

Non-Thermal Effects of Far-Infrared Ray (FIR) on Human Hepatocellular Carcinoma Cells HepG2 and their Tumors

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Abstract

Background: We developed a cell culture CO₂ incubator and a mice rack that can continuously irradiate cells or murine with FIR. Our goal is to make clear the non-thermal effect of FIR on HepG2 with these instruments morphologically.

Methods: By using them, *in vitro*, we examined the proliferation of cultured HepG2 cells with hemacytometer, BrdU assay, WST-1 assay, HE staining, Toluidine blue staining and microarray studies. And *in vivo*, we measured the tumors, observed the sections by IHC, DAPI staining with light microscopes and performed microarray studies.

Results: Proliferation of HepG2 cells were suppressed (e.g., cell count declined by 34% after 10 days of FIR irradiation), tumor volumes reduced by 86% after 30 days of FIR irradiation, mRNA of Vascular Endothelial Growth Factor (VEGF) decreased by 48%, vascular area in cross sections from the tumors decreased 60% compared with the control. More frequent properties in apoptosis were observed by TUNEL and DAPI staining in FIR-treated groups. Body weight of mice increased compared with the control. Oxidation and Reduction (Redox) reactions by H⁺ (proton and electron)/O₂⁻ (a kind of Reactive Oxygen Species (ROS)) were induced by FIR.

Conclusions: These results clarified that FIR inhibited the proliferation of HepG2 at non-thermal circumstances (at 25±0.5, 37±0.5°C). FIR will serve as a tool against diseases induced by HepG2.

Keywords: Far-Infrared Radiation (FIR); VEGF; HepG2; ROS; Redox

Introduction

Recently, adding to the heat effect, biological effects of FIR have been reported. For instance, at 39~41°C, mammary tumor growth in mice was inhibited (Udagawa et al., 1999; Udagawa and Nagasawa, 2000; Udagawa, 2000). Furthermore, even at normal temperature, at 25.5°C, it was also inhibited (Nagasawa et al., 1999). As we had FIR incubator and animal raiser made, we had carried out the experimental study as former reported (Hosokawa et al., 2005; Yamashita et al., 2005; Ishibashi et al., 2008) about HSC3 (human tongue cancer cell), Sa3 (human gum cancer cell), A549 (human lung cancer cell), A431 (human vulva cancer cell) and MCF7 (human breast cancer cell) (Udagawa, 200). Exceedingly, in this article we

have performed experimental study of effects of FIR on cultured hepatocellular carcinoma cells HepG2 to detect another cancer responsible for its anticancer effects.

Materials and Methods

Instruments

We fabricated an FIR radiant-paneled cell incubator and a murine keeping rack by coating ceramics consist of carbon/silica/aluminum oxide/titanium oxide (radiating efficiency>97% compared with an ideal blackbody of 100%) using a polycarbonate printing technique (Bloodissue Co. Tokushima Japan). They have a stably irradiate system with FIR at wavelength between 4 and 20μm (maximum at 7-12μm) under conditions of 100% humidity, at 37.0±0.5°C degrees and 5% CO₂ in air.

Cell line and cell culture

Human hepatoma HepG2 cell line was purchased from Riken Cell Bank (Tsukuba, Japan). HepG2 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma, St. Louis, MO, USA). The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100μg/mL penicillin G and 100μg/mL streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ in 95% air at 37°C for 24 hours. The medium were replaced every 3 days.

Measurement of cell number and growth

Cells (5×10⁴) were plated in triplicate in 24-well plates (Nunc, Roskilde, Denmark). The attached cell populations were measured on day 0, 2, 4, 6, 8, 10, 12, 14 using 0.3% Trypan blue (Wako Junyaku, Osaka) and a hemocytometer (SLGC, Tokyo). And also WST-1 assay was performed. Cells (1×10³ cells/well) were scattered into 96-well plates (Nunc, Roskilde,

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Denmark). Having confirmed the cells in the non-confluent stage, we administered 10mL WST-1 (Roche, Mannheim, Germany) into 100 mL medium. After 4 hours reaction at 37°C, Absorbance values (at 405nm) were measured by an ImmunoMini NJ-2300 plate reader (System Instrument, Tokyo, Japan).

