Simultaneous Treatment with P53 Overexpression and Interferon γ Exerts a Dramatic Increase in Apoptosis Induction of U87 Cells

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Abstract

Background: Gliomas possess low immunogenicity, which is an inevitable hinder in front of cancer immunotherapy. Different interferons (IFNs) may proceed apoptosis instead in p53-dependent or independent pathways. P53 induces the anti-inflammatory programmed cell death in cancer cells; on the other hand, IFN gamma (IFNγ) is a modulatory/pro-inflammatory cytokine. There are contradictory reports of whether this cytokine can possess an anti- or pro-cancerous impact on tumors. Hence, we aimed to investigate the possible cooperative apoptotic effect of the P53 and IFNγ overexpressions on the U87 glioblastoma cell line. Materials and Methods: The P53 expressing vector was amplified by Escherichia coli BL21. This vector was confirmed by the aid of sequencing. At the next step, U87 cells were transfected using lipofectamine. Cells were treated with P53 vector and/or IFNγ. The type of cellular death investigated by flow cytometry and the expression level of cleaved caspase-3 protein was also precisely demonstrated by western blotting. Results: Sequencing results revealed that inserted P53 was identical with human P53. Western blot results revealed that both IFNγ and P53 overexpression could up-regulate cleaved caspase-3 protein expression in this cell line. Interestingly, flow cytometry data determined that concurrent treatment with P53 exogenous overexpression and IFNγ induces about 70% apoptosis in U87; more than the sum of cell death occurs after IFNγ or P53 overexpression alone (~18%+21%=39%). Conclusion: The present study results showed that p53-overexpression and IFNγ could ultimately induce up-regulation of the caspase-3 and ultimately significant apoptosis increasing in the U87 cell line. Although IFNγ is believed to be a pro-inflammatory cytokine and P53 is an anti-inflammatory agent, our results demonstrated that they could act synergistically to induce apoptosis in U87 cells.

Keywords: Glioblastoma; Caspase-3; Interferon γ; P53
Introduction

Glioblastoma is the most aggressive and challenging type of brain tumor; it is a high-grade astrocytoma that can develop in adults and children [1]. The median 5-year survival of malignant glioblastoma patients is about 2%, and their overall survival is usually not more than one year and has not been improved significantly during the last three decades [1]. Meanwhile, 17000 patients are reported to be new cases each year in the United States, which is believed to uniformly result in mortality [2]. New approaches delineate a bright future in treating patients suffering glioblastoma; however, they substantially exert side effects and offer palliative outcomes rather than final cures [3]. It is important to notice that after introducing temozolomide as a new effective chemotherapy agent, adjuvant or concurrent prescription of it with chemotherapy or radiotherapy respectively, improved survival for just two months [2-5]. This highlights how critical it is to investigate new approaches for the treatment of this disease. One of the renowned compounds, which seems to play the role of Hecate for tumors, is a cytokine named interferon-gamma (IFNγ) [6]. IFNγ is commonly secreted from natural immune cells, including T-cells found in the tumors’ microenvironment and the tumor cells at low concentrations [7]. This master regulator can be beneficial in the immunotherapy of cancers with poor immunogenicity, as it is a pro-inflammatory cytokine and, according to the literature, up-regulates major histocompatibility (MHC) class I [7]. At first glance, of course, this precious anti-cancer agent may have other sides and can be somehow believed to be a double-edged sword, which confirms that the dark side should be investigated more deeply. By affecting fundamental characteristics of a tumor cell, IFNγ has the potential to interfere with important tumorigenesis properties, including proliferation and angiogenesis; moreover, it can increase immunogenicity [6-8]. On the other hand, repeated vaccination with IFNγ may cause immunoediting in tumor cells, as reported in a study by Rearden et al. [9]. This phenomenon delineates double-face of IFNγ for cancer treatment, which can cause resistance of tumor cells to the therapy, and even more emerges the importance of studies on the underlying pathways related to IFNγ signaling in the tumor cells [10, 11]. P53 is a gatekeeper gene that acts as a tumor suppressor and is an obstacle against different cancer formation and development features, including angiogenesis inhibition, maintenance of genomic stability, etc. [10]. This famous anti-tumor gene is mutated in more than half of different cancers. Most of the tumors obtained from patients with glioblastoma multiform suffer deregulation of the P53 pathway [11]. Opposite to the IFNγ, P53 is an anti-inflammatory agent [12]. Based on the important anti-tumor role of the P53 and the high potential of the IFNγ in apoptosis induction in tumor cells, this study aimed to assess the possible cooperative cytotoxic effect of both agents in a U87 glioblastoma cell line.

