Effect of Interferon-Beta (IFN-β) on tumor suppressor and apoptotic markers in hepatocellular carcinoma cell line

Hend Okasha¹, Marwa Hassan², Tarek Aboushousha³, Safia Samir¹

¹Biochemistry and Molecular Biology Department, Theodor Bilharz Research Institute, Giza, Egypt
²Immunology Department, Theodor Bilharz Research Institute, Giza, Egypt
³Pathology Department, Theodor Bilharz Research Institute, Giza, Egypt

ABSTRACT

IFN-β (Rebif) is a drug with valuable cancer therapeutic potential as it mediates anti-proliferation and apoptosis induction. Therefore, this study aimed to evaluate the anti-proliferation of IFN-β through assessing gene expression of p53 and caspase-3 in hepatocellular carcinoma cell (HCC) line (HepG2). The 50% inhibition viability concentration (IC50) of IFN-β was calculated by cytotoxicity assay, and it was tested on normal cell line (Vero). After treating HepG2 cells with IFN-β, p53 and caspase-3 expression was analyzed, at time intervals of 2, 4, 6, and 24 hrs, by real-time PCR, histopathology and immunohistochemistry. IC50 of IFN-β was 420 ng/ml, which wasn't cytotoxic on normal cells. P53 expression was gradually up-regulated by time, and then, it was decreased after 24 hrs incubation. However, no expression of caspase-3 was detected compared to cell control. Histopathologically, cells degeneration was remarkable after 24 hrs while no change was noticed in control. Immunohistochemical analysis revealed a decline of p53 and caspase-3 expression in treated cells with IFN-β after 24 hrs, to 30% compared to cell control (75% and 60%, respectively). In conclusion, IFN-β has the potential to be utilized as a suppressor of HCC proliferation and inducer of malignant cells apoptosis.

INTRODUCTION

Hepatocellular Carcinoma (HCC) is a primary cancer of liver cells which causes more than 670,000 deaths around the world. It is the sixth most common cancer in the world and the third most common cause of cancer-related fatality. In Egypt, HCC is the second most common cancer in men and the sixth most common cancer in women (Zekri et al., 2016; Mourad et al., 2018).

Because of the high hepatotoxic effect, classical chemotherapy has failed to cure metastatic hepatoma. The most broadly used medication for liver cancer treatment is the doxorubicin alone or in combination with other chemotherapeutics. (Raoul et al., 2019). However, their severe toxicity on normal hepatocytes leads to their limited usage. Consequently, finding alternative therapeutic agents without or with low hepatotoxicity are highly desirable (Li et al., 2014).

Interferons (IFNs) are pleiotropic cytokines that have important antiviral, antiproliferative, antitumor, and immunomodulatory activities (Zhao et al., 2014). According to previous studies, all three types of IFNs can induce apoptosis of tumor cells. The apoptotic classical features of certain types of tumor cells were definitely apparent when exposed to IFNs such as cell shrinkage, chromatin condensation, and
DNA fragmentation. These signs led to utilizing the expression of apoptotic markers or the suppression of tumor markers as an indicator to IFN-induced apoptosis (Testoni et al., 2011). Interferon beta (IFN-β) is considered a signaling molecule with important therapeutic potential in cancer management since it induces gene transcription which mediates anti-proliferation and cell death induction (Kazaana et al., 2019).

IFN-β has been known to cause apoptosis in certain cell types, such as pro-B cells, human adrenocortical carcinomas, and hepatoma cell lines. The IFN-β signaling pathways comprises STAT1 homodimers and STAT1/STAT2 heterodimers. The pro-apoptotic effect of STAT-1 is dependent on transcription-self-reliant interactions with p53, TRADD, and NF-κBp65 and on transcription-dependent activation of caspases and death receptors and ligands. Many studies have concluded that STAT1 was related to the expression of the inflammatory caspases-1 and -11, leading to activation of the effector caspases (Liu et al., 2018).

This study aims to examine the anti-cancer therapeutic effect of interferon-β on human HCC cell line (HepG2) through analyzing the expression levels of p53 and caspase-3 as a tumor suppressor and apoptotic markers, respectively.