Morphological observation of HepG2 cells *in vitro*

Cells were incubated on cover slips of 22×22mm (Matsunami Glass Inc. Osaka) on 35mm dishes (Nunc). After 4 days of incubation irradiated by FIR, they were rinsed with PBS (-), fixed by 4% formalin in PBS on ice for 30 minutes. Then Hematoxylin and Eosin staining was performed. Living cells were photographed by CK40 upside down Microscope (Olympus, Tokyo) and PM-BP35 photo instrument (Olympus).

A study of effects of FIR on tumors of mice

2×10⁶ HepG2 cells in PBS containing OD matrigel were implanted in dorsal panniculus layer against 8-10 week CB17/Icr-Prkdc/CrjCrj SCID mice. Immediately after infusion they were divided into with and without FIR treatment groups. Their cages were made of plastics such as to penetrate FIR. And raised in FIR-irradiating mice rack for 50 days. For the duration of the experiment we measured their body weight, shortest and longest axis of tumors every 5 days and calculated the tumor volume by the next equation.

$$\text{Tumor volume} = 0.5 \times (\text{longest axis}) \times (\text{shortest axis})^2$$

Mice were fed with solid food irradiated by sterilizing radiation (Oriental Kobo Industrial Inc., Tokyo, Japan) and sterilized water. After 50 days they were euthanized subjected to the Tokushima University animal care and use guidelines. Tumor tissues were excised and mRNA of them was extracted. During these experiments temperature condition of with and without FIR were set precisely the same.

Histological investigation of tumor tissue *in vivo*

After 50 days mice with and without FIR treatment were euthanized and only tumors were excised and fixed with 4% paraformaldehyde with PBS at 4°C for 4 hours. Then dehydrized by ethanol and embedded in paraffin, produced tissue sections at 4-µm-thick tissue sections and Hematoxylin and Eosin staining was performed.

cDNA microarray assay and data analysis

cDNA microarray analysis was performed in order to elucidate the influence of FIR *in vitro* and *in vivo* on the expression of genes. Total RNA was extracted from the 4 day incubated cells of HepG2 with and without FIR and the tumors from 50 day raised tumor-bearing mice with and without FIR according to the protocol of Qiagen Rne mini kit (Qiagen, Valencia, CA, USA). After ascertained A260/A280 rate was over 1.8 with UV spectrophotometer DC530 (Beckman Coulter, Fullerton, CA, USA), we ascertained the no fragmentation of RNA with Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Next amplification of each RNA and labelling of Cy3 (control group) and Cy5 (FIR group) was carried out using Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). Hybridization of these RNA was performed according to the protocol of Agilent human 1A ver.2 microarray

slides (Agilent Technologies) which carries 19,000 typical human genes. After rinsing and drying, fluorescent light of Cy3 and of Cy5 was scanned by 670 and 770 nm by Agilent Technologies Microarray Scanner (Agilent Technologies). GeneSpring 7.0 software (Silicon Genetics, CA, USA) was used for data analysis and extracted the genes whose expression was significant. And we made a list of FIR-sensitive genes by per spot and per chip intensity-dependent (LOWESS) normalization by using raw data. Then we extracted the data whose value was

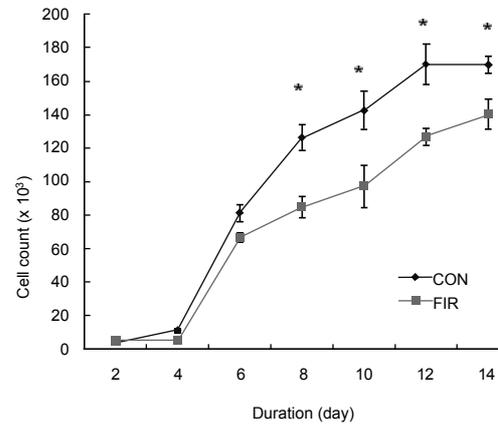


Figure 1A: Cell number were counted using a hemocytometer. Cell count were significantly lower compared with controls on 8, 10, 12, 14 days.