Materials and Methods

Cell Culture

U87 cells (purchased from Pasteur Institute, Iran) were cultured in high glucose dulbecco's modified eagle's medium (DMEM; Gibco, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Germany) and 1% penicillin-streptomycin as antibiotics (Gibco, Germany). IFNγ was obtained from Sigma (Germany).

Transformation and Plasmid Extraction

The pCMV-Neo-Bam-H1-p53 plasmid (P53 plasmid), expressing human wild-type P53, was kindly provided by Dr. Michael Resnick (NIEHS, NIH). P53 cDNA was inserted into the pCMV-NeoBam vector from Addgene (Cambridge, Massachusetts, USA), with the aid of cloning by the restriction enzyme BamHI (vector map is depicted in Figure-1). To amplify the plasmid, competent E. coli BL21 (Gibco, Germany) was transformed and cultured on Luria broth (LB) agar (Sigma, Germany) with 0.1% ampicillin (Gibco, Germany).
To reach appropriate absorption, single colonies were isolated and cultured in LB medium.
Plasmids were isolated by BioNEER kit (South Korea, Figure-2).

**Transfection**
For transfection, Lipofectamine® 2000 was utilized. Cells were seeded into 12-well plate 24 hours prior to the transfection without antibiotics; at the time of transfection, cells’ confluency was about 70-80%. The transfection was optimized, and the concentration of transfected plasmid was determined to be 100 ng/μl (we exploited a eukaryotic green fluorescent protein (GFP) containing plasmid to control the transfection). Plasmid with transfection reagents was incubated for 30 min to form plasmid:lipofectamine complex. This complex was added to each well after this time.

**Study Design**
Our main aim in this study was to determine whether P53 and IFNγ act synergistically and exert anti-tumor activity on U87 cells; with this regard, we designed the experiment with four groups as follow:
1. Control group: untreated U87 cells that cultured in normal condition.
2. IFNγ group: U87 cells that received IFNγ (100 ng/ml) for 48 hours.
3. P53 group: U87 cells transfected with P53 plasmid according to the protocol mentioned above (72 hours).
4. FNγ+P53 group: U87 cells transfected with the P53 plasmid and 24 hours later with 100 ng/ml of IFNγ (so the time P53 plasmid had 72 hours to be expressed, same time as the P53 group, and cells treated with IFNγ for 48 hours, same as the IFNγ group).

**Flow Cytometry**
To elaborately investigate whether cellular death induced post-treatment was necrosis or apoptosis, and to quantify apoptosis percent, Annexin V/PI (Roche, Switzerland) kit was performed, and cells' fluorescence was detected by flow cytometry device (FACScan flow cytometer, BD FacsCanto II, USA).

**Western Blotting**
Protein expression of the cleaved caspase-3 was determined by western blotting in all groups. Cells were lysed, and the same amount of protein from each sample was utilized for this test. Firstly, proteins were separated by the aid of 10% SDS-PAGE and transferred to nitrocellulose membranes. These membranes were then incubated for 1 hour with 5% nonfat milk at room temperature. Rabbit monoclonal antibodies against cleaved caspase-3 proteins (Abcam, United Kingdom) were utilized as the primary antibody (membranes were incubated with primary antibody at 4°C overnight). A rabbit monoclonal antibody against β-actin
was used as the control. Post washing step (washing buffer contained Tris-buffered saline and Tween 20), the membranes were incubated with the secondary antibody goat Anti-Rabbit IgG H&L (HRP; Abcam, United Kingdom). The complexes were visualized with an Immobilon Western Chemiluminescent HRP substrate (Millipore, USA).

Ethical Consideration
This study has been approved by the ethics committee of Tehran Islamic Azad University of Medical Sciences (IR.IAU.TMU.REC.1399.373).

