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Ultrasound-mediated interferon β gene transfection inhibits growth of malignant melanoma

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ABSTRACT

We investigated the effects of ultrasound-mediated transfection (sonotransfection) of interferon β (IFN-β) gene on melanoma (C32) both in vitro and in vivo. C32 cells were sonotransfected with IFN-β in vitro. Subcutaneous C32 tumors in mice were sonicated weekly immediately after intra-tumor injection with IFN-β genes mixed with microbubbles. Successful sonotransfection with IFN-β gene in vitro was confirmed by ELISA, which resulted in C32 growth inhibition. In vivo, the growth ratio of tumors transfected with IFN-β gene was significantly lower than the other experimental groups. These results may lead to a new method of treatment against melanoma and other hard-to-treat cancers.

1. Introduction

Malignant melanoma is one of the most malignant tumors known to man. It causes the majority of skin cancer related deaths. Although there is a chance of cure in early surgical resection of localized tumor, disseminated form of the disease remained very difficult to treat. Several treatment modalities have been investigated. The more recent of these include gene therapy under the premise that some form of cancers, melanoma included, are somehow gene-related [1,2]. Gene therapy is carried out by introducing recombinant genes into somatic cells to alter the course of a disease process. Several strategies have been designed to introduce functional genes and to allow integration into the nucleus of target cells. Viral-mediated gene transfer is efficient for the task [3], but cytotoxicity, cytopathy and antigenicity are among the limiting factors in therapeutic application. Non-viral methods offer an alternative method for gene transfection. These include electro-transfection and liposome-mediated transfection. Electro-transfection is a physical method of gene delivery [4,5], whereas liposome-mediated transfection is considered a chemical method [6]. These two methods are considered to be relatively safer than the viral method [4,5,7]. Although gene transfer via electroporation in vivo (DNA injection followed by application of electric fields) is effective to some extent, the tissue damage caused by the electric pulse remains a major issue [8].

The clinical use of ultrasound in the treatment of tumors is now gaining popularity [9]. The use of ultrasound in therapy [10,11] and also in gene transfection (sonotransfection) [12–16] has been investigated both in vitro and in vivo. Ultrasound induces cell-membrane porosity, and enhances the delivery of naked DNA into cells in vitro. Reversible membrane damage induced by ultrasound can occur and then followed by resealing of the holes (or pores) and eventual survival of the cells [17]. During such poration, large molecules in the medium enter into the cells and remain trapped there after resealing [11,16]. Understanding how these mechanisms work has lead us to an experimental system that optimizes an in vitro sonotransfection [2]. Moreover, recent studies have demonstrated the enhanced permeability of naked plasmid DNA into tumors in vivo, as well as the enhanced effect of ultrasound by microbubble echo-contrast agents [8,18]. These phenomena are related to the US-stimulated bubble activity commonly called acoustic cavitations [19,20]. Although several studies have been published about the use of ultrasound in sonotransfection against tumors [21,22], no investigation has been made on the sonotransfection with therapeutic gene against melanoma tumors. In this study, we investigated the microbubble-facilitated sonotransfection of interferon β (IFN-β) gene, a cytokine known to inhibit tumor growth, into malignant melanoma cells in vitro and in vivo [22]. We then investigated whether or not sonotransfection of IFN-β gene to human malignant melanoma (C32) in nude mice can suppress tumor growth.

2. Materials and methods

2.1. Cell line

The human melanoma cell line (C32) was cultured in minimum essential medium (MEM) with Earle's salts and L-glutamine

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supplemented with 1% essential amino acids and 10% fetal bovine serum (Gibco, Invitrogen Corporation, Groningen, The Netherlands) under humidified air and 5% CO_2.

2.2. Plasmid-DNAs and interferon β protein

We used three types of plasmid-DNAs. One was pEGFP-N1 cDNA containing CMV promoter and another was pCMV (Hu beta) inserted human interferon β (IFN-β) cDNA containing CMV promoter. IFN-β plasmid was kindly provided by Riken BRC (Ibaragi, Japan). Human IFN-β was kindly provided by Daiichiseiyaku Co. Ltd. (Tokyo, Japan) and Toray Co. Ltd. (Tokyo, Japan). The other was a GFP-tagged IFN-β cDNA (Human TrueORF™; OriGene Technologies, Inc., MD).

