ethics Committee (approval number: 53/2019, date: 25.12.2019).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: B.M., T.B., Concept: B.M., A.E.C., Design: B.M., T.B., A.E.C., S.A., Z.S.A., O.Y., Data Collection or Processing: B.M., T.B., Analysis or Interpretation: B.M., S.A., Z.S.A., O.Y., Literature Search: B.M., T.B., A.E.C., S.A., Z.S.A., O.Y., Writing: B.M., A.E.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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