MATERIALS AND METHODS

Cell lines and cell culture

The human liver cancer (HepG2) and African Green Monkey kidney Vero cell lines were obtained from the Department of Cell Culture, Vacsera, Egypt. Cells were cultured in D-MEM w/o PYR (Thermo Fisher Scientific) and RPMI 1640 medium (Biowest, USA), respectively, supplemented with 10% inactivated fetal bovine serum (FBS) filter sterilized (LONZA), antibiotics (penicillin, streptomycin), fungizone solution (LONZA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer 1M (Biowest, USA).

Cytotoxic and Anti-cancer activity

According to Feoktistova et al. (2016), the cytotoxicity of IFN-β (88 mcg/ml); Rebif, was tested on both the normal cell line (Vero cells) versus the cancer cell line (HepG2). HepG2 and Vero cell lines have been harvested, inoculated in 96 well plates (7000 cells/100 μl medium/well), and cultured with RPMI 1640 medium and D-MEM, respectively. The media were supplemented with 10% FBS, 1% antibiotic fungizone and 1% HEPES buffer. The plates were incubated at 37°C for 24hrs with 5% CO2 in a humidified chamber. After 24 hrs, the media were removed from the monolayer cultures. IFN-β was diluted, in the corresponding media supplemented with 2% FBS, at different concentrations starting from 5 μg/ml and then 2-fold serial dilutions. The diluted IFN-β was added to the cells, and the plate was incubated for 24 hrs. Then, IFN-β was aspirated from all wells. Twenty μl of crystal violet solution (0.5%) was added to each well, and the plate was incubated at room temperature for 10 min. Each well was washed 3 times with 200 μl distilled H2O. After washing, the plate was inverted on filter paper to remove any remaining liquid. Two hundred μl of methanol was added to each well, and the plate was incubated with for 20 min at room temperature. Using ELISA reader, the optical density was measured at 570 nm (OD570). The IFN-β efficiency was calculated according to the following equations:

% cell viability = (treated cells O.D / control cells O.D) x 100

Percent of cell cytotoxicity (%) = 100 – percent of cell viability (%)

After measurements were performed, the concentration required for a 50% inhibition of viability (IC50) was determined graphically. Standard Graph was done by plotting the concentration of IFN-β on X-axis and the relative cell viability on Y-axis.

All experiments were performed in triplicate, and the mean values were used for calculation.

Selectivity index (SI)

The SI indicates the cytotoxic selectivity (i.e. safety). The selectivity index (SI) defined as the proportion of the IC50 obtained from the experiment on normal cells vs. cancer cells (Senthilraja and Kathiresan, 2015).

SI = IC50 of a compound in a normal cell line/ IC50 of the same compound in a cancer cell line.

Anti-proliferative effect of IFN-β

After detecting the optimum concentration of IFN-β at which 50% of cells died (IC50), this concentration was added to a 6-well tissue culture plates containing 7x10^5 HepG2 cells per ml of RPMI, supplemented with 10% FBS, 1% antibiotic fungizone and 1% HEPES buffer. The plates were cultured at 37°C for 24hrs with 5% CO2 in a humidified chamber. After that, media were aspirated from monolayer cultures and stimulated with a concentration of 420 ng/ml of IFN-β. Incubation was done for 2, 4, 6 and 24 hrs. After incubation, HepG2 cells were subjected to total RNA extraction.

Extraction of total RNA

According to the modified protocol of Chomczynski and Mackey (1995), the monolayer cells were
rinsed with ice-cold PBS once. Then, they were directly lysed by adding 1 ml of Trizol reagent (Invitrogen, USA) per 3.5 cm diameter dish and cells were scraped with a scraper. Two hundred μl of chloroform was added to the mixture which was suspended by vortex for 15 seconds and was incubated at room temperature for 2 to 3 minutes. Centrifugation was done at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred to a new tube. The RNA was precipitated from the aqueous phase by mixing with 500 μl of isopropanol alcohol. The sample was incubated at 15 to 30°C for 10 minutes and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed completely, then the RNA pellet was washed once with an equal volume of 75% ethanol and was mixed using vortex followed by centrifugation at 7,500 x g for 5 minutes at 4°C. The washing procedure was repeated, and all the ethanol was removed. The pellet of RNA was dried with vacuum for 5-10 min. Finally, the RNA was dissolved in DEPC-treated water (Ambion).