2.3. Gene transfection and growth inhibition of melanoma cell line

About 10,000 cells were plated in each well of the 24-well lumox™ MULTIWELL with 50 μm thin gas-permeable transparent bottom (Greiner bio-one, Germany), and incubated overnight. For sonication, lumox MULTIWELL was placed on top of the ultrasound transducer. After sonication, cells were put back into the incubator. Sonications were done by varying the parameters such as intensity, pulse rate and DF. After 100 μg of pEGFP-N1 and/or IFN-β gene and/or 100 μl of echo-contrast agent composed of microbubbles (Sonazoid; Daiichiseiyaku Ltd., Tokyo, Japan) were added, cells in the MULTIWELL were sonicated using an ultrasound device (SonoPore KTAC-4000, NepaGene, Chiba, Japan). Using transducers designed at 1.011 MHz oscillation frequency; the potential ideal conditions include intensity of 0.17 W/cm^2 at burst frequency of 0.5 Hz, 25% DF, 30 s sonication, rectangular pulse type, and with sweeping frequency type 1 at 12% sweep width and 100 ms sweep interval. Cell viabilities were assayed at 3 and 6 days after the sonication. The growth inhibition effect of IFN-β gene transfection to melanoma cell line was evaluated by counting the number of viable cells.

2.4. Detection of IFN-β in vitro

The amount of IFN-β in the culture medium of the cultured cells was determined by enzyme-linked immunoassay (ELISA) using a Hu IFN-β ELISA kit (Kamakura Technoscience Ltd., Kamakura, Japan) 1 and 3 days after the treatments.

2.5. Measurement of cell viability

The Trypan blue dye exclusion test was performed by mixing 200 μl of cell suspension with an equal amount of 0.3% Trypan blue solution (Sigma, St. Louis, MO) in PBS. After 5 min incubation at room temperature, the number of cells excluding Trypan blue (unstained) was counted using a Burker Turk hemocytometer to estimate the surviving viable cells immediately after sonication.

2.6. Gene transfection and in vivo growth inhibition of melanoma tumors in nude mice

Female nude mice, BALB-C (nu/nu), aged 6 weeks, purchased from Nippon SLC Co., Ltd. (Hamamatsu, Shizuoka, Japan) were used in the in vivo experiments. C32 cells (2×10^6) were collected, suspended in Hank's balanced salt solution (HBSS) (Gibco, Invitrogen, Japan) and inoculated subcutaneously into both of flanks of female nu/nu immunodeficient nude mice (6 weeks old) purchased from Nippon SLC Co., Ltd. (Hamamatsu, Shizuoka, Japan). Mice were housed three to five per cage in microisolator cages placed in laminar flow shelving to maintain sterility. Animals were handled within the sterile confines of a clean room. The mice were otherwise maintained under standard vivarium conditions. Tumor studies were conducted under a protocol approved by the animal care and use committee of the university.

A total of 47 subcutaneous tumors were divided into six groups, (1) tumor-bearing but untreated as control (4 mice: 8 tumors), (2) tumor-bearing treated with US (4 mice: 8 tumors), and (3) tumor-bearing treated with US and MB (4 mice: 8 tumors), (4) tumor-bearing treated with US, IFN-β (gene) and MB (4 mice: 8 tumors), (5) tumor-bearing treated with IFN-β (4 mice: 7 tumors), (6) tumor-bearing treated with IFN-β (gene) and MB (4 mice: 8 tumors).

Two weeks after melanoma inoculation, tumors were subsequently exposed to 1.011 MHz ultrasound using a 2 cm (diameter) transducer after intra-tumor injection with IFN-β genes mixed with microbubbles. Sonications (I_{SATA} 0.22 W/cm^2, burst frequency of 0.5 Hz, 50% duty factor) were performed for 3 min once a week for 4 weeks, a total of four treatments. Tumor sizes (maximum diameter × width) of the transfected group were measured twice a week and compared with that of the un-transfected group.

