Microenvironmental Alterations by Radiation in Murine Hepatocarcinoma

Ik Jae Lee

Department of Medicine
The Graduate School, Yonsei University
Microenvironmental Alterations by Radiation in Murine Hepatocarcinoma

Directed by Professor JinsilSeong

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Medicine

Ik Jae Lee

December 2010
This certifies that the Doctoral Dissertation of Ik Jae Lee is approved.

Thesis Supervisor: Jinsil Seong

Thesis Committee Member#1: Nam Hoon Cho

Thesis Committee Member#2: Kyung Sik Kim

Thesis Committee Member#3: NaeChoon Yoo

Thesis Committee Member#4: Jeong-Sik Yu

The Graduate School
Yonsei University

December 2010
ACKNOWLEDGEMENTS

First I give thanks to my God for allowing me to write this dissertation, and a very special gratitude is owed to my supervisor Prof. Jinsil Seong for her guidance and encouragement. Her extraordinary intelligence and insights are qualities I admire. Without her guidance and persistent help this dissertation would not have been possible. I also want to give my thanks to Prof. Nam Hoon Cho, Prof. Kyung Sik Kim, Prof. Nae Choon Yoo, and Prof. Jeong-Sik Yu for their encouraging words and thoughtful criticism.

In addition, I appreciate Prof. Sang-Jun Ha, Prof. Beom Jin Lim, Hyo Jin Park, Wonwoo Kim, and Yoo Keun Shin for helpful discussions and technical assistance. With their help, my thesis has been greatly improved.

Finally, I would like to give thanks to my family for their love, sacrifice and encouragement.

I would like to dedicate this paper to all of you with all of my heart. I hope this will be a small step to help patients who suffer from cancer.

December 2010
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>4</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>8</td>
</tr>
<tr>
<td>1. In vivo tumorigenicity</td>
<td>8</td>
</tr>
<tr>
<td>2. Establishment of heterotopic and orthotopic murine hepatocarcinoma</td>
<td>8</td>
</tr>
<tr>
<td>3. Radiation to the heterotopic and orthotopic murine tumor</td>
<td>8</td>
</tr>
<tr>
<td>4. Comparative analysis of metastatic potential between heterotopic and orthotopic murine tumor</td>
<td>9</td>
</tr>
<tr>
<td>5. Immunohistochemical staining of tumor microenvironmental molecules</td>
<td>10</td>
</tr>
<tr>
<td>6. Tumor microenvironmental molecules by Western blot</td>
<td>10</td>
</tr>
<tr>
<td>7. Host response to different microenvironment by enzyme-linked immunosorbent assay (ELISA)</td>
<td>12</td>
</tr>
<tr>
<td>8. Immune response of the normal peritumor liver and tumor by flow cytometry</td>
<td>12</td>
</tr>
<tr>
<td>9. Statistical analysis</td>
<td>13</td>
</tr>
</tbody>
</table>
III. RESULTS ........................................................................................................... 14

1. Tumor progression and radiation response by different TME compartments ................................................................. 14
   A. Establishment of different TME animal models for murine hepatocarcinoma .............................................................. 14
   B. Tumor growth assay by different TME models ......................... 15
   C. Difference in metastatic involvement in lung by different TME ...... 16
   D. Difference in microvessel density by different TME models .......... 18
   E. Difference in VEGF expression by different TME models .......... 19
   F. Difference in TGF-β1 expression by different TME models ........ 21
   G. Difference in COX-2 expression by different TME models .......... 23

2. Tumor microenvironmental response to radiation .............................. 25
   A. VEGF expression after radiation at tumor and peritumor normal liver ................................................................. 25
   B. TGF-β1 expression after radiation at tumor and peritumor normal liver ..................................................................... 27
   C. COX-2 expression after radiation at tumor and peritumor normal liver ................................................................. 29

3. Alterations of cytokine after radiation by different TME models ........ 31

4. Immune cell expression after radiation ......................................................... 33
IV. DISCUSSION ........................................................................................................... 36
V. CONCLUSION ......................................................................................................... 48
REFERENCES ........................................................................................................... 49
ABSTRACT (IN KOREAN) .......................................................................................... 59
LIST OF FIGURES

Figure 1. Schematic illustration of the tumor microenvironment ........................................ 6

Figure 2. Tumor growth in the thigh (heterotopic) and liver (orthotopic) of C3H mice  ..................... 14

Figure 3. Tumor growth pattern in orthotopic and heterotopic tumor models .................................. 15

Figure 4. The numbers of metastatic lung nodules in heterotopic and orthotopic tumor models ........... 17

Figure 5. (A) Microvessel density (MVD) in heterotopic and orthotopic tumor models. (B) Western blot assays with antibody to CD31 in orthotopic and heterotopic tumor models ........................................ 18

Figure 6. (A) Immunohistochemical staining with antibody to VEGF in orthotopic and heterotopic tumor models (B) Western blot assay of VEGF ............. 20
Figure 7. (A) Immunohistochemical staining with antibody to TGF-β1 in orthotopic and heterotopic tumor models. (B) Western blot of TGF-β1. (C) Serum TGF-β1 by ELISA ................................. 22

Figure 8. Immunohistochemical staining (A) and Western blot (B) with antibody to COX-2 in orthotopic and heterotopic tumor models................................. 24

Figure 9. Immunohistochemical staining (A) and Western blot (B) with antibody to VEGF in orthotopic and heterotopic tumor models after radiation .......... 25

Figure 10. Immunohistochemical staining (A) and Western blot (B) with antibody to TGF-β1 in orthotopic and heterotopic tumor models after radiation ⋯ 27

Figure 11. Immunohistochemical staining (A) and Western blot (B) with antibody to COX-2 in orthotopic and heterotopic tumor models after radiation .......... 29
Figure 12. Serum collection from orthotopic and heterotopic tumor models for measurement of serum VEGF (A), TGF-β1 (B) and IL-6 (C) levels to evaluate the cytokines after radiation by ELISA 31

Figure 13. Immune cells in normal peritumor liver tissues and tumors in the orthotopic tumor model 34
ABSTRACT

Microenvironmental Alterations by Radiation in Murine Hepatocarcinoma

Ik Jae Lee

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Jinsil Seong)

**Background:** Radiotherapy (RTx) technology for hepatocellular carcinoma (HCC) has rapidly developed and enables more successful treatment. However, RTx has been challenging due to the potential for intrahepatic and extrahepatic metastasis. These occurrences suggest that the tumor microenvironment (TME) and surrounding adjacent normal tissues might be important modulators of RTx. For murine hepatocarcinoma, heterotopic tumor
Implantation into the thigh or dorsal skin has become a standard method for establishing murine hepatocarcinoma models. Although these types of models help in understanding the nature of cancers and their therapeutic approaches, they are not sufficient for investigating the interactions of tumor cells with the surrounding microenvironment of adjacent normal tissues, including immunological responses. For this reason, we established a syngenic murine hepatocarcinoma model via orthotopic implantation. The goal of this study was to evaluate the biological features of orthotopic murine hepatocarcinoma and to determine the response of TME molecules to radiation.