Quantitative real-time PCR (qRT-PCR)

Using SuperScript™ IV First-Strand Synthesis Kit (Invitrogen™, Cat number: 18091050), 0.1 ug of total extracted RNA was used in order to obtain cDNA. qRT-PCR using QuantiTect SYBR Green PCR kit (Qiagen, USA, Cat Number: 208052) was performed on Applied Biosystems StepOne™ Real-Time PCR System (AB Applied Biosystems, Foster City, CA) using following cycling parameters: 95°C for 30 seconds, then 55°C for 15 min followed by 40 cycles of (95°C annealing for 30 seconds), followed by a melt curve. The sequence of primers (Thermo Scientific) was designed for each gene, as shown in Table 1 (Huang et al., 2017). The ratio of expression of each marker was denoted as the mean of three experiments. The gene expression levels were detected by the relative comparative quantitation method.

Table 1: Sequence of primers for gene expression analysis using qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>GCCCAACAAC-</td>
<td>CTTGGGCATC-</td>
</tr>
<tr>
<td></td>
<td>CACAGCCTCTCT</td>
<td>CTTGAGTTCC</td>
</tr>
<tr>
<td>Caspase3</td>
<td>CTCGGTCTGCTG</td>
<td>CATGCCCTCA</td>
</tr>
<tr>
<td></td>
<td>GTCAGATGTCGA</td>
<td>GAAGCACAACAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAGTCACAAC-</td>
<td>GACAAGCCTTC-</td>
</tr>
<tr>
<td></td>
<td>GATTGGCTCGT</td>
<td>CGTTCTCAT</td>
</tr>
</tbody>
</table>

Histopathological Study

According to Koh et al. (2013), The HepG2 cells were grown on culture plates till reaching 80% confluence. Then, cells were removed by trypsinization. In order to harvest the cells, centrifugation was performed at 1,000-3,000 x g for 1 minute. Washing of cells with 20 ml of 1x PBS (Biowest, USA) was carried out twice. After repeating centrifugation, cells were resuspended in 20 ml of 10% Neutral Buffered Formalin (VWR, Cat. #VW3239-4) and were incubated for 15 min at room temperature. Centrifugation and washing steps were repeated. Carefully, the cell pellet was released from the bottom of the tube with a pipette and 0.5 ml of 3% low melting agar solution (Sigma, USA, Cat Number: 39346-81-1) has been added. After solidification at room temperature, excess agarose from the cells was removed.

The agarose pellet was then placed into a tissue cassette for tissue processing. Four microns thick sections were stained with hematoxylin and eosin stain (H and E stain) for routine histopathological examination.

Immunohistochemical analysis of p53 and Caspase-3 expression

Removal of agarose from the cells sections was done by immersing the slides in Xylene with two changes for 10 min each was done, followed by rehydration of tissue sections by immersing the slides in decreasing grades of ethanol.

Antigen retrieval was established by immersing the slides in a microwave compatible tray containing 10 mM Sodium Citrate buffer (pH 6.0) with 0.05% Tween 20. Then, boiling the slides and maintaining the sub-boiling temperature for 5 minutes in the microwave was carried out. Cooling in room temperature was done for 30 min. The slides were washed three times; 3 min each, by immersing them in TBST (Tris Buffered Saline having 0.05% Tween 20) (Cuevas et al., 1994).

Quenching of Endogenous Peroxidase was done by incubating the slides in 3% hydrogen peroxide prepared in methanol for 15 minutes in dark condition. Then, the slides were washed in TBST 3 times, 3 min each (Radulescu and Boenisch, 2007).

According to Nogueira et al. (2013), each antibody for p53 (Mouse monoclonal anti-P53 antibody, ROCH Cat Number: Bp53-11) and caspase-3 (Recombinant Anti-Caspase-3 antibody, Abcam, Ventana, Cat Number: ab195905) was used at a dilution 1:100 in 1x PBS. Sections were incubated with diluted primary antibody overnight, at 4°C, in a humidified chamber. After washing the sections 4 times, 5 min each with TBST, they were then incu-
hated for 30 min with the secondary biotinylated antibody followed by avidin-peroxidase complex (Avidin peroxidase Universal Detection Kit, Dako, Denmark) for another 30 min according to the manufacturer’s instructions. A brown color was developed with diaminobenzidine for 2-4 min, washed in distilled H2O, and counterstained with Mayer’s hematoxylin for 1 min. The entire procedure was performed at room temperature. In addition, negative controls, in which the primary antibody was omitted and replaced by 1x PBS, were also used.

The expression level of p53 and caspase-3 in tissue cells was judged according to the percentage of p53 and caspase-3 positive cells in each sample. Specifically, a percentage of ≤10 % was judged negative, and >10 % was positive.

All sections were evaluated and recorded. The sections were examined by using light microscopy [Scope A1, Axio, Zeiss, Germany]. Photomicrographs were taken using a microscope-camera [AxioCam, MRc5, Zeiss, Germany].

RESULTS AND DISCUSSION

Cytotoxic activity of IFN-β on normal and cancer cell lines

Cytotoxic activity was tested in vitro against Vero cells and human cancer cell line HepG2 to determine IC50. The results of the cytotoxicity assay are presented in Table 2. IFN-β was found to be more toxic to cancer cells than normal cells and was able to inhibit the proliferation of cancer cells HepG2 at a concentration of 420 ng/ml. (Figure 1).

### Table 2: The percent of cytotoxicity of IFN-β on Vero and HepG2 cell lines using a different concentration of IFN-β

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Vero</th>
<th>% Cytotoxicity</th>
<th>HepG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µg/ml</td>
<td>13.75</td>
<td>90.4</td>
<td></td>
</tr>
<tr>
<td>2.5 µg/ml</td>
<td>12.75</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td>1.25 µg/ml</td>
<td>12.5</td>
<td>70.1</td>
<td></td>
</tr>
<tr>
<td>0.625 µg/ml</td>
<td>8.25</td>
<td>57.6</td>
<td></td>
</tr>
<tr>
<td>0.3125 µg/ml</td>
<td>4.75</td>
<td>49.5</td>
<td></td>
</tr>
<tr>
<td>0.156 µg/ml</td>
<td>2.25</td>
<td>47.25</td>
<td></td>
</tr>
<tr>
<td>0.078 µg/ml</td>
<td>0</td>
<td>44.5</td>
<td></td>
</tr>
<tr>
<td>0.039 µg/ml</td>
<td>0</td>
<td>38.13</td>
<td></td>
</tr>
<tr>
<td>0.0195 µg/ml</td>
<td>0</td>
<td>34.14</td>
<td></td>
</tr>
</tbody>
</table>

Selectivity index (SI)

According to the IC50 of IFN-β on normal cells (Vero) and cancer cells (HepG2), the cytotoxic selectivity (therapeutic) index was calculated where the IC50 of Vero cells was 16.6 µg/ml and the IC50 of HepG2 cells was 420 ng/ml. Accordingly, the therapeutic index was IC50 of IFN-β in Vero cell line/its IC50 in HepG2 cell line = 39.52. The protocol of Moustafa et al. (2014) evaluated the cytotoxic activity as remarkable activity when affecting >75% of cell population, moderate activity when affecting 75-40 % of cell population, low activity when affecting 40-0.1% of cell population, or no cytotoxic activity when affecting 0% of cell population. IFN-β had low cytotoxic activity against normal Vero cells and had remarkable cytotoxic effect against cancerous liver cells. The data obtained in the current study revealed that the half inhibitory effect of IFN-β on HepG2 cancer cell growth occurred at a concentration of 420 ng/ml. Senthilraja and Kathiresan (2015) stated that a drug is considered to be worthy if it has a therapeutic index value of 16 or greater. The present study detected a therapeutic index value of IFN-β of about 39.52 in HepG2 cells.

Apoptotic genes expression (P53 and Caspase-3)

The relative expression levels of p53 and caspase-3 were evaluated in HepG2 cell line using GAPDH as a reference gene. The results indicated cumulative up-regulation of p53 after incubation of cells with IFN-β in comparison to control cells with the highest level of expression of p53 was detected after 6 hrs of incubation (Figure 2). However, no expression of caspase-3 was detected in both treated and control HepG2 cells. The calculated IC50 was used to detect the relative expression of the tumor suppressor p53 and the apoptotic marker caspase-3 at time intervals of 2, 4, 6, and 24 hrs, using real-time PCR. A marked increase in the expression of p53 time-dependently was noticed as Takaoka et al. (2003) and Dierckx et al. (2017) who demonstrated that IFN-α/β induce the transcription of the p53 gene. However, no expression of caspase-3 was detected in the current experiment. In contrast, Murata et al. (2006) have found that caspase-3 increased in HCC cell lines after the addition of IFN-β.

Histopathological examination

Histopathological examination of sections prepared from the cell control group and stained by H&E stain revealed sheets of malignant hepatocytes (50 to 150 cells per sheet), with preserved morphology. The number of cells per sheet was decreased over the 24 hrs after application of IFN-β to the media, being only 5 to 15 cells per sheet after 24 hrs.

Morphological changes were also evident, with remarkable cellular degeneration and loss of nuclei was noticed, especially after 24 hrs from the time of IFN-β application, as shown in Figure 3.
Figure 1: (a): Percentage of viability of normal Vero cells at different concentrations of IFN-β. (b): Percentage of viability of HepG2 cells treated with various concentrations of IFN-β to detect the median inhibitory concentration (IC50).

Figure 3: Sections in HepG2 cells at different incubation periods (2hrs, 4hrs, 6hrs, and 24hrs) showing degeneration of cells after 24h compared to cell control (CC). (Hematoxylin and eosin stain)

**Immunohistochemical analysis**

Studying the expression of both p53 and caspase-3 in tissue sections at 24 hrs after applying the IFN-β revealed that HCC cells of the control group showed a mean value of 75% positive cellular expression of p53 which was evident as a nuclear brownish coloration of the cultured cells. The percentage of positive cells decreased gradually giving a mean of 30% in cultured cells after 24 hrs from the time of applying IFN-β (Figure 4 and Figure 5).

In addition, the cell control group showed a mean positive caspase-3 expression of about 60% in cultured cells. However, the percentage of positive cells, treated with IFN-β, accounted for approximately 30% after 24 hrs (Figure 6 and Figure 7).

Histopathological examination revealed that IFN-β has induced degeneration of HepG2 cells after 24 hrs of incubation compared to the cell control during the same incubation period. 75% expression of p53 was detected in cell control and this percentage was decreased in IFN-β-treated cells to 30%. This decline of p53 expression might be due to the
cell degeneration caused by IFN-β treatment for 24 hrs. In addition, marked expression of caspase-3 (60%) in cell control was observed, in comparison with IFN-β-treated cells (30%). This could be also a result of apparent cell degeneration. The results of both real-time PCR and immunohistochemistry were the same for p53 expression. On the other side, caspase-3 gene expression was not detected by real-time PCR, and this may be explained by the late expression of caspase-3 in tumor cells (Jin et al., 2016) that could be detected immunohistochemically using a specific antibody to the expressed protein in the localized tissue.
CONCLUSIONS

In conclusion, the results of the present study reveal the therapeutic potential of IFN-β as an agent that can be utilized for suppressing tumor proliferation and inducing apoptosis of liver cancer cells. Further studies will be warranted for the assessment of IFN-β alone or in combination with chemotherapeutic drugs (such as doxorubicin) on experimental models.

Clinical significance

In spite of discovering novel chemotherapeutic drugs such as doxorubicin, the frequency of cure is still limited for hepatocellular carcinoma (HCC). Hence, IFN-β was investigated as a potential therapeutic agent. In the current study, it was found that IFN-β has an anti-cancer effect on HCC cells by suppressing tumor proliferation and promoting apoptosis.

REFERENCES


paralog DNp73 oncogene is repressed by IFN-alpha/STAT2 through the recruitment of the Ezh2 polycomb group transcriptional repressor. Onco
gene, 30:2670–2678.