2.7. Histopathological analysis

Tumors were excised 28 days after the first treatment. Tumors were fixed in 10% neutral buffered formalin, routinely processed and paraffin embedded. Histopathological analysis was evaluated with conventional hematoxylin and eosin staining of tissue sections. The detection of apoptotic cells in tissue sections were performed using TdT-mediated dUTP nick end labeling (TUNEL assay) method.

2.8. Detection of IFN-β, GFP and caspase 3 by immunohistological staining in vivo

Melanoma tumor was excised 3 days after sonotransfection with GFP-tagged IFN-β cDNA (Human TrueORF™; OriGene Technologies, Inc., MD). The tumor tissue was fixed in 10% neutral-buffered formalin and 4 μm sections were mounted onto glass slides. Immunohistochemistry was performed using standard protocols for dichromatic staining of paraffin-embedded sections. Antibodies used were, rabbit anti-human IFN-β polyclonal antibodies (FL-187) and mouse anti-human caspase 3 monoclonal antibody (3CSP03) (Santa Cruz Biotechnology, Inc.), and animal GFP antibody (Cell Signaling Technology). Detection antibodies and secondary detection reagents used from Biocare Medical were horse anti-mouse IgG, alkaline phosphate anti-rabbit IgG, diaminobenzinedine (DAB) and Vulcan Fast red.

2.9. Statistical analysis

Data from these study were analyzed using unpaired *t*-test including Welch's correction. Results were considered to be significant when the corrected *p*-value is less than 0.05, indicated as $p < 0.05$ in the manuscript and figure legends.

3. Results and discussion

To determine successful sonotransfection, first C32 cells were sonicated after adding pEGFP-N1 genes into the in vitro culture. Sonotransfected cells glows green under fluorescence microscopy owing to GFP expression in cells. Transfection rates were a little more than 10% in the samples belonging to a group treated with ultrasound in the presence of microbubbles and no transfection was observed in the other groups, particularly the unsonicated sample (Fig. 1A). However, fewer GFP-positive cells were observed when a GFP-tagged IFN-β cDNA was used in sonotransfection. This reduced transfection rate is consistent with the apoptosis inducing