**Methods:** Murine hepatocarcinoma (HCa-I) models were established in the liver (orthotopic) and thigh muscle (heterotopic) of male C3H/HeJ mice. In these models, tumor growth and lung metastasis were assessed. To evaluate the tumor microenvironmental alterations by different TME and radiation, the tumor models were irradiated with 10 Gy. Immunohistochemical and Western blot analysis were then performed for vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), transforming growth factor beta1 (TGF-β1) and clusters of differentiation 31 (CD31). Serum sampling was conducted for evaluation of VEGF, TGF-β1 and interleukin-6 (IL-6) level. The presence of immune cells was evaluated in peritumor liver tissues and in tumors in the orthotopic TME after irradiation.
**Results:** The number of lung metastasis was higher in the orthotopic tumor model than in the heterotopic tumor model. VEGF, CD31, COX-2, and TGF-β1 expression increased in the orthotopic tumor model. This expression was prominent at the peripheral tumor margin. The expression of angiogenic factor (VEGF) and key regulatory molecules (TGF-β1 and COX-2) in the tumor tissue decreased following radiation. However, the expression in peritumoral normal liver also increased after radiation. Levels of secreted factors (VEGF and TGF-β1) decreased after radiation, but IL-6 levels increased. The immune cell response of CD8 cells and programmed death 1 (PD-1) in CD8 T-cells were increased after radiation. However, numbers of regulatory T cells decreased after radiation.

**Conclusion:** The tumor microenvironment in murine hepatocarcinoma was more aggressive for orthotopic than for heterotopic tumors in terms of lung metastasis. Radiation enhanced the expression of VEGF, COX-2, and TGF-β1 at peritumoral normal tissue in the orthotopic tumor model. This model is useful for investigating tumor microenvironments and radiation effects.

---

Key words: tumor microenvironment, hepatocarcinoma, radiation
Microenvironmental Alterations by Radiation

in Murine Hepatocarcinoma

Ik Jae Lee

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Jinsil Seong)

I. INTRODUCTION

The use of radiotherapy (RTx) for hepatocellular carcinoma (HCC) has long been overlooked because of the low tolerance dose of radiation for the entire liver.\textsuperscript{1,2} Recently, irradiation techniques have been rapidly developing for high precision RTx, and these have enabled more successful treatment of HCC by delivering a substantial dose of radiation to the tumor with acceptable levels of toxicity.\textsuperscript{1-7}
Radiation treatment for HCC also has been challenging due to frequent incidences of intrahepatic metastasis, as well as risk of radiation-induced injury to adjacent organs. Chung et al. suggested that RTx to eradicate a primary HCC might result in the outgrowth of previously dormant microtumors not included in the RTx field.\textsuperscript{8} In our preliminary clinical study, we investigated the correlation between COX-2 expression at irradiated non-tumor liver sites and examined patterns of failure in HCC patients.\textsuperscript{9} The findings suggested that the tumor microenvironment (TME) and surrounding adjacent normal tissues might be important modulators of RTx. The TME is a complex of extracellular matrix and various cell types including carcinoma cells, endothelial cells, fibroblasts and immune cells, as well as extracellular matrix molecules, and is known to contribute to the carcinoma process (Figure 1).\textsuperscript{10} Recent studies have provided convincing evidence that the TME might be involved in regulating metastasis and tumor progression.\textsuperscript{11,12} The TME and surrounding adjacent normal microenvironment of the tumor-host interface promote tumor progression through extensive remodeling of surrounding adjacent normal tissues to provide a supportive environment for tumor growth, angiogenesis, invasion, and metastasis of cancer cells.\textsuperscript{13-15}

Heterotopic tumor implantation into the thigh or dorsal skin has been used as a standard method to establish murine hepatocarcinoma models. Although
these types of models help in understanding the nature of cancers and their therapeutic approaches, they are not sufficient for the investigation of interactions of tumor cells with the surrounding microenvironment of adjacent normal tissues including immunological responses. Therefore, we established a syngenic murine hepatocarcinoma model via orthotopic implantation. The goal of this study was to evaluate the biological features of orthotopic murine hepatocarcinoma and the response of TME molecules to radiation.

Figure 1. A schematic illustration of the tumor microenvironment (TME). Tumor cells orchestrate effects directly through the release of factors such as vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), transforming growth factor beta 1 (TGF-β1), and interleukin-6 (IL-6) or indirectly promote (through the induction of tissue hypoxia, angiogenesis or
appearance of necrosis) the modification of the TME by attracting or activating other non-tumor cells. Immune response of the TME is inhibited by the presence of CD25+Foxp3 regulatory T (Treg) cells and programmed death 1 (PD-1) cells.
II. MATERIALS AND METHODS

1. In vivo tumorigenicity

Male C3H/HeJ mice, 7-8 weeks old, were used for this study. Animal experiments were conducted in accordance with the Animal Research Committee’s Guidelines at Yonsei University Medical College guidelines, and all the facilities were approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). A murine hepatocarcinoma (HCa-I) was evaluated for this study. This transplantable murine tumor is syngenic to C3H/HeJ and is a highly radioresistant tumor with a TCD 50 (radiation dose yielding 50% tumor cure rate) of > 80 Gy.16

2. Establishment of heterotopic and orthotopic murine hepatocarcinoma

A heterotopic tumor model was generated by inoculating $1 \times 10^6$ HCa-I cells into the muscles of the right thighs of the mice. Tumor cell suspensions were prepared as previously described.17

To evaluate the changes of the tumor and normal peritumor liver by radiation, $1 \times 10^6$ HCa-I cells were injected directly into the subcapsular parenchyma of the left lobe of the liver. Two weeks after tumor cell implantation, mice were randomly assigned to a radiation treatment group or
control group.