Statistical Analysis
All statistical analysis was accomplished by Graphpad Prism V8. For comparing whether differences between two and more samples were statistically significant, student t-test and one-way ANOVA were carried out, respectively. Also, P-value less than 0.05 was considered as significant.

Results

Plasmid Extraction
Competent E. coli BL21 transformed for amplification of P53 plasmid. After transformation, single colonies (red circles in Figure-1) were isolated and cultured in LB broth medium containing 0.1% ampicillin as a selection marker.

After reaching proper optical density, plasmid extraction was performed with the BioNEER plasmid extraction kit (Figure-2).

Vector Map and Sequencing
To confirm the P53 sequence inserted in the plasmid, sequencing was performed for this cDNA with the universal primers of the vector. The inserted sequence was equal to the human P53 cDNA (Figure-3).

Transfection Control
To ensure that the transfection was performed functional, we utilized a eukaryotic GFP containing plasmid and transfected U87 cells with this plasmid as the same protocol used for the P53 plasmid. More than 70% of transfected cells were GFP positive under a fluorescent microscope (Figure-4).

Flow Cytometry
Annexin V/PI flow cytometry results for different samples implicated that the IFNγ group had about 18% cell death (100-cell viability), and the P53 group had about 21% cell death. Interestingly when applied together in the P53+IFNγ group, cell death was calculated to be about 70% (Figure-5).

Western Blotting
The expression level of cleaved caspase-3 protein, as an execution caspase and related downstream of both Bak and Bax up-regulation—which can be induced by IFNγ and p53, respectively—determined by western blotting.
As it is shown in Figure-5, the protein level of the cleaved caspase-3 normalized to the internal protein β-actin increased in treated groups, including the U87 cells that were transfected with a P53-overexpressed plasmid as well as groups treated with IFNγ or concurrent treatment with both (IFNγ and P53-overexpressed plasmid). Furthermore, this expression level was much higher in the P53+IFNγ group than in the other groups (Figure-6).

**Discussion**

High-grade astrocytoma is called glioblastoma, which is the most malignant of all brain tumors and accounts for the majority of gliomas [1]. Glioblastoma symptoms are subtle at first, and some types of gliomas do not cause any symptoms, which results in high mortality and calls for more demand of studies at detection and/or treatment steps [12, 13]. Gliomas are low immunogenic, which obstruct...
immunotherapy. With this regard, different studies performed struggle to increase immunogenicity in these tumors [14].

IFNγ is a renowned master regulator and possesses pro-inflammatory characteristics that can up-regulate MHC class I and increase immunogenicity; however, its cytotoxicity does not fulfill for treatment of tumors; mainly pieces of evidence exist that in repeated vaccination of IFNγ in NHL mice models, it can act reversely and causes tumor resistance [8, 9, 11].

With this regard, we exploited IFNγ treatment in a U87 cell line. Our study revealed IFNγ treatment alone caused about 20% of cell death, which was not satisfactory at first glance. For increasing the efficiency of the treatment, we intended to investigate whether overexpression of P53 can elevate the apoptosis rate in these cells. Hence, at the next step, we assessed the possible synergistic apoptotic outcome of the IFNγ and P53 overexpression on U87 cells. Interestingly, we found out when treating merely with just IFNγ or overexpressing P53, about 20% of cell death occurs; and in cells treated with both, the rate of cellular death increased up to about 80%. In the following steps, we tried to shed light on the possible joint molecular mechanism of action of these two treatments.

Qian et al. [15] assessed the role of IFNγ in immune evasion of orthologous glioma tumors in mice models. They concluded that IFNγ is important in the up-regulation of PD-L1 (an immune suppression receptor that was expressed on the surface of many cancer cells and by interaction with PD1 causes dysfunction of T-cells) even in the tumor microenvironment and act as tumor progressive agent. Furthermore, it is reported that CD8+ T-cells secret IFNγ near the tumor sites, sequentially affecting the JAK/STAT pathway and IRF-1 leading to the up-regulation of PD-L1 in cancer cells [15].

Chemotherapy agents may induce apoptosis with two somehow related predominant pathways, including extrinsic and intrinsic [16].

