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Evaluation of 2-[18F]-fluoro-2-deoxy-D-glucose positron emission tomography /computed tomography in rat models with hepatocellular carcinoma with liver cirrhosis[1]

S.I. Park[a] , J.H. Lee[b], H.J. Ham[a], Y.J. Jung[a], M.S. Park[a], J. Lee[c], L.S. Maeng[a], Y.A. Chung[a,d] and K.S. Jang[a,e,*]
[a]*Institute of Catholic Integrative Medicine, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Incheon 403-720, The Republic of Korea*
[b]*Laboratory of Molecular Pathology, Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul 151-742, The Republic of Korea*
[c]*Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064, USA*
[d]*Department of Radiology, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea , Incheon 403-720, The Republic of Korea*
[e]*Department of Neurosurgery, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea , Incheon 403-720, The Republic of Korea*

Abstract. Liver cirrhosis is a predominant risk factor for hepatocellular carcinoma (HCC). However, the exact mechanism of the progression from cirrhosis to cancer remains unclear. The uptake of 2-[^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) is widely used as a marker of increased glucose metabolism to monitor the progression of cancer with positron emission tomography (PET)/computed tomography (CT). Here we investigated the feasibility of using ^{18}F-FDG PET/CT in the diethylnitrosamine (DEN) mediated experimental hepatocellular carcinoma model. Rats received weekly intraperitoneal injections of DEN for 16 weeks for induction of HCC. We recorded starting from 0 days or 0 weeks after the last DEN injection. The weight and survival rate of rats were then measured. Also, an ^{18}F-FDG PET scan and serum analysis were performed at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection. The body weight of rats was maintained between 350 g and 370 g during 14 and 20 weeks, and the rats were euthanized at 35 days after the last DEN injection. The serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphate (ALP) were significantly higher at zero weeks after the last DEN injection. The ^{18}F-FDG uptake for the quantitative evaluation of HCC was done by measuring the region of interest (ROI). At minus two weeks after the last DEN injection, the ROI of rats had significantly

[1] S.I. Parka and J.H. Lee contributed equally to this paper.

*Address for correspondence: K.S. Jang, Institute of Catholic Integrative Medicine, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Incheon 403-720, The Republic of Korea. Tel.: 82-32-280-5243; Fax: 82-32-280-5244; E-mail: nucmedkr@gmail.com.

increased compared to the normal group, in a time-dependent manner. These results suggest that FDG uptake serves as a good screening test to evaluate the feasibility of DEN-induced HCC.

Keywords: Hepatocellular carcinoma, PET/CT, ^{18}F-FDG

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer death worldwide [1, 2]. The majority of patients have pre-existing cirrhosis at the time they develop HCC. The prognosis for patients with HCC is generally poor, with a less than 10% 5-year survival after diagnosis, and a median survival period of six months for patients with unresectable tumors [3, 4]. Various treatment options are available clinically, which include surgery, chemotherapy, and radiotherapy. However, the patients are at high risk for recurrence and the progressing mechanism of HCC, including long-lasting inflammation in hepatocytes leading to cirrhosis, has not been fully elucidated [5, 6].

Chemical and cell line-induced HCC models in rodents are used to investigate HCC. The cell line-induced HCC, such as HepG2 and N1S1cells, are used as experimental models. However, these models have to be made by xenotransplantation or without cirrhosis. In this study, we used diethylnitrosamine (DEN)-induced HCC, as it enables the sequential formation of cirrhosis and HCC. Also, these results suggest that liver function after the increase of serum components such as ALT, AST, and ALP progresses from cirrhosis to HCC by affecting the liver function after DEN treatment.

Also, the number of studies that investigate effects of the drug therapy has been assessed by the histology of the removed livers from sacrificed animals in DEN-induced liver cancer models. However, the size of the progressing cancer and newly generated tumors could not be observed time- dependently. Therefore, time-dependent tumors in DEN-induced liver cancer are necessary for monitoring via non-invasive methods.

Positron emission tomography (PET)/computed tomography (CT) are nuclear imaging modalities that have been widely used to investigate cancer [7, 8]. PET imaging provides information on the metabolic alterations in masses. The tumors are known to have increased consumption of glucose, which provides energy for cell growth and lipid synthesis. The glucose analogue, 2-[^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG), is an indicator of the utilization and the degree of specific functions in the cancer. This imaging will contribute to the refinement of cancer bioassays and offer additional information, such as identifying metastatic spread and secondary tumor growth in a timely fashion [9, 10].