3. Radiation to the heterotopic and orthotopic murine tumor

The tumor-bearing legs were treated with a single dose of 10 Gy using a clinical linear accelerator (Varian Medical Systems Inc., Palo Alto, CA, USA) for the heterotopic tumor model. In the orthotopic tumor model, the radiation was delivered to the abdomen as a single dose of 10 Gy. Radiation administration was followed by tumor inoculation 2 weeks later. Irradiated orthotopic and heterotopic tumors were sampled at the first and 3rd day after radiation. Non-irradiated orthotopic and heterotopic tumors were also obtained on the same days for comparison with the irradiated groups.

4. Comparative analysis of metastatic potential between heterotopic and orthotopic murine tumor

HCa-I, grown in thighs, develops spontaneous lung metastasis in 10–20 days after tumor implantation. The number of lung metastatic nodules was evaluated for different TME. Mouse lungs were taken at 6, 12, and 18 days after tumor implantation and were fixed with Bouin’s solution. Metastatic lung nodules were counted under a polarizing microscope (×4). Tumor size for the different TME was measured every 3 days for a period of 15 days.
5. **Immunohistochemical staining of tumor microenvironmental molecules**

Immunohistochemistry was used to assess the effect of radiation on microenvironmental molecules in heterotopic and orthotopic tumors including:

1. Angiogenic factors such as vascular endothelial growth factor (VEGF) and clusters of differentiation 31 (CD31); and
2. Key regulatory molecules for TME such as cyclooxygenase-2 (COX-2), and transforming growth factor beta 1 (TGF-β1). Microvessel density (MVD) was assessed using the criteria described by Weidner *et al.* The areas of highest neovascularization were identified as regions of invasive carcinoma with the highest numbers of discrete microvessels stained for CD31.

6. **Tumor microenvironmental molecules by Western blotting**

The effects of radiation on tumor microenvironmental molecules were analyzed semiquantitatively by Western blotting. Tumor tissue was obtained from the heterotopic and orthotopic tumor models, and normal peritumor liver tissue was collected from the orthotopic tumor model. The tissues were washed three times in ice-cold phosphate-buffered saline (PBS), and lysed for 1 hour in a cold buffer containing 100 mM HEPES, 200 mMNaCl, 20%
glycerol, 2% NP40, 2 mM EDTA, 40 mM Ð-glyceraldehydephosphate, 2 mM sodium fluoride, 1 mM DTT, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 5 µg/m upeptin, and 2 µg/m aprotinin. The samples were centrifuged at for 5 min at 4°C and the supernatant fraction was collected. Total cellular protein was determined with the Bradford protein assay (Bio-Rad, Hercules, CA, USA). The protein samples (50 µg) were subjected to SDS-PAGE as described previously. To detect specific proteins, the following antibodies were used at the concentrations recommended by the manufacturers: a mouse COX-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit polyclonal antibodies to TGF-β1 and VEGF (Abcam® , Cambridge, MA, USA). After washing in TBST, the membranes containing blotted proteins were incubated for 1 hour at room temperature with either an anti-sheep or anti-mouse (Cell Signaling Technology, Beverly, Massachusetts, USA) immunoglobulin (IgG) antibody conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Detected proteins were quantified by densitometry (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) after chemiluminescence detection (Fuji photo film, Tokyo, Japan) using the ECL western blot detection system (Amersham Pharmacia Biotech). 21,22
7. Host response to different microenvironments by enzyme-linked immunosorbent assay (ELISA)

Serum was collected in orthotopic and heterotopic tumor models for measurement of serum VEGF, TGF-β1 and interleukin-6 (IL-6) levels by ELISA using mouse VEGF, TGF-β1, and IL-6 Quantikine ELISA Kits (R&D System, Minneapolis, USA) according to the supplier’s instructions. Blood was obtained via closed cardiac puncture by means of a 22-gauge hypodermic needle and a subxyphoid approach.

8. Immune response of the normal peritumor liver and tumor by flow cytometry

For flow cytometry, the normal peritumor liver and tumor samples were obtained and spleen tissues were sampled as a control. All antibodies were obtained from BD Biosciences (San Diego, CA, USA). Lymphocytes were isolated from the spleen, liver, and tumor tissues, as follows: After incubation with 0.25 mg/ml collagenase B (Boehringer Mannheim) and 1 U/mlDNase (Sigma, St. Louis, Mo.) at 37°C for 45 min, the digested tissue was centrifuged and the pellet was resuspended in 5 to 10 ml of 44% Percoll (Sigma). This suspension was underlaid with 56% Percoll and spun at 850 × g for 20 min at 20°C. The lymphocyte population was harvested from the
interface, and red blood cells were lysed using 0.83% ammonium chloride.

9. **Statistical analysis**

Results are expressed as means ± SE. The $t$-test was used to evaluate the significance of the differences. All tests were two-sided, and a $p$ value less than 0.05 indicated statistical significance.
III. RESULTS

1. Tumor progression and radiation responses in different TME compartments

A. Establishment of different TME animal models for murine hepatocarcinoma

Successful tumor formation was obtained in both the heterotopic and orthotopic models after tumor cell inoculation. None of the animals died during the inoculation. Features of the heterotopic and orthotopic tumor model after tumor cell implantation are shown in Figure 2.

A                           B

Figure 2. Tumors grown in the thigh (heterotopic) and liver (orthotopic) of C3H mice. Tumor cells were implanted in the thigh muscle in the heterotopic tumor model (A) and at a subcapsular site in the liver in the orthotopic tumor model (B).
B. Tumor growth assays in the different TME models

Tumor size was first compared in the heterotopic and orthotopic tumor models. Tumors grew at a faster rate in the orthotopic tumor model than in the heterotopic model at early stages but at a similar rate at 15 days (Fig. 3). In the present study, little or no difference was noted between the different tumor models. In the early stages, tumors formed in the injected liver lobe. Tumors expanded to fill the abdominal cavity and other lobes of liver, and some tumors were externally visibly by the late stages.

Figure 3. Tumor growth pattern in orthotopic and heterotopic tumor models. Tumors grew at a faster rate in the orthotopic than in heterotopic model at early stages and then showed similar growth rates by 15 days. In the present study, little or no difference was noted between the two different tumor
models.