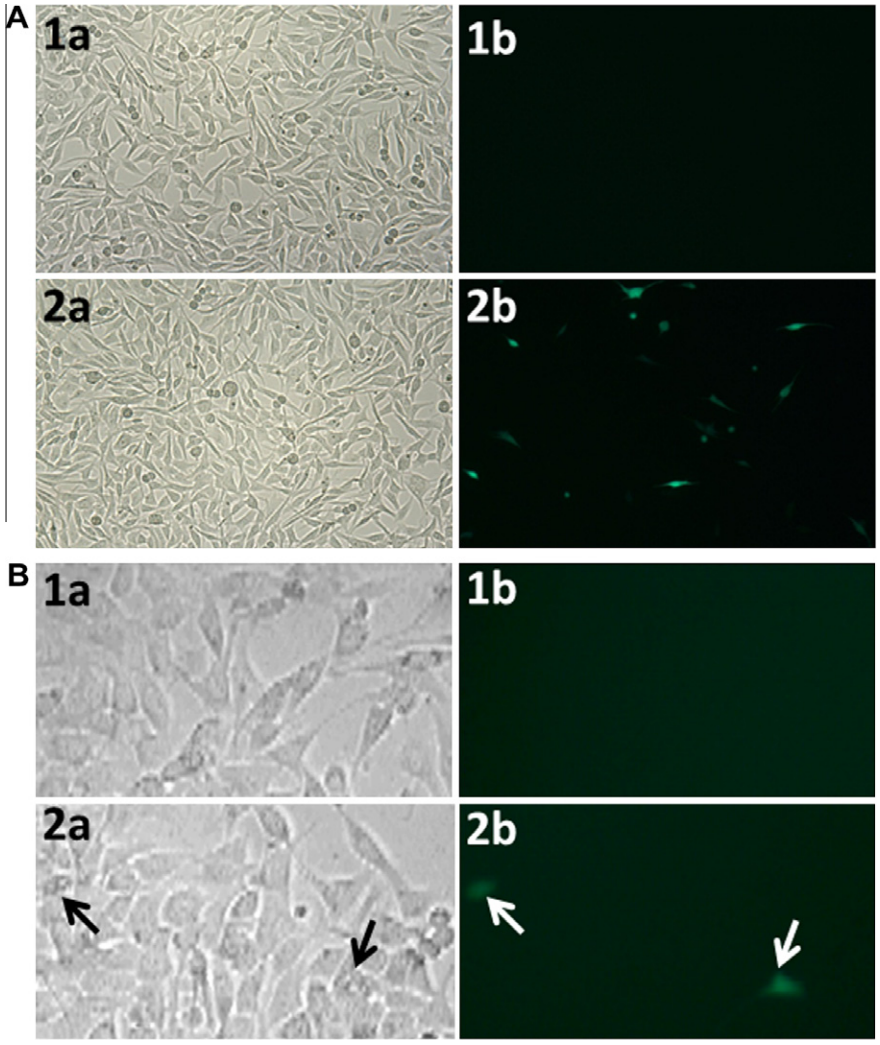

Fig. 1. Microscopy after pEGFP-N1 transfection (A) or sonotransfection with GFP tagged IFN-β cDNA (TrueORF™) (B). Micrograph (100×) of unsonicated control sample (A-1a), and its fluorescence micrograph (1b). No GFP expression was observed with the unsonicated samples. Fluorescent microscopy (100×) revealed GFP expressions after sonotransfection with pEGFP-N1 (A-2a and -2b). Micrograph (200×) of TrueORF™-sonotransfected cells (arrows in B-2b) showing morphological changes consistent with apoptosis (B-2a).

effect of IFN-β on the sonotransfected cells. Fig. 1B (2a and 2b) shows GFP positive cells with morphological changes consistent with apoptosis. Then we measured IFN-β produced by C32 cells after sonotransfection with IFN-β genes. IFN-β was detected by ELISA in the supernatant of cultured melanoma cell line at 1 and 3 days after sonotransfection with IFN-β gene with levels of IFN-β at 85.7 (±2.67 SD) IU and 103.9 (±3.08 SD) IU, respectively. Significant amount of IFN-β was only detected in sonicated group with IFN-β gene and microbubbles. No IFN-β detected in samples from other experimental groups that include those unsonicated and those sonicated in the absence of microbubble and/or IFN-β genes. The results confirmed that C32 cells can be sonotransfected by either pEGFP-N1 gene or IFN-β gene.

In monitoring the number of viable cells in each sample, viable cultured melanoma cells were counted 3 and 6 days after sonication with IFN-β gene and microbubbles. The cell growth ratio of melanoma sonicated with IFN-β gene and microbubbles was significantly lower than the other experimental groups (Fig. 2A). The group sonicated in the presence of microbubbles and the group treated with IFN-β also showed inhibition of cell growth compared with the control group or other unsonicated groups. The numbers of melanoma cells sonicated with IFN-β gene and microbubbles were 0.96×10^4 (±0.49 SD) cells 3 days after treatment and 1.26×10^4 (±0.64 SD) cells 6 days after treatment. Those of unsonicated samples were 5.56×10^4 (±0.53 SD) cells 3 days after treatment and 19.70×10^4 (±2.19 SD) cells 6 days after treatment. The cell number of melanoma sonicated with IFN-β gene and microbubbles was significantly lower than group sonicated in the presence of microbubbles alone and group with IFN-β alone ($p < 0.05$). Number of cells was significantly reduced in IFN-β gene transfection group than unsonicated group 3 and 6 days after treatment ($p < 0.05$) (Fig. 2B). These data suggest that there are synergistic effects between the direct effect of sonication in the presence of microbubbles and the IFN-β generated by the sonotransfected cells against the C32 cells.

Antitumor effect of sonotransfection with IFN-β gene and microbubbles in subcutaneous tumors was also confirmed. The growth ratio of melanoma tumors transfected with IFN-β gene was significantly ($p < 0.05$) lower than the other experimental groups (Fig. 3A). Sonication alone also showed inhibition of tumor growth compared with the unsonicated group. The growth ratio of the transfected group was lower than that of sonication alone and that of the group treated with IFN-β alone, which also showed significant tumor growth inhibition compared with the control with no treatment.

The tumor sizes were blindly measured twice a week, and treatments, including sonication, were repeated every week. The

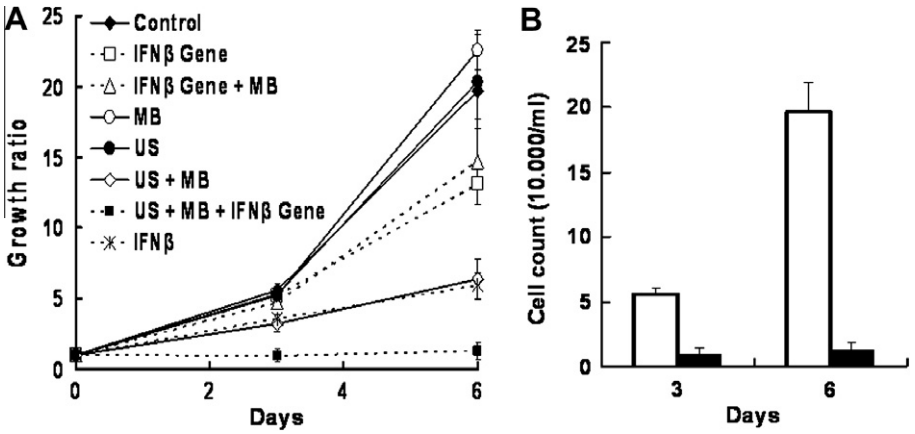

Fig. 2. Effect of ultrasound-mediated IFN-β gene transfection in melanoma cell line. (A) The cell growths of melanoma transfected with IFN-β gene (US + MB + IFN-β gene) were significantly lower than the other experimental groups. Group sonicated in the presence of microbubbles (US + MB) and group added with IFN-β (IFN-β) also showed inhibition of cell growth compared with the control group or other unsonicated groups. The growth of US + MB + IFN-β gene was lower than that of US + MB or IFN-β. (B) Number of cells was significantly reduced in IFN-β gene transfection group (black bars, US + MB + IFN-β gene) than those unsonicated (white bars, control) 3 and 6 days after treatment. Data are expressed as means ± SD. Control, unsonicated; IFN-β gene, IFN-β gene added but not sonicated; IFN-β gene + MB, IFN-β gene and microbubbles were added but not sonicated; MB, microbubbles added but not sonicated; US, sonication only; US + MB, sonication after adding microbubbles; US + IFN-β gene, sonicated in the presence of IFN-β gene but no microbubbles added; US + MB + IFN-β gene, sonicated in the presence of IFN-β gene and after addition of microbubbles; IFN-β, IFN-β added but not sonicated.

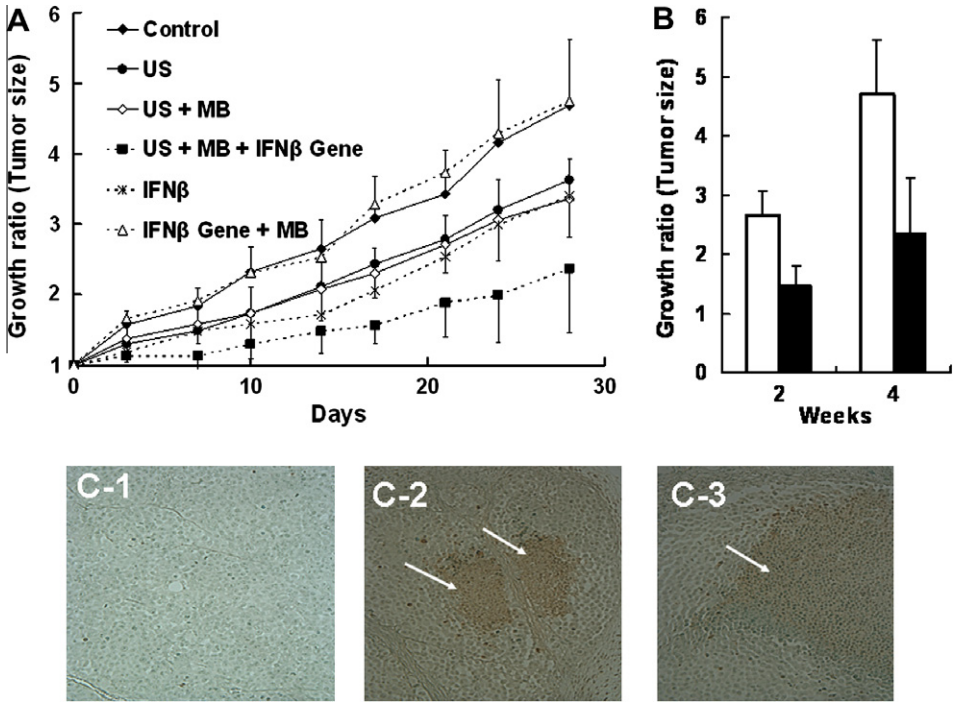

Fig. 3. Effect of IFN-β gene sonotransfection in subcutaneous tumors. (A) The growth ratios of melanoma tumors transfected with IFN-β gene were significantly lower than the other experimental groups. Sonication alone (US) also showed inhibition of tumor growth compared with the unsonicated group (control). The growth ratio of the transfected group (US + MB + IFN-β gene) was lower than that of sonication alone (US) and that of IFN-β treated group (IFN-β). (Control) unsonicated; (US) sonication only; (US + MB) sonication after adding microbubbles; (US + MB + IFN-β gene) sonicated in the presence of IFN-β gene and after addition of microbubbles; (IFN-β) IFN-β added but not sonicated; (IFN-β gene + MB) IFN-β gene and microbubbles were added but not sonicated. (B) Growth ratios of tumors significantly reduced in IFN-β gene transfection group (black bars, US + MB + IFN-β gene) than those unsonicated (white bars, control) 2 and 4 weeks after treatment. Data are expressed as means ± SD. (C) Micrographs (100×) of tumor tissue slides stained with TUNEL assay to determine presence of apoptosis. (C-1) Samples from unsonicated tumor did not show positive staining. Histopathological examination of tumors revealed significant apoptotic induction in the tissue samples from the treated groups. Brownish staining of cells (TUNEL positive indicated by white arrows) indicates apoptosis of melanoma cells after sonication with IFN-β gene and microbubbles (C-2) or IFN-β (C-3).

growth ratios of melanoma tumors sonicated with IFN-β gene and microbubbles were 1.49 (±0.31 SD) 2 weeks after treatment, and 2.37 (±0.90 SD) 4 weeks after treatment (Fig. 3B). Those of unsonicated were 2.65 (±0.41 SD) 2 weeks after treatment, and 4.69 (±0.92 SD) 4 weeks after treatment ($p < 0.05$). The growth ratio of melanoma sonicated with IFN-β gene and microbubbles was lower than the group sonicated in the presence of microbubbles without IFN-β gene or group treated with IFN-β alone. These findings suggest that successful sonotransfection of IFN-β gene resulted in tumor growth inhibition in vivo.

Histopathologic examination of tumors revealed significant apoptotic induction in the tissue samples from the treated groups

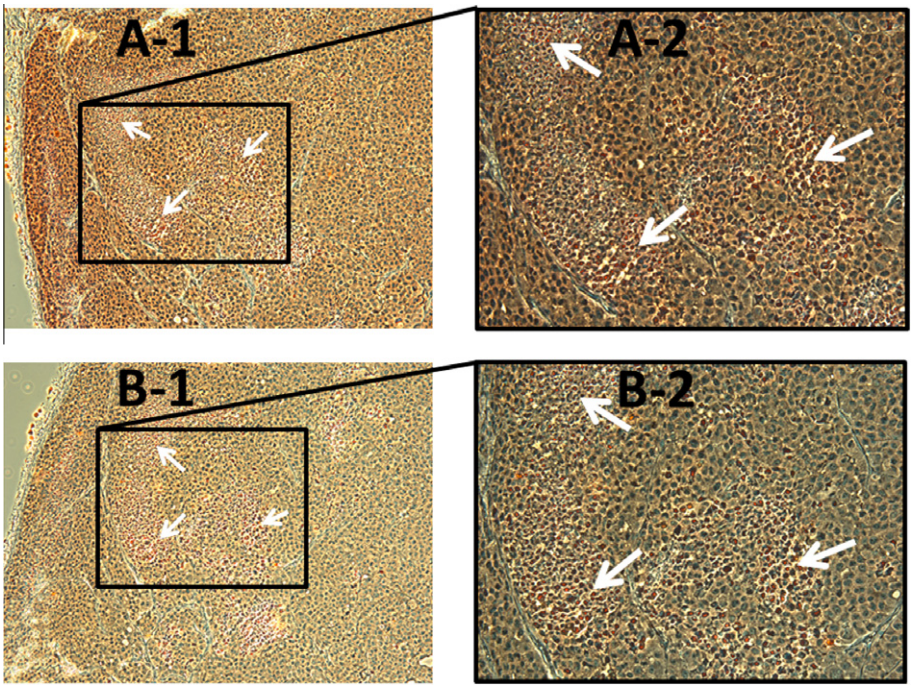

Fig. 4. Immunohistochemistry showing the presence of IFN-β, GFP and caspase 3 in the tumor tissue sonotransfected with GFP tagged IFN-β cDNA. Patchy areas with light reddish coloration (arrows) indicate presence of IFN-β protein and caspase 3 (A). In similar regions of the tumor tissue, the presence of GFP protein and caspase 3 were also detected (B).

(Fig. 3C). Brownish staining of cells (TUNEL positive cells) indicates apoptosis of melanoma cells after sonication with IFN-β gene and microbubbles, or IFN-β treatments.

Furthermore, immunohistochemical analysis of tumor tissue 3 days after sonotransfection with GFP-tagged IFN-β cDNA showed regions in the tumor which are both positive for IFN-β protein, GFP protein, and caspase 3 (Fig. 4). This result confirmed that tumor regions positive for IFN-β in the tumor are the result of sonotransfection, and the relative increase of caspase 3 activity in the same regions further suggests enhanced apoptotic activity in those regions.

In this study, we focused on the sonotransfection of IFN-β into malignant melanoma (C32 cell line) in vitro and in vivo with Sonazoid microbubbles. The result showed that gene transfection can be facilitated by sonication in the presence of microbubbles. Sonotransfection of IFN-β gene resulted into inhibition of cell growth in vitro and tumor growth in vivo that is more effective than just treating cells with IFN-β proteins (Figs. 2 and 3). These findings confirmed previous findings of IFN-β gene transfection by means of cationic liposomes that resulted in tumor growth inhibitions [22]. Successful transfection was also confirmed by fluorescence microscopy visualizing GFP production in cells transfected with GFP genes (Fig. 1). Considering that the ultrasound parametric condition used in the experiments were not directly lethal to the cells, safety of this method for clinical applications is implied. Moreover, use of exogenous IFN-β has been shown to have anti-proliferative effects but not cytotoxic activity against melanoma cells [22], a finding which was also observed in our experiments (data not shown). Data from our in vivo experiments showed that inhibited tumor growth was partly due to apoptosis induction (Figs. 1 and 4), a finding consistent with previous published results showing that IFN-β can induce apoptosis on certain cancer cell lines [23]. Since the level of apoptosis induction was higher in the IFN-β gene treated cells than those directly treated with IFN-β protein, it could be suggested that IFN-β gene transfection is more effective in inducing apoptosis of melanoma cells. Because production of IFN-β is limited only to cells sonotransfected with IFN-β genes, the effects is more likely localized than IFN-β protein treatment.

The prospect of using sonotransfection as a safer method in gene therapy and the ability of ultrasound to be used in a targeted gene transfer, offer greater potential for this method to eventually find its way into clinical use in the treatment of cancer, especially the most difficult ones to treat like melanoma.

Acknowledgments

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