In this study, a rat model with DEN-induced liver injury that reproduces the progression of cirrhosis to HCC was established. Therefore, we investigated the relationship of time-dependent ^{18}F-FDG uptake and histological examination in DEN-induced HCC.

2. Results and discussion

DEN toxicity is primarily associated with an excessive production of free radicals in the liver and proceeds to oxidative stress pathway to liver damage [11, 12]. Also DEN induces post-necrotic hepatocellular proliferation that contributes to enhancing the number of initiated cells and is associated with the development of HCC through a multistep carcinogenesis process that involves long-lasting inflammation in hepatocytes, leading to cirrhosis [13]. In this study, we used a DEN-induced HCC

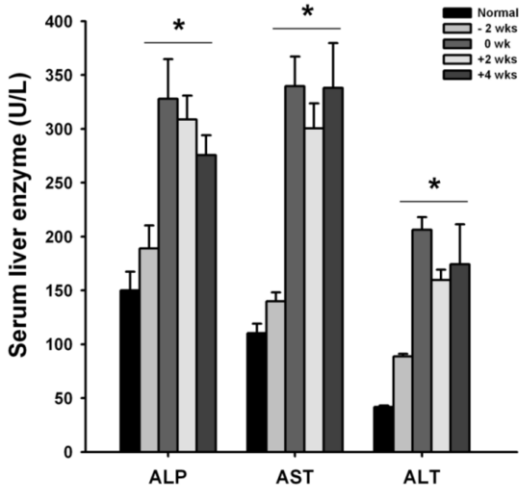

Fig. 1. The level of serum ALT and AST, and ALP after DEN-induced HCC. Rats were administered with a single intraperitoneal injection of DEN for 16 weeks. The serum was collected at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection. At zero weeks after the last DEN injection, the levels of ALT, AST, and ALP had significantly increased compared to the negative control. Data are expressed as mean ± SD, *$P < 0.05$. wks, weeks; ALP, Serum alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase

model that could establish HCC and cirrhosis simultaneously, because a malignant tumor progresses from liver cirrhosis. This model may show the similarities between experimental and human HCC and be a better scheme for studying human HCC than the implanted HCC models with cirrhosis.

2.1. Levels of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) in serum on DEN-induced HCC

Serum components such as ALT, AST, and ALP are markers of hepatic function, and their increased concentration in blood indicates liver damage by the cytotoxic effects of DEN [14, 15]. The increase of hepatic function-related factors is due to the production of free radicals during the course of DEN metabolism, which damages the hepatocellular membrane. We found that hepatic function-related factors could change time-dependent components in the serum level on DEN-induced HCC. We collected the serum at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection and analyzed the components of related liver functions. At minus two weeks after the last DEN injection, the rats showed increased levels of ALP, AST, and ALT compared to the normal rats (ALP: AST: ALT; -2 wks: 189 ± 21.3: 140 ± 8.1: 88.6 ± 2.3 vs. normal: 150 ± 17.3: 110 ± 8.6: 41 ± 1.4 U/L) (Figure 1). Levels of ALP, AST, and ALT had significantly increased from 0 to plus 4 weeks after the last DEN injection (ALP: AST: ALT; 0 wk: 328 ± 36.7: 339.6 ± 27.4: 206.1 ± 11.8 vs. +4 wks: 275.6 ± 18.3: 338 ± 41.6: 174 ± 36.9 U/L). These factors show an increase of over two fold compared to the normal values. Taken together, these results showed that increased liver damage takes place between minus 2 and plus 4 weeks.

2.2. Survival and body weight on DEN-induced HCC

We observed the spontaneous deaths of rats after the last DEN injection. The survival rate began at 16 weeks after confirmation of HCC and continued until the rats were dead. We recorded starting from 0 days after the last DEN injection. Rats started dying at the seven days status after the last DEN