C. Differences in metastatic involvement of the lungs in response to different TME

Each murine HCa-I model tumor grew rapidly and began to spread to the lung. When tumor behavior was compared between heterotopic and orthotopic model, the average number of metastatic lung nodules was found to significantly increase in the orthotopic tumor model at 9 and 12 days after tumor implantation (p=0.03, 0.02, respectively). Fifteen days after tumor implantation, the average numbers of lung nodules in heterotopic and orthotopic tumor models were 10.6±4.0 and 27.3±12.8, respectively (p=0.04, Fig. 4). Thus, the orthotopic tumor model had higher metastatic potential than the heterotopic tumor model.
Figure 4. The number of metastatic lung nodules in heterotopic and orthotopic tumor models at 6 (*, p=0.04), 12 (**, p=0.02), and 18 days (***, p=0.03) after tumor implantation. (A) Mice lungs were fixed with Bouin’s solution and tumor numbers were counted under a polarizing microscope (x4). The white nodules on the lung surface indicated metastatic lung nodules (arrows). (B) The number of metastatic lung nodules was higher in the orthotopic tumor
D. Differences in microvessel density (MVD) in the different TME models

The MVD in each experimental group was evaluated by immunohistochemical staining for CD31 (Fig. 5). The MVD was significantly higher in the orthotopic than in the heterotopic tumor model (p=0.001), and an increase in CD31 expression was observed by Western blotting in the orthotopic tumor model. These data indicate that the orthotopic tumor model might have a higher metastatic potential than the heterotopic tumor model.
Figure 5. (A) Microvessel density (MVD) calculated in heterotopic (H) and orthotopic (O) tumor models. Microvessels were stained using immunostaining for the endothelial cell marker, CD31. MVD significantly increased in the orthotopic tumor model (*, p=0.001). (B) Western blot assays with antibody to endothelial cell marker, CD31 in the orthotopic and heterotopic tumor models. CD31 expression was higher in the orthotopic than in the heterotopic tumor model.

E. Difference in VEGF expression by different TME models

VEGF was expressed in both the heterotopic and orthotopic tumor models, whereas only a scant expression occurred in the non-tumor liver tissues (Fig. 6A). VEGF expression was higher in the orthotopic than in the heterotopic tumor model as determined by immunohistochemical staining. Semi-quantitative analysis Western blot analysis also demonstrated that VEGF expression was higher in the orthotopic than in the heterotopic tumor model (Fig. 6B). Higher serum VEGF levels were also measured in the orthotopic
than in the heterotopic tumor model, but the difference was not statistically
significant (p > 0.05, Fig. 6C).

A

Heterotopic

Orthotopic

B

H

O

VEGF (43, 24 kDa)

GAPDH (36 kDa)

C

Serum VEGF (pg/ml)

Heterotopic

Orthotopic
Figure 6. (A) Immunohistochemical staining with antibody to VEGF in orthotopic (O) and heterotopic (H) tumor models. The orthotopic tumor model showed intense cytoplasmic immunoreactivity (brownish staining, magnification 400x). (B) Western blot assay of VEGF. VEGF protein levels were elevated in the orthotopic tumor model compared to the heterotopic tumor model. (C) Serum was collected from the orthotopic and heterotopic tumor models for measurement of serum VEGF levels by ELISA. Higher serum VEGF levels occurred in the orthotopic model compared to the heterotopic tumor model (p > 0.05).

F. Differences in TGF-β1 expression in the different TME models

Expression and location of TGF-β1 protein were determined by immunohistochemistry in tissue specimens from each tumor model. TGF-β1 showed high expression in both the heterotopic and the orthotopic tumor models (Fig. 7A). TGF-β1 expression was more prominent in the peripheral tumor area in the orthotopic tumor model. Western blot and ELISA assays showed that TGF-β1 expression and serum levels were slightly higher in the orthotopic mode than in the heterotopic tumor model (p > 0.05, Fig. 7B and 7C).
A

Heterotopic

Orthotopic

B

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 (12.5 kDa)</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH (36 kDa)</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

C

![Bar graph showing Serum TGF-β1 (pg/ml) for Heterotopic and Orthotopic groups.]
Figure 7. (A) Immunohistochemical staining with antibody to TGF-β1 in orthotopic (O) and heterotopic (H) tumor models. Both tumor models demonstrated intense cytoplasmic immunoreactivity (brownish staining, magnification 400x). (B) Western blot of TGF-β1. (C) Serum was collected from orthotopic and heterotopic tumor models for measurement of serum TGF-β1 levels by ELISA. Western blot and ELISA assays showed that TGF-β1 expression and serum levels were slightly higher in the orthotopic tumor model than in the heterotopic tumor model (p > 0.05).

G. Differences in COX-2 expression in the different TME models

To determine the cellular localization of active COX-2 in each tumor model, frozen sections were stained for COX-2 (Fig. 8A). In heterotopic tumors, only slight staining for total COX-2 was detected in frozen sections. In the orthotopic tumor model, staining for COX-2 was increased in the peripheral tumor tissue compared to the weak staining in the central tumor area and in normal tissue. Semi-quantitative analysis by Western blotting analysis demonstrated a higher COX-2 expression in the orthotopic tumor model than in the heterotopic tumor model (Fig. 8B).
Figure 8. (A) Immunohistochemical staining of COX-2. The orthotopic tumor model showed predominant expression of COX-2 in the cytoplasm (brownish staining) of the peripheral tumor tissue (magnification x400). (B) Western blot assays with antibody to COX-2 in orthotopic (O) and heterotopic (H) tumor models. Immunohistochemical staining and Western blottings showed that higher COX-2 expression occurred in the orthotopic model than in the heterotopic tumor model.
2. Tumor microenvironmental responses to radiation

A. VEGF expression after radiation in tumors and normal peritumor liver tissues.

Tumor microenvironmental responses after radiation exposure were evaluated by expression of VEGF, a potent angiogenic factor among the non-neoplastic cellular compartments of the TME. VEGF expression in tumor tissue decreased in both orthotopic and heterotopic tumor models after radiation (Fig. 9). In contrast, Western blots of the normal peritumoral liver tissue from the orthotopic tumor model indicated an increase in VEGF expression after radiation.