In the extrinsic pathways, death receptors like the tumor necrosis factor (TNF) family, DR4, DR5, and CD95/APO-1/Fas ligands play important roles [17]. When stimulated, they cause conversion of inactive pro-caspases-8 and -10 into active initiator, which can envisage activation of Bid (a pro-apoptotic Bcl2 family protein) and release of cytochrome C from mitochondria into the cytoplasm [17]. In the intrinsic pathway, mitochondria play a crucial role; and after stimulation, cytochrome C, Apaf-1, and caspase-9 are released to the cytoplasm, where the association of calcium ions forms apoptosome and in turn results in active caspase-3 [16-21]. Similarly, our western blot results revealed that after

Figure 7. The schematic figure for a possible relationship between IFNγ and P53 apoptosis induction. IFNγ can induce the oligomerization of the Bak proteins (a pro-apoptotic member of the bcl-2 family), P53 can induce the oligomerization of the Bax proteins (another pro-apoptotic bcl-2 family member). Both the oligomerized Bak and Bax can induce the permeabilization of the mitochondrial outer membrane (MOM). This leads to the release of cytochrome C into the cytoplasm. The cytoplasm cooperates with other proteins (e.g., Apaf-1 and caspase-9) to produce an apoptosome complex. Apoptosome can induce the pro-caspase-3 into the cleaved/activated form of the caspase-3. This caspase is an execution caspase and can trigger other caspases (e.g., 6 and 7) activation and lead cell towards apoptosis.
treatment of U87 cells with IFNγ and/or P53 overexpression, the expression level of cleaved caspase-3 increased (this may be a reliable joint venture between these two treatments). Tekautz et al. [22] demonstrated that IFNγ induces up-regulation of caspase-8 (an essential initiator caspase in the extrinsic pathway) in neuroblastoma cell lines [22]. Similarly, Afshar et al. [23] in gliomas cell lines without wild-type P53, radiation induces caspase-8 dependent, P53 independent apoptosis [23]. According to the literature, the U87 cells that possess wild-type P53 are more resistant to radiotherapy [23, 24]. Our results indicate that overexpression of P53 in the U87 cell line can potentially sensitive it to treatments.

On the other hand, IFNγ can induce different pro-apoptotic pathways in cancer cells [25-34]. A study performed by Ruiz-Ruiz et al. [35] demonstrated that IFNγ sensitive breast cancer cells to death regardless of P53 status by activating CD95 death receptor, up-regulation of caspase-8, and release of cytochrome C from mitochondria. In the current study, we assessed whether overexpression of P53 can synergistically act with IFNγ in the U87 cell line. Interestingly, flow cytometry data determined that concurrent treatment with P53 exogenous overexpression and IFNγ induces about 70% apoptosis in U87 cells; more than the sum of cell death occurs after IFNγ or P53 overexpression alone (approximately 39%).

Regarding western blotting results, the U87 cells that received IFNγ and P53, the protein expression level of the cleaved caspase-3 was higher than the untreated control or other groups, which received merely IFNγ or p53 overexpression treatment alone. Interestingly, our results confirm that both IFNγ and P53 can lead to the same cell destiny, while the direct targets of them were different. IFNγ does not induce Bax, a pro-apoptotic target for P53 [36]. Bak and Bax are the building blocks that can permeabilize mitochondrial outer membrane (MOM) by oligomerization [36-40]. However, both two compounds (IFNγ and p53) could induce a common pathway of cytochrome C release from MOM into the cytoplasm [38]. Consequently, assembly of apoptosome complex with the aid of Apaf-1 and caspase-9, which results in activation (cleavage) of the execution caspase, caspase-3, and finally caspase cascade (Figure-7) [40].

Conclusions

According to our results, IFNγ and P53 can act cooperatively to induce apoptosis in U87 cells with the aid of caspase-3 activation. Hence simultaneous treatment with both IFNγ and P53 can be exploited to elevate the treatment efficiency, especially in cancers with wild-type P53. However, complementary investigations are an obvious demand prior to the instigation of the bench to the bedside.

Conflict of Interest

The authors declare that there are no conflicts of interest.

References

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