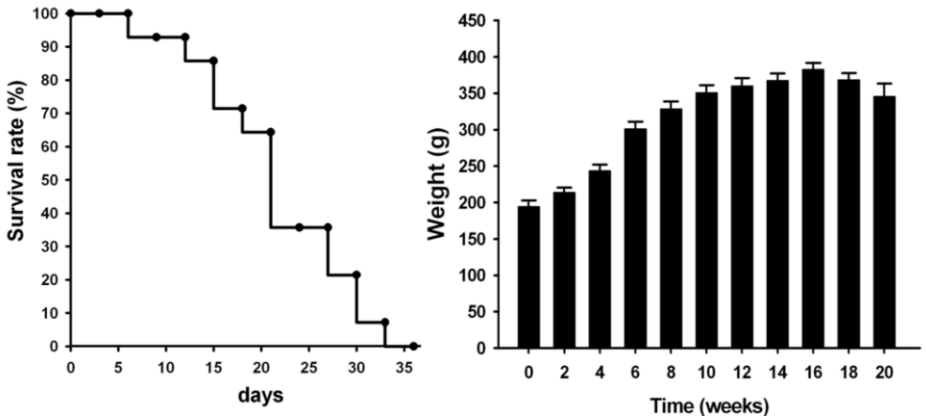

Fig. 2. Graph of the mean survival and body weight after DEN-induced HCC. A, the survival of HCC was analyzed by the log-rank test based on the Kaplan-Meier method. We recorded starting from 0 days after the last DEN injection. B, the body weight changes assessed every 2 weeks during 20 weeks.

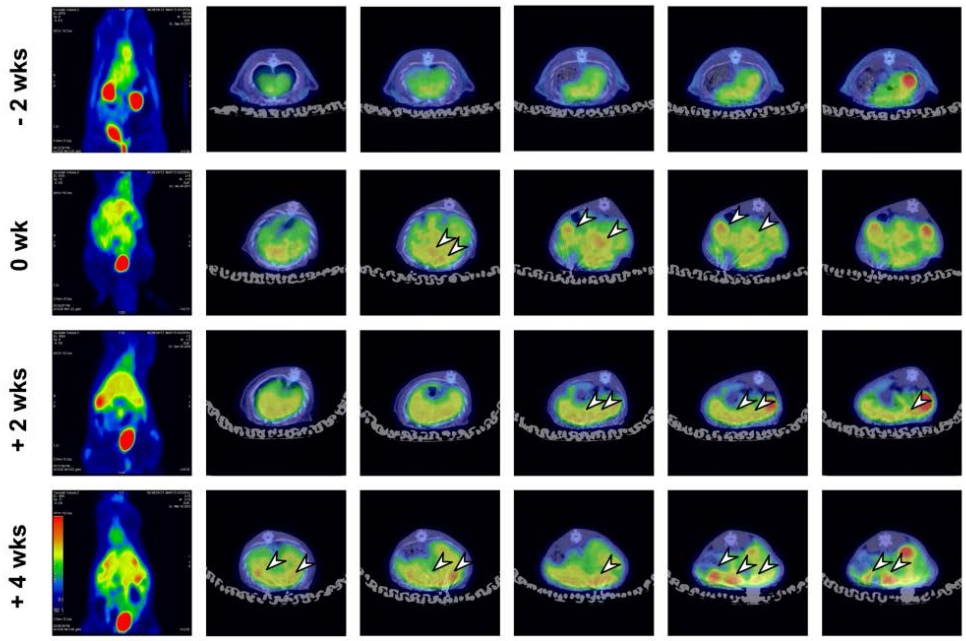

Fig. 3. ^{18}F-FDG PET of the liver after DEN-induced HCC. Axial slices of ^{18}F-FDG PET were scanned at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection. At minus two weeks after the last DEN injection, cirrhosis of the liver had progressed and uptake of ^{18}F-FDG had increased due to enhanced glucose metabolism. At zero weeks after the last DEN injection, the rat's liver had significantly increased the uptake of ^{18}F-FDG into generated HCC and showed multi-nodular HCC. Uptake of ^{18}F-FDG had increased time dependent after the DEN injection.

injecttion, and 40% of rats were dead between 15 days and 20 days. The longest survival time was 36 days (Figure 2). Also, body weight changes were assessed every 2 weeks during the 20 weeks after initiating DEN injection. Also, body weight was the greatest at 16 weeks after initiation of DEN treatment (383 ± 8.7 g). However, the body weight of the rats reduced time-dependently after the last DEN injection. These results suggest that HCC took a serious turn by tumor growth. The histological examination showed progressing HCC, such as cirrhosis, malignant nodules, and dysmorphia at a time de-

pendent between minus 2 and plus 4 weeks after the last DEN injection.