A.
Figure 9. (A) Immunohistochemical staining with antibody to VEGF in orthotopic and heterotopic tumor models after radiation. (B) Western blot assays of VEGF in heterotopic and orthotopic tumor models after radiation. VEGF expression in tumor tissues decreased in both orthotopic and heterotopic tumor model after radiation (magnification 400x). In contrast, Western blot assays of the normal peritumoral liver tissue from the orthotopic tumor model showed an increase in VEGF expression after radiation.
B. TGF-β1 expression after radiation in tumors and normal peritumor liver tissues

TGF-β1 is a key regulatory molecule in the non-neoplastic cellular compartments of the TME. TGF-β1 expression in tumor tissues after radiation decreased in both the orthotopic and heterotopic tumor models (Fig. 10). Semi-quantitative Western blot analysis demonstrated an increase in expression of TGF-β1 protein in normal peritumor liver tissues in the orthotopic tumor model after radiation. Tumor compartmentation showed no significant response after radiation in either model.
Figure 10. Immunostaining (A) and Western blot assays (B) with antibody to TGF-β1. The orthotopic tumor model exhibited an elevated expression of TGF-β1 in normal peritumor tissues after radiation (B). TGF-β1 expression in the tumor tissue after radiation decreased in both orthotopic and heterotopic tumor models (magnification 400x). Western blotting demonstrated an increase in expression of TGF-β1 protein in normal peritumor liver tissues in the orthotopic tumor model after radiation. Tumor compartmentation in heterotopic and orthotopic tumor models revealed no significant responses after radiation.
C. COX-2 expression after radiation in tumors and normal peritumor liver tissues

COX-2 is also a key regulatory molecule among the non-neoplastic cellular compartments of the TME. COX-2 expression by a tumor decreased following radiation in both orthotopic and heterotopic tumor models (Fig. 11) and the normal peritumoral liver tissue showed an increase in COX-2 expression in the orthotopic model after irradiation. Tumor compartmentation in the orthotopic model revealed a slight decrease in COX-2 expression after radiation.

A
Figure 11. (A) Immunostaining with antibody to COX-2. COX-2 expression by tumors decreased in orthotopic and heterotopic tumor models following radiation (magnification 400x). (B) Western blot assay of COX-2 in the heterotopic and orthotopic tumor models. The normal peritumor tissue demonstrated an increase of COX-2 expression after irradiation in the orthotopic tumor model.
3. Alterations in cytokines after radiation in different TME models

Secreted factors are important molecules among the non-neoplastic cellular compartments of the TME. The serum TGF-β1, VEGF, and IL-6 levels were evaluated because local RTx may have a significant effect on systemic cytokine levels, which may show altered radiation responses in different TME (Fig. 12). The serum TGF-β1 and VEGF concentrations were reduced after irradiation in both heterotopic and orthotopic tumor models. In contrast, IL-6 increased after irradiation in both models.

A.
B

![Graph showing Serum TGF-β1 (pg/ml) for Heterotopic and Orthotopic groups for Control, RT Day 1, and RT Day 3.]

C

![Graph showing Serum IL-6 (pg/ml) for Heterotopic and Orthotopic groups for Control, RT Day 1, and RT Day 3.]

32
Figure 12. Serum was collected from orthotopic and heterotopic tumor models for measurement of serum VEGF (A), TGF-β1 (B) and IL-6 (C) levels to evaluate cytokine responses by ELISA after radiation. No significant changes (p > 0.05) were noted, but serum TGF-β1 and VEGF concentrations were reduced after irradiation in both heterotopic and orthotopic tumor models. IL-6 increased after irradiation in both tumor models, but the differences were not statistically significant (p > 0.05).

4. Immune cell expression after radiation

The presence of immune cells in normal peritumor liver tissues and tumors in the orthotopic tumor model was evaluated. In naive mice, which were untreated and had no tumors, the frequency of CD8 and CD25+Foxp3 Treg cells expression showed no significant alteration, and PD-1 expression was decreased after radiation. The frequency of CD8 cells increased in the tumor and normal peritumor liver tissue, and these cells also increased after radiation (Fig. 13). PD-1 expression in CD8 T-cells also increased after radiation. The number of CD25+Foxp3 Treg cells in the tumor and normal peritumor liver tissues increased. However, CD25+Foxp3 Treg cell expression was decreased after radiation.
Figure 13. The immune cells in normal peritumor liver and tumor tissues in the orthotopic tumor model. (A) In naive mice, which were untreated and had no tumors, the frequency of CD8 and CD25+Foxp3 Treg cell expression showed no significant alteration, whereas PD-1 expression was decreased after radiation. The number of CD8 cells increased in tumor and normal peritumor liver tissues and (B) PD-1 expression on CD8 T-cells also increased after radiation. (C) The number of CD25+Foxp3 Treg cells in tumor and normal peritumor liver tissues increased. However, the numbers of CD25+Foxp3 Treg cells were decreased after radiation.
IV. DISCUSSION

Histological examination of tumors shows that many non-epithelial cell types are present in tumors, which comprise the tumor stroma. This suggests that the tumor microenvironment (TME) is not defined only by the properties of the malignant cell population, but rather is a product of the interaction between tumor and non-neoplastic tissue.

A number of non-neoplastic cells help comprise the tumor microenvironment. Some of the more well studied examples include cancer associated fibroblasts, tumor endothelial cells, pericytes, bone marrow progenitor cells, and a number of immune cells including tumor associated macrophages.\textsuperscript{11,12,23} These non-neoplastic cells play important roles in the secretion and bioavailability of pro- and anti-metastatic factors and thus may either facilitate or suppress metastasis. Cretu et al. suggested an expanding array of components that contribute to metastasis.\textsuperscript{24}

These metastasis effectors can be organized into at least four broad categories, including: (A) malignant tumor cells, (B) non-neoplastic cells (endothelial cells and immune cells), (C) secreted soluble factors (growth factors and chemokines) and, (D) non-cellular solid-state structural features such as the extracellular matrix (ECM). These distinct groups help compose
what many have termed the TME.