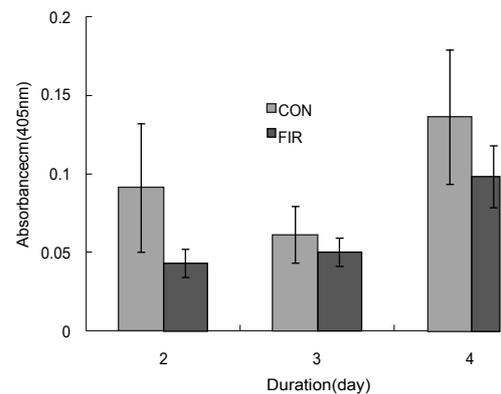


Figure 1B: Synthesis of DNA was examined by BrdU incorporation at days 2, 3, 4 after FIR exposure. It is decreased significantly at days 2, 4.

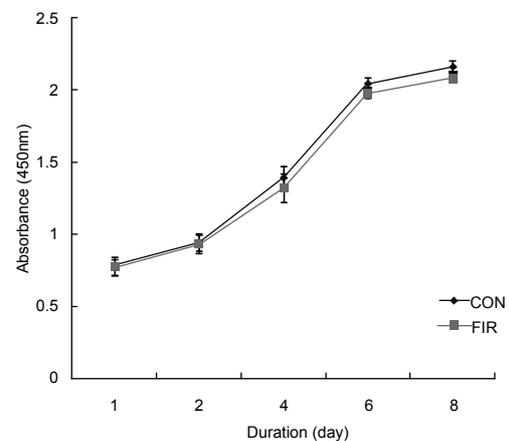


Figure 1C: Cell proliferation was estimated by WST-1 assay. This Water Soluble Tetrazolium salt was cleaved to formazan by mitochondrial dehydrogenases in the viable cells. There were few differences between groups with and without FIR exposure.

Gene Name	Description	Fold change	Control	Heater
VEGF	Homo sapiens vesicular endothelial growth factor (VEGF)	-1.848	108	37
ALB	Homo sapiens albumin (ALB)mRNA	-1.383	101888	83142
AFP	Homo sapiens alpha-fetoprotein (AFP), mRNA	-1.383	101833	83141
ASGR1	Homo sapiens Asialoglycoprotein Receptor 1(ASGR1), mRNA	-1.092	22343	20830
ASGR2	Homo sapiens Asialoglycoprotein Receptor 2(ASGR2), transcript	-10443	8082	4200

VEGF (Vesicular Endothelial Growth Factor) mRNA decreased by 40%.

ALB (Albumin) mRNA decreased by 36%.

AFP (Alpha-Fetoprotein, a marker of liver cancer usually produced only in fetallife) mRNA decreased by 36%.

ASGR1 (Asialoglycoprotein) mRNA decreased by 8%, ASGR2 by 31%.

Table 1: microarray assay of HepG2 in vivo.

at once p -value <0.01 against the control group and expression rate $\geq \pm 1.5$ against the control groups, and then except even one condition as follows---genes whose fluorescence were filled, genes whose fluorescent value were differences with background fluorescence were within $+2.6 \times SD$. We classified these FIR-sensitive genes according to GO term information (<http://www.godatabase.org>).

Detection of tumor cell apoptosis by TUNEL staining

Isolated tumor tissue from euthanized mice was fixed in 4% paraformaldehyde in PBS at 4°C for 4 hours. Dehydrated by ethanol, embedded in paraffin, we produced 4- μ m-thick tissue sections. Then In Situ Detection Kit (Takara Shuzo, Osaka) was carried out. After deparaffinization, treated by proteinase K for 15 minutes at room temperature, rinsed with PBS (-). Labeling reaction was performed with TdT enzyme, and labeling FITC, dUTP at 37°C for 90 minutes. After rinsing with PBS (-), observation of apoptosis was performed with BX51 fluorescent microscope (OLYMPUS).

