

Comparison of Antitumor Effects of Native and Recombinant Human Interferon- α on Non-small Cell Lung Cancer Cells

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Abstract. *Background:* In the present work, we compared the antitumor effects of native human interferon- α (IFN- α) (nHuIFN- α) and recombinant human IFN- α (rHuIFN- α) on human lung adenocarcinoma A549 cells. *Materials and Methods:* The antitumor activity was determined by measuring cell viability and apoptosis, while the abundance of mRNA, measured by polymerase chain reaction (PCR), determined the potential role of p21 and survivin in antitumor activity of nHuIFN- α . *Results:* The results show that nHuIFN- α significantly reduced A549 cell viability, compared to rHuIFN- α . The most potent effect of nHuIFN- α was also observed when apoptosis was measured. A549 cells treated with nHuIFN- α expressed a significantly higher amount of p21 mRNA, while the amount of survivin mRNA was significantly reduced. *Conclusion:* Considering both the anti-proliferative and anti-apoptotic effects of each IFN- α , we conclude that further elucidation of the mechanisms of the antitumor activity of nHuIFN- α will help in producing more effective and less toxic therapeutic protocols and preparations.

Interferon-alpha (IFN- α) represents a family of glycoproteins with a broad spectrum of activities, including antitumor effects (1). It is well-known that IFN- α has immunomodulatory, anti-proliferative, pro-differentiating, cytotoxic and anti-angiogenic effects (2, 3). The anti-proliferative effect of IFN- α has been suggested to play a major role in its chemotherapeutic effect (4), although the role of IFN- α in the induction of the apoptotic pathway has been also shown (3).

IFN- α is administered as the standard treatment of some malignancies (1). Unfortunately, non-small cell lung cancer

is often resistant to chemotherapy. Results from pre-clinical and clinical studies revealed greater activity when a combination of different chemotherapeutics is used, compared with the activity of single-drugs alone. This effect is further increased by addition of various interferon types, especially IFN- α (5-7). However, the benefits of therapy with IFN- α remain controversial because of discordant results from different clinical trials, and significant dose-dependent toxicological side-effects (8).

Previously we reported that native human IFN- α (nHuIFN- α), which contains several subtypes of IFN- α and other cytokines in traces, has a better anti-proliferative and pro-apoptotic effect on non-transformed cells than does recombinant human IFN- α (rHuIFN- α) (9) suggesting the additive effect of the constituents of the nHuIFN- α . In the present study, we showed that better effects of nHuIFN- α on proliferation and apoptosis could be also expected when tumor cells are treated.

Materials and Methods

Cell culture. A549 cells (human lung adenocarcinoma cell line) were purchased from the American Type Culture Collection (LGC Standards GmbH, Wesel, Germany) and cultivated in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (Moregate Biotech, Bulimba, Australia) and neomycin 50 μ g/ml (Gibco Invitrogen Corporation, Carlsbad, CA, USA). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells were sub-cultivated by splitting them 1:6 twice a week.

Cell proliferation/viability and apoptosis assays. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO, USA) was added at concentration of 0.5 mg/ml and incubated at 37°C for 4 h. The medium was removed and insoluble crystals were dissolved in 200 μ l of dimethyl sulfoxide (DMSO). The absorbance was measured at 560 nm with a reference wavelength of 670 nm (10). Apoptosis was measured with FACScan cytometer using propidium iodide (PI) (Sigma Chemical Co.) DNA staining (9).

Reagents and antibodies. nHuIFN- α was produced at the Institute of Immunology (Zagreb, Croatia) by infection of human peripheral blood leucocytes (hPBLs) with Sendai virus, according to

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standardized production method (11). rHuIFN α was purchased from AbDSerotec (Kidlington, UK). Actinomycin D (ActD) was obtained from Sigma Chemical Co.

Isolation of RNA and reverse transcription and quantitative PCR. Isolation of RNA, reverse transcription and quantitative PCR were carried out as in (10). The primer pair used for the quantification of *p21* mRNA was 5'gga cct gtc act gtc ta 3' and 5'ggc ttc ctc ttg gag aag at 3' and for quantification of survivin mRNA was 5'cca ctg aga acg agc cag ac 3' and 5'gcg caa ceg gac gaa tgc tt 3'. The amount of the transcript was normalized against that for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene transcript amplified with specific primers: 5'agaacatcatcctgctctactg3' and 5'tgtcgtctgtgaagtcagaggaga3'.

Statistical analysis. The statistical significance of the results was determined using the Student's *t*-test. The results are reported using *p*-value, mean and standard deviation of the mean. Differences with a *p*-value less than 0.05 were considered significant.

Results and Discussion

A number of studies have examined effects of nHuIFN- α or rHuIFN- α on different tumor cells. A possible stronger effect of nHuIFN- α in comparison to rHuIFN- α on tumor proliferation and apoptosis has not been described previously. As we described in our previous work on non-transformed cells (9, 10), nHuIFN- α has a stronger anti-proliferative and significant pro-apoptotic activity when compared to rHuIFN- α .

nHuIFN- α more strongly affects the viability and apoptotic rate of A549 cells than does rHuIFN- α . To examine the effects of nHuIFN- α and rHuIFN- α on the viability of tumor cells, A549 cells were incubated for 72 h in a complete growth medium with and without the addition of either of the two IFN- α preparations at different concentrations. Figure 1A shows a dose-dependent decrease in cell viability in A549 cells treated with either type of IFN- α . The decrease was not statistically significant at an IFN- α concentration of 100 IU/ml, compared to non-treated cells ($p>0.05$, $n=3$). rHuIFN- α concentrations of 1,000 and 10,000 IU/ml statistically significantly reduced cell viability when compared to non-treated cells ($p<0.05$, $n=3$). At the same concentrations nHuIFN- α reduced cell viability significantly more potently compared to rHuIFN- α ($p<0.05$, $n=3$) and non-treated cells.

To determine whether IFN- α affects their apoptotic rate, A549 cells were incubated with increasing concentrations of either nHuIFN- α or rHuIFN- α for 72 h, stained with PI and analyzed by flow cytometry. Apoptosis was increased by both IFN- α preparations in a dose-dependent manner (Figure 1B). Both IFNs statistically significantly increased the apoptotic rate at all concentrations compared to non-treated cells ($p<0.05$). A statistically significant increase in the number of apoptotic cells was found when cells were exposed to 1,000 and 10,000 IU/ml nHuIFN- α , in

comparison to cells treated with an equal concentration of rHuIFN- α ($p<0.05$, $n=3$).

A pro-apoptotic response probably also depends on the cell line sensitivity, and the role of IFN- α in it is still far from clear. However, if IFN- α alone is not sufficient for the induction of apoptosis in all tumor cell lines, this would be a strong argument for the addition of other cytokines or usage of polycytokine preparations such as nHuIFN- α . This could also explain why nHuIFN- α significantly more potently induces apoptosis of A549 cells compared to rHuIFN- α .

nHuIFN- α more strongly induces p21 and suppresses survivin expression in A549 cells than does rHuIFN- α . To further elucidate and compare the activity of two IFN- α preparations we measured the induction of anti-proliferative and pro-apoptotic regulator *p21* and pro-apoptotic factor survivin, described as important factors in IFN- α activity (12-14).

Cells were treated with different concentrations of either nHuIFN- α or rHuIFN- α to test whether these preparations are able to modulate the expression of the *p21* gene. The abundance of *p21* mRNA in treated cells was quantified by PCR. Treatment with both IFN- α preparations increased *p21* mRNA synthesis in a dose-dependent manner (Figure 2A). nHuIFN- α significantly increased *p21* mRNA synthesis at every concentration applied compared to non-treated cells ($p<0.05$, $n=3$). rHuIFN- α statistically significantly increased *p21* at a concentration of 10,000 IU/ml ($p<0.05$, $n=3$), but not at 100 and 1,000 IU/ml ($p>0.05$, $n=3$). The induction of *p21* mRNA by nHuIFN- α was stronger than by the same dose of rHuIFN- α at all three concentrations ($p<0.05$).

According to a recently published work, up-regulation of *p21* was correlated to induction of apoptosis through the suppression of survivin (15). It has been reported that the inhibition of survivin induces apoptosis in a number of malignant cell lines and in primary tumor cells (16, 17).

Decrease of *survivin* mRNA expression in cells treated with nHuIFN- α is shown in Figure 2B. Interestingly enough, a dose-dependent increase of *survivin* mRNA expression was observed in cells treated with rHuIFN- α (Figure 2B). There was no statistically significant difference between *survivin* mRNA expression in cells treated with 100 IU/ml nHuIFN- α or 100 IU/ml rHuIFN- α , compared to non-treated cells ($p>0.05$, $n=3$). A549 cells treated with 1,000 and 10,000 IU/ml of nHuIFN- α revealed a statistically significant decrease compared to non-treated cells ($p<0.05$, $n=3$), while equal concentrations of rHuIFN- α led to a statistically significant increase in *survivin* mRNA synthesis compared to the same control cells ($p<0.05$, $n=3$). These findings suggest that down-regulation of survivin may, at least in part, account for the enhancement of nHuIFN- α -mediated apoptosis in comparison to rHuIFN- α .

In conclusion, the findings of this study demonstrate that nHuIFN- α exerts significantly stronger induction of