Heterotopic tumor implantation has been used as a standard method to establish animal tumor models. Although these types of models help in understanding the nature of human cancers and their therapeutic approaches, many problems still remain unresolved. One major concern is that a tumor derived from a patient and implanted subcutaneously into an immunodeficient animal no longer behaves as it did in the patient. Holfman\textsuperscript{25} developed an orthotopic implantation model utilizing intact tissues obtained directly from surgery. This approach has yielded a high survival rate and frequent metastases in cancers of the colon,\textsuperscript{26} bladder,\textsuperscript{27} lung,\textsuperscript{28} pancreas,\textsuperscript{29} prostate\textsuperscript{30} and stomach.\textsuperscript{31} These models of human cancer in nude mice could show various manifestations similar to tumor behavior in patients. Gao et al. established a nude mouse model of human hepatocellular carcinoma via orthotopic implantation of histologically intact tissue.\textsuperscript{32} Although various orthotopic HCC models have been established in nude mice, the present study established a syngenic orthotopic tumor model to evaluate the microenvironmental alterations, including immunologic responses. This model also represents the features of a mouse model with murine HCC, and could be an interesting tool for exploring therapeutic strategies of HCC and microenvironmental effects.
Several research studies have reported that heterotopic and orthotopic tumors differ in terms of both angiogenesis and metastasis. Morikawa et al.\textsuperscript{33} showed that implantation of KM12 cells into the cecal wall (orthotopic) or a subcutaneous site (ectopic) of nude mice produces tumors with different metastatic potentials: cecal (orthotopic) tumors are invasive and metastatic, whereas subcutaneous (ectopic) tumors are not, suggesting that different organ environments may differentially influence the expression of metastasis-related genes. Onogawa et al. reported that contents of the epidermal growth factor receptor (EGFR), basic fibroblast growth factor (bFGF), IL-8, VEGF-C and VEGF-D protein were higher in orthotopic than in ectopic tumors.\textsuperscript{34,35} Camphausen et al. reported that cells grown under orthotopic conditions are more susceptible to radiation-induced changes in gene expression.\textsuperscript{36} They concluded that the influence of an orthotopic environment on radiation-induced modulation of gene expression in glioma cells was not only quantitative but qualitative. Taking into account these types of environmental influences will likely be important in defining the putative functional significance of radiation-induced changes in gene expression. In our present study, a more aggressive potential for tumor growth and metastasis was seen for the orthotopic than for the heterotopic tumor model. In addition, angiogenic factors (VEGF and CD31), key regulatory molecules (COX-2 and
TGF-β1), and serum cytokines (VEGF, TGF-β1, and IL-6) increased to a greater extent in the orthotopic than in the heterotopic tumor model. Lung metastasis may therefore depend on changes in the tumor microenvironment or on tumor cell-intrinsic genetic events.

Angiogenesis is balanced between secreted pro- and anti-angiogenic factors. The contribution of the tumor stroma to the angiogenesis is induced by transforming signaling pathways and by tumor hypoxia. Several cell types of the tumor stroma appear to contribute in a significant way: (i) endothelial cells by secreting angiogenic factors, such as angiopoietin-2, which affect the activation status of the endothelium and its differentiation into mature vessels, (ii) infiltrating macrophages and mast cells by secreting additional angiogenic factors, including VEGF-A, FGF-2, TGF-β1 and IL-8, and matrix metalloproteinase (MMP) that activate latent forms of these growth factors, (iii) cancer-associated fibroblasts (CAFs) by secreting additional growth factors, cytokines and chemokines and by modulating the extracellular matrix and (iv) additional cells of the innate and adaptive immune system or of tissue homeostasis. During tumor outgrowth, tumor cells and cells of the tumor stroma are stimulated for example by tumor hypoxia or lack of nutrition to produce angiogenic factors, such as VEGF-A and placental growth factor (PIGF). These factors, together with various inflammatory chemokines and
cytokines and other stimuli secreted by both neoplastic and tumor stroma cells, tilt the balance between angiogenesis inhibitors and inducers towards the stimulation of angiogenesis (angiogenic switch). Therefore, tumor cell and tumor stroma-induced angiogenesis not only promotes tumor growth, but also the hematogenous dissemination of tumor cells and the outgrowth of metastasis. Several clinical studies demonstrate a direct correlation of high primary tumor blood microvessel densities (MVDs) with increased incidence of metastases, and tumor MVD was a significant and independent prognostic indicator for relapse-free and overall survival of cancer patients.\textsuperscript{40-42} Similarly, elevated tumor or serum level of the angiogenic factors VEGF and IL-6 were associated with an increased incidence of metastasis in cancer patients.\textsuperscript{43,44} Xenograft animal models confirm the correlation of tumoral overexpression of angiogenic growth factors, increased MVD of primary and secondary tumors and metastasis formation.\textsuperscript{45,46} Kuperwasser et al. reported that tumor cells expressed high levels of FGF-2 and secreted type IV collagenase, resulting in the development of highly vascularized metastatic tumors when transplanted orthotopically.\textsuperscript{47} In our tumor model, angiogenic factors (VEGF and CD31) were higher in the orthotopic than in the heterotopic tumor model. These findings suggested that VEGF expression might be associated with an increased lung metastatic potential in the orthotopic tumor model compared to
the heterotopic tumor model.

Overexpression of COX-2 has recently been reported in HCC, and upregulation of COX-2 enzyme promotes tumor proliferation and growth, as well as tumor spread via the mediation of angiogenesis and immune function.\textsuperscript{48-52} In addition, COX-2 has a vital role in inhibiting apoptosis and stimulating tumor angiogenesis, both of which play an important role in cancer metastasis.\textsuperscript{53,54} It remains controversial whether COX-2 is overexpressed in HCC compared with matched adjacent liver tissues.\textsuperscript{50,55,56} The mean intensity of COX-2 expression in cirrhotic liver specimens is significantly higher than in normal livers and in moderately-differentiated HCC.\textsuperscript{57} COX-2 mRNA is overexpressed in adjacent liver tissue compared with in HCC.\textsuperscript{56}

Although there are some data on COX-2 expression in normal peritumor liver tissues in HCC, little is known about their alterations by radiation. Chung et al. suggested that RTx to eradicate a primary HCC might result in the outgrowth of previously dormant microtumors not included in the RTx field.\textsuperscript{8} In our preliminary clinical study, significant differences were found in terms of recurrence and extrahepatic recurrence between high and low intensity COX-2 patients (p=0.034 and 0.017, respectively) in an irradiated liver tissue group.\textsuperscript{9} These findings suggest to the importance of
also developing a pre-clinical tumor model for evaluating the efficacy of RTx in HCC that takes into consideration both the tumor and the normal peritumor liver. In our animal study, staining for active COX-2 in the orthotopic tumor was increased in the peripheral tumor tissue compared to the weak staining observed in the central tumor area and the normal tissue. Increased expression of COX-2 protein was also observed in normal peritumor liver tissue in the orthotopic model after irradiation. These findings suggest that increased COX-2 expression at normal peritumor liver after radiation could promote tumor growth and metastasis.