Statistical analysis

Data are means \pm SE of replicate samples in single experiments or replicate experiments as described in the figure legends. Student's t -test was used for comparisons between two groups-treated groups and control groups. And significant threshold was set to $P<0.05$. Multiple group comparisons were performed by one-way ANOVA, followed by the Tukey-Kramer multiple group comparisons test. All statistical analysis was carried out using Statcel 2 software (OMS publishing, Saitama, Japan).

Results

Effects of FIR on HepG2 Cell Proliferation

To evaluate the effects of FIR on HepG2 cell proliferation, we performed assays as described in the Material and Methods. Figure 1A shows viable cell counts at days 2, 4, 6, 8, 10, 12 respectively after FIR exposure using hemacytometer. Cell counts of FIR-treated group decreased 36, 34, 26, 21% at days 8, 10, 12, 14 respectively compared with control one significantly. Similar result was obtained by BrdU assay. Synthesis of DNA by incorporated BrdU of FIR-treated group decreased by 53, 20, 28% at days 2, 3, 4 compared with control one significantly (Figure 1B). WST-1 assay, however, exhibited similar levels. Absorbance of FIR-treated cells decreased only 0.0, 5.4, 3.3% after 2, 4, 6 days (Figure 1C). This means FIR Water Soluble Tetrazolium salt was cleaved into formazan by

oxidation-reduction (redox) reaction or disproportionation reaction by O_2^- in the FIR-treated tumor compared to the control.

Morphological analysis of HepG2 cells

We were not able to find any difference between experimental and control groups.

Effects of FIR on the expression of HepG2 genes

mRNA of albumin (function marker of hepatocell), alpha-fetoprotein(AFP) and asialoglycoprotein receptor 2 (ASGR2) decreased by 36.1%, 36.0% and 30.7%. VEGF decreased 36.1% (Table 1).

Effects of FIR on implanted HepG2 tumor

Cell count of FIR-treated group decreased 18.4% compared to the control. We measured HepG2 cell-size ($n=1000$) with and without FIR (Figure 2A). Longest axis and volume of cells decreased but the volume of nucleus increased by 5 '16% with FIR group. Therefore nucleus to cell (N/C) ratio of FIR group increased 13 '14%. Disruption of microtubule cytoskeleton of control groups was also observed (Figure 2B). And we could observe cell apoptosis more frequently in FIR-treated groups (Figure 2C). Tumor volume of HepG2 of FIR groups decreased significantly after 30 days. On the other hand body weight decreased slightly compared to control groups (Figure 3A,

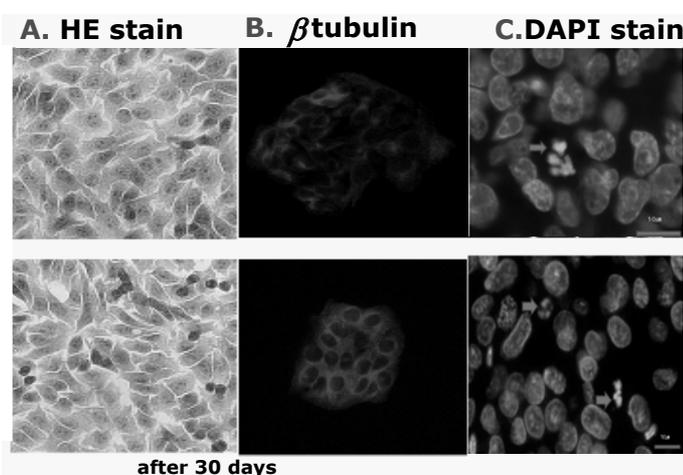


Figure 2: Upper row is shown control group, lower row is shown FIR group; A. HE staining, Cell size is relatively smaller in FIR group than control; B. Disruption of microtubule cytoskeleton in control group and shrinkage of microtubule in FIR group were observed; C. We observed cell apoptosis more frequently in FIR-treated group.