2.3. PET/CT imaging and uptake of 18F-FDG of HCC

A number of studies have reported that tumors can be proven for both experimental and human studies with PET/CT imaging of ^{18}F-FDG [16, 17]. ^{18}F-FDG can provide a useful indicator of tumor growth and metastasis because ^{18}F-FDG accumulates in tumor mass with increased glucose metabolism [18]. Also, sequential PET/CT scanning of the same animal can provide additional information, such as tumor growth and development, as well as metastasis and secondary tumor growth [10]. In this study, we investigated whether quantitative evaluation of HCC was analyzed using PET/CT with the time-dependent ^{18}F-FDG, without sacrificing animals after DEN-induced HCC. PET/CT imaging was performed at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection. Specific areas of the liver were identified with increased uptake of ^{18}F-FDG in PET imaging (Figure 3). The uptake of ^{18}F-FDG was expressed in regions of interest (ROI). We think that the increased uptake of ^{18}F-FDG in the specific area was generated from multiple tumors over 3 mm. At zero weeks after the last DEN injection, the ^{18}F-FDG uptake of specific areas into the liver was higher in certain areas compared to minus two weeks (0 wk: 8.3 ± 1.2 vs. -2 wks: 4.6 ± 0.5 ROI). Also, the quantitative image analysis of ^{18}F-FDG revealed the values of ROI (Figure 4). The values of ROI were increased between minus 2 weeks and plus 4 weeks after the last DEN injection. Our data show that the process of tumor formation is associated with hepatic function-related factors in blood and ^{18}F-FDG uptake. These results suggest that ^{18}F-FDG imaging could be a useful tool to measure tumor growth and metastasis and may serve as a better guide for monitoring experimental therapies.

2.4. Histology of the cirrhosis and HCC in DEN treatment

The cirrhosis and HCC in DEN treatment were confirmed by its gross appearance and histology over time, following the DEN injection (Figure 5). At minus two weeks after the DEN injection, microscopic fibrosis became obvious, and distinct cirrhosis appeared. The malignant nodules and a dysmorphic and dyschromic aspect were detected on the surface of the liver at 0 weeks after the DEN injection. Also, malignant nodules over 3 mm in diameter and a tumor boundary consistent with cirrhosis were achieved between 0 and plus 4 weeks after the last DEN injection. Taken together, these results ensured the identification of well-differentiated HCC on histological examination (Figure 5).

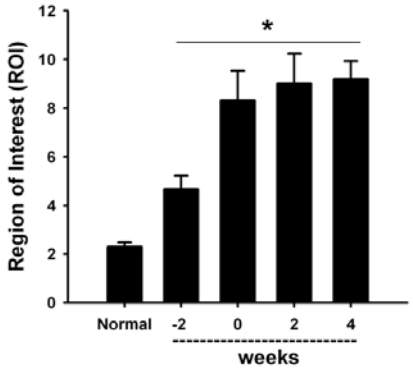

Fig. 4. Quantitative analysis of ^{18}F-FDG uptake after DEN-induced HCC. The ROI by ^{18}F FDG uptake had significantly increased at minus two weeks compared to the negative control. The ROI values had increased time dependent between 0 and plus 4 weeks after the last DEN injection. Data are expressed as mean ± SD, $*P < 0.05$.

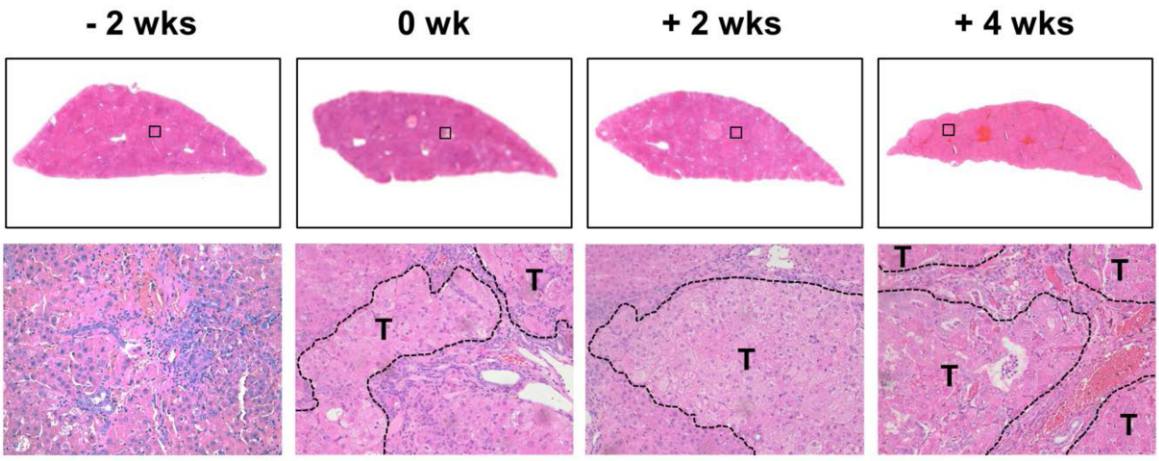

Fig. 5. Histopathology analysis after DEN-induced HCC. The H&E staining of the tumor was performed at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection. H&E staining showed few connective tissues by cirrhosis at minus two weeks after the last DEN injection. Tumor tissues increased stage by stage between 0 and plus 4 weeks. (wks, weeks; T, tumor)