TGF-β1 is one of the key players involved in the communications between CAF and tumor cells, and it is also expressed by multiple cell types, including the stromal fibroblasts, the inflammatory cells, and carcinoma cells. TGF-β1 represents a major growth-inhibitory signal in normal cells, especially epithelial cells with either a cytostatic or apoptotic response. TGF-β1 also has much more complicated effects on tumorigenesis. TGF-β1 is immunosuppressive when acting on inflammatory cells, thereby promoting carcinogenesis through inhibition of the immune response against the tumor. Overexpression of TGF-β1 by fibroblasts stimulates neoplastic growth of human breast epithelium in vivo. Transfection of primary human prostate tumor cells with the TGF-β gene stimulated anchorage-independent growth
and promoted tumor growth, angiogenesis, and metastasis after orthotopic implantation in severe combined immunodeficiency mice. Increased secretion of TGF-β1 in irradiated mammary stroma may be part of the mechanism by which irradiated stroma stimulate tumorigenesis. In the present study, TGF-β1 showed high expression in both the heterotopic and orthotopic tumor models. Orthotopic tumor tissue in the peripheral zone of the tumor seemed to be more involved and an increase was seen in expression of TGF-β1 protein in normal peritumor liver tissue in the orthotopic model after irradiation. Radiation-induced TGF-β1 in normal tissue causes persistent and specific changes in cell signaling and may compromise therapeutic responses. Therapeutic inhibition of TGF-β1-specific inhibitors, based on blockade of synthesis, ligand/receptor binding, or receptor kinase signaling, might therefore increase the radiation response, thereby promote immunogenicity and preventing metastasis.

IL-6 exhibits functional pleiotropy and redundancy. It is involved in the immune response, inflammation, and hematopoiesis. This molecule has been variously referred to as interferon-β2, B-cell stimulatory factor 2, human plasmacytoma growth factor, or hepatocyte stimulatory factor. Radiation exposure results in an inflammatory reaction, with endothelial cells playing the key role and involving IL-6, IL-8, and expression of intercellular adhesion
molecule 1.\textsuperscript{61} Mouthon et al. found that IL-6 was increased in plasma from intestine- and liver-irradiated mice.\textsuperscript{62} Serum IL-6 was significantly increased in advanced liver cancer patients 24 and 48 h after treatment with yttrium-90 microspheres.\textsuperscript{63} Cheng et al. reported increased levels of IL-6 in the conditioned medium of irradiated endothelial cells.\textsuperscript{64} They also confirmed the nonspecific increase of IL-6 in patients undergoing RTx to part of the liver. In contrast, serum IL-6 did not increase in most patients irradiated for non-liver sites.

IL-6 is also considered one of the major triggers of VEGF, and the mechanism of VEGF induction by IL-6 may be critically involved in the progression of tumor cell growth.\textsuperscript{65,66} Adachi et al. reported that IL-6 induces both tumor cell growth and VEGF production in malignant mesotheliomas.\textsuperscript{67} The Janus kinase-signal transducers and activators of transcription (JAK-STAT3) pathway are involved in VEGF induction by means of IL-6 stimulation. Our orthotopic tumor model showed a higher level of IL-6 than did the heterotopic tumor model and the irradiated orthotopic tumor model showed increased serum IL-6. Although serum VEGF decreased after irradiation, VEGF expression in peritumoral normal liver tissue increased. These findings suggested that IL-6 might promote VEGF production in the peritumoral normal liver.
The increased incidence of cancer in immunosuppressed patients and intense research into tumor immunology over the past years has provided evidence that the immune system can recognize and eliminate tumor cells.\textsuperscript{68,69} Nevertheless, an increasing body of evidence indicates that immune cells represent a double-edged sword during tumorigenesis. Tumor-induced immunotolerance not only allows primary tumor outgrowth but also allows metastasis. In contrast, some of immune cells may have the opposite effect and suppress metastasis. For example, immune cells such as natural killer cells and dendritic cells facilitate tumor cell killing.\textsuperscript{11,12,23,70} Thus, alterations in the structural, cellular, biochemical, and molecular composition of a particular TME may play important roles in controlling tumor dissemination.

Given the new appreciation of the TME in controlling metastasis and the complexity of its various compartments, the orthotopic animal model is a useful tool due to its retention of a similar natural environment. Pang et al. analyzed the immune status within HCC.\textsuperscript{71} They demonstrated that the representation of CD\textsuperscript{8+} T cells, NK cells, NK-T cells, and gamma delta (γδ) T cells was significantly reduced in tumor infiltrating lymphocytes (TILs) in HCC patients. The CD\textsubscript{4+} T population was found to be substantially expanded in TIL and a significant increase in CD\textsubscript{25+}Foxp3 Treg cells were observed in the tumor tissue. Several groups have reported that reduced CD\textsuperscript{8+} T cells and
increased Foxp3\(^+\) cells in HCC are coupled with poor prognosis.\(^{72,73}\) Yang et al. investigated the numbers of CD25+Foxp3 Treg cells in tumor-involved and non-involved areas of the liver of patients with HCC.\(^{74}\) They observed large numbers of CD25+Foxp3 Treg cells in the marginal region of HCC, and found evidence that these cells might act to control CD8+ CTL activity. Demaria et al. described the effects of radiation on the immunological environment of cancer.\(^{75}\) Tumor-associated macrophages (TAM) frequently infiltrated the tumor stroma, where they promote angiogenesis and tumor growth. Abnormal distribution, architecture, and function of tumor vessels limit the infiltration by anti-tumor CTL mostly present at the periphery of tumors. Their function is inhibited by the presence of CD25+Foxp3 Treg cells and immature myeloid cells (IMC).

Medical application of ionizing radiation to cancer has focused on dose escalation for improving the killing of the cancer cells, while assuring the recovery of normal tissue. Some evidence now indicates that radiation can affect the immune system; thus, radiation can trigger cellular and stromal effects in addition to its cytotoxic results.\(^{76,77}\) Recently, some of the factors regulating the immunogenicity of dead cells have begun to be understood although reports about the radiation effects on the immunological microenvironment of cancer are still insufficient.\(^{78,79}\) Radiation causes death
of a fraction of the cancer cells by apoptosis and necrosis. Dead cancer cells then serve as a source of tumor antigens for uptake by dendritic cells, and release danger signals that promote dendritic cell maturation into effective antigen-presenting cells. Normalization of the tumor vasculature following RT also improves the recruitment of CTL that can penetrate deeper and in larger numbers into the tumor. RTx-induced up-regulation of MHC class I molecules in surviving cancer cells enhances their recognition and killing by CTL. Overall, the extent of tumor regression versus re-growth after RTx will depend on the relative balance between anti-tumor CTL and immunosuppressive mechanisms, such as CD25+Foxp3 Treg cells and IMC.