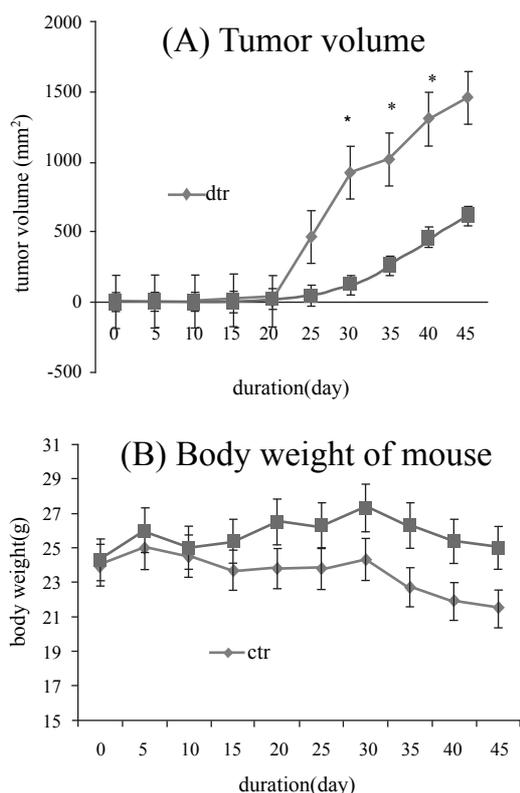
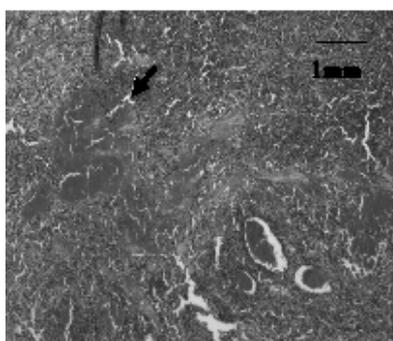


Figure 3: A. From 30 days tumor volume of HepG2 in FIR group is significantly decreased; B. Body weight is decreased slightly compared to control group.

A control
Many vessels grew in the tumor (after 30 days)



B FIR
A few vessels were observed (after 30 days)

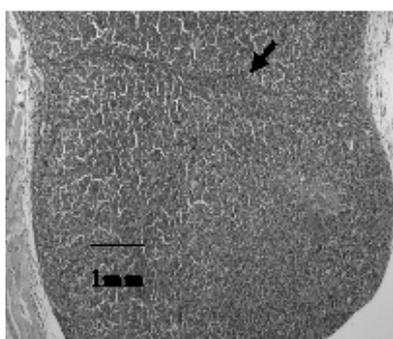


Figure 4: A,B Vascularization area is distinctly fewer in FIR group than in control group.

Figure 3B). Vessel areas also decreased with FIR-treated groups (Figure 4A, Figure 4B).

Discussion

Considering several literatures (Udagawa et al., 1999; Udagawa and Nagasawa, 2000; Udagawa 2000) and our past experimental studies (Hosokawa et al., 2005; Yamashita et al., 2005; Ishibashi et al., 2008), we can experiment and report the results of quite new combination of FIR (4~20mm), HepG2 and mice in this study. New findings are 1) FIR inhibits the growth of HepG2 cultured cells and solid tumor cells 2) H/O_2^- were produced by FIR 3) Cell volume decreases but nuclear volume increases by FIR - therefore N/C ratio increases. 4) Vascular area of tumor was reduced 5) mRNA of VEGF decreased 6) mRNA of ATP-related genes increased

- 1) We have performed these xenograft between human cancer cell and murine four times. And nearly same results have been gotten. So we can't deny this phenomenon. FIR (4~16mm) inhibited HeLa cell growth [14], too. FIR (3~25mm) inhibited Vascular Endothelial Inflammation (Lin et al., 2008).
- 2) As manufacturer's instructions said WST-1 resulted in merely reduction by H, but it is a complex disproportionation reaction producing O_2^- (Ishiyama et al., 1993; Uketa, 2004). Researchers have demonstrated that cells like mitochondrial dehydrogenase produce H/O_2^- (Kohashi, 1996; Kohashi, 1993; Friedman, 2007). But we newly found that without cells they are produced by FIR irradiation.
- 3) This facts are pretty commonly observed. I think this is not only a cause but also a result of cell death. Swelling of nucleus means disruption of nuclear lamina. This was induced by FIR and affect next results.
- 4), 5), 6) We should not decide increase of mRNA don't always related to those protein or their functions.

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