3. Experimental

3.1. Experimental design

The experimental protocols used in this study were designed according to the animal experimental guidelines established by the Institutional Animal Care and Use Committee of Catholic University Medical School. The liver cancer model procedure was performed as described previously [19, 20]. Male Sprague-Dawley (200 g) rats received intraperitoneal injections of DEN (Sigma, St. Louis, MO, USA) at 50 mg/kg body weight once a week for 16 weeks to induce HCC. We recorded from 0 days or 0 weeks after the last DEN injection. Two rats were sacrificed to confirm the successful development of HCC after the PET/CT scan at intervals of two weeks.

3.2. PET/CT imaging

Images were taken at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection, using a PET scanner and a General Electric discovery STE (Waukesha, WI, USA). Subjects were deprived of food for about 12 ~ 16 hr preceding the ^{18}F-FDG injections, but had access to drinking water at all times. ^{18}F-FDG (1.1 ± 0.04 mCi) was injected intravenously into the caudal vein, followed by a 30 min uptake period, during which the subjects remained conscious in a warm environment with a heating pad for optimal brain ^{18}F-FDG uptake. During brain scanning, anesthesia was maintained with ketamine and xylazine, and body temperature was kept at 37 °C with a heating pad on the scanner bed.

3.3. PET/CT imaging data analysis

PET imaging data was analyzed as described in previous literature [21, 22]. For semi-quantitative analysis of ^{18}F-FDG uptake, the ROIs in the lesion area were identified in images of the coronal and horizontal sections. Each ROI was defined on the coronal tomograms that showed the highest uptake in the middle of the tumor. The mean of ^{18}F- FDG in the ROI was calculated as the averaged nCi/cc

after calibration for both livers of insulated areas and muscle in the same images. Radioactivity was used as a reference to normalize data obtained in the normal tissue.

3.4. Liver function tests

Serum ALT (Siemens, Sacramento, CA, USA), AST (Siemens), and ALP (Siemens) determinations were analyzed at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection. Briefly, the blood samples were collected after the PET-CT. The samples were centrifuged at 3000 rpm for 20 min, and the plasma was collected in tubes. Plasma samples were stored at -70°C until use. ALT, AST, and ALP concentrations were estimated using the ADVIA 1650 bioassay (Siemens, USA).

3.5. Histological examination

After taking the PET-CT scan at minus 2, 0, plus 2, and plus 4 weeks after the last DEN injection, animals were sacrificed for histological examination. The rats (n=5, for each week) were deeply anesthetized with 15% urethane and sacrificed by decapitation. Then the liver was immediately removed and fixed in 10% formalin for 24 hr. The liver tissues were embedded in a paraffin block and cut into slices with a thickness of 4 μm for hematoxylin and eosin (H&E) staining. The sections were dewaxed in a histochoice clearing agent (Sigma) and rehydrated through a graded alcohol series. The slides were stained with H&E, and images were acquired using a slide scanner (3D Histech, Budapest, Hungary) and a microscope equipped with a spot digital camera (Nikon, Chiyoda-ku, Tokyo, Japan) (× 200).

3.6. Statistical analysis

The value of ALT, AST, ALP, and ROI were subjected to the paired t-test of the rats. Data are presented as mean values ± SD. Probability values less than 0.05 were considered significant. The statistical analysis of survival was carried out using a log-rank test.

4. Conclusion

We have shown that PET/CT imaging could provide *in vivo* detection and quantification of HCC. Also, the uptake of ^{18}F-FDG showed similar results compared to the histological examination. This may suggest that the progressing tumor and newly generated tumors in DEN-induced HCC can be observed with non-invasive methods, without sacrificing rats. Also, it would be very useful for the monitoring experimental therapies. Further studies are needed to identify the PET/CT imaging of the progressing tumor in the therapeutic effects of drugs on HCC.

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