Ganss et al. reported that radiation markedly enhanced homing of the activated T cells to the tumors, an effect associated with remodeling of the tumor vasculature.\textsuperscript{80} In another tumor model (the experimental melanoma B16 transduced with a reporter antigen), tracking to the tumor by tumor-specific T cells was enhanced by tumor irradiation, and was associated with radiation-induced up-regulation of vascular cell adhesion molecule 1 (VCAM-1) on the tumor vessel.\textsuperscript{81} These data support the hypothesis that radiation can increase the permeability of solid tumors to immune cells. Schaue et al. demonstrated that the levels of CD25+Foxp3 Treg cells in colorectal cancer patients increased upon completion of chemoradiotherapy, whereas this did not
happen in prostate cancer patients. Our present study demonstrated that orthotopic tumor models showed increased immunologic responses, such as PD-1 and CD25+Foxp3 Treg cells in tumor and peritumor liver tissues. After radiation, numbers of regulatory T cells decreased, but numbers of PD-1 cells increased.

With the development of molecular target therapies, the use of several drugs, antibodies, or fusion proteins to reduce or eliminate human suppressor cells is also currently being clinically evaluated. Thus, there is considerable support for a rationale for potential clinical trials that employ multimodal tumor microenvironment-based therapies. These results remind us of the potential importance of innate immunity in antitumor responses, which needs to be further explored.

V. CONCLUSION

An orthotopic tumor microenvironment in a murine hepatocarcinoma model was more aggressive than a heterotopic tumor microenvironment in terms of lung metastasis. Radiation enhanced the expression of VEGF, COX-2, and TGF-β1 in normal peritumoral tissue in orthotopic tumor model. This model provides is useful for investigating the tumor microenvironment and radiation effects.
REFERENCES


29. Fu X, Guadagni F, Hoffman RM. A metastatic nude-mouse model of


37. Folkman J. Fundamental concepts of the angiogenic process. Curr


46. Rofstad EK, Halsor EF. Vascular endothelial growth factor,


63. Wickremesekera JK, Chen W, Cannan RJ, Stubbs RS. Serum proinflammatory cytokine response in patients with advanced liver tumors following selective internal radiation therapy (SIRT) with


마우스 간암 모델에서 방사선조사 시 종양 미세환경의 변화

<지도교수 성 진 실>
연세대학교 대학원 의학과
이익재

방사선치료 기술이 발달하여 종양에 방사선 조사량을 높이는 동시에 정상 조직이 받는 방사선을 최소한으로 제한하는 것이 가능하게 되면서 간암에 방사선치료를 적용한 임상 경험들이 빠르게 축적되고 있다. 그러나,빈번한 간내 외 전이와 주변경상장기의 방사선치료 부작용의 문제가 있어, 이를 극복하고자 많은 연구가 진행되고 있다. 방사선치료에 있어서 종양뿐만 아니라 종양 주변 정상조직을 포함한 종양 미세환경 (tumor microenvironment)이 중요한 역할을 하며, 이 분야의 연구가 필수적이다. 지금까지는 간암의 마우스 동물모델에서 주로 다리나 등에 종양세포를 형성하는 이소성 모델을 만들었지만,
종양미세환경을 연구하기 위한 동물모델로는 동소성 동물모델이 좀 더 적합하다. 따라서 이번 연구에서는 동소성 간암 동물모델을 만들었고, 이러한 동소성 간암동물모델의 생물학적 특징들을 방사선에 의한 종양 미세환경의 변화를 관찰하기 위해 동물실험을 시행하였다. C3H/HeJ 웅성 마우스에 방사선 치료에 강한 내성을 보이는 마우스 간암인 HCa-I 세포주를 단측 대퇴부 근육 내에 이식하여, 이소성 (heterotopic) 종양 모델과, 간의 좌측엽에 이식한 동소성 (orthotopic) 종양 모델을 만들어 상이한 종양 미세환경을 가지고 있는 동물모델을 만들었다. 동물모델 연구에서는 상이한 미세환경을 가진 환경에 따른 종양의 성장과 폐전이 양상을 분석하였고, 동소성종양에서 이소성 종양에 비해 폐전이가 증가하였다. 동소성종양에서 면역화학염색법과 Western blot 에서 VEGF, CD31, COX-2 와 TGF-β1의 발현이 증가하였다. 방사선에 의한 종양미세환경의 변화를 관찰하기 위해 방사선 조사를 10 Gy 시행하였고, 종양과 주변 정상간조직의 변화를 관찰하였다. 방사선을 조사받은 경우 종양에서 VEGF, COX-2와 TGF-β1의 발현이 감소하였으나, 동소성종양모델에서 주변 정상 간조직의 Western blot 에서는 오히려 VEGF, COX-2와 TGF-β1의 발현이 증가하였다. VEGF와 TGF-β1 사이토카인은 동소성종양
모델에서 증가되어 있었고, 방사선 조사 후 감소하였다. 하지만 IL-6의 경우는 동소성종양 모델에서 증가되어 있는 양상은 동일하였으나 방사선조사 후 IL-6의 수치는 증가하였다. 면역세포의 발현은 종양조직에서 대조군에 비해 CD8 발현세포가 많이 증가되어 있었고, 이는 방사선 조사 후 증가하였다. Programmed death-1(PD-1) 세포 또한 방사선 조사 후 증가하였으나, 규제 T 세포(regulatory T-cell)는 방사선 조사 후 감소하였다. 결론으로, 본 연구에서 동소성종양 모델의 경우 폐전이 양상이 이소성 종양 모델에 비해 증가된 양상을 보였다. 방사선 조사 후 동소성 종양모델의 주변 정상 간조직에서 VEGF, COX-2와 TGF-β1의 발현이 증가하였다. 이러한 동소성 종양모델은 종양 미세환경과 방사선의 영향에 관련된 연구를 하는데 좋은 모델이 될 것으로 사료된다.

핵심되는 말: 방사선, 종양 미세환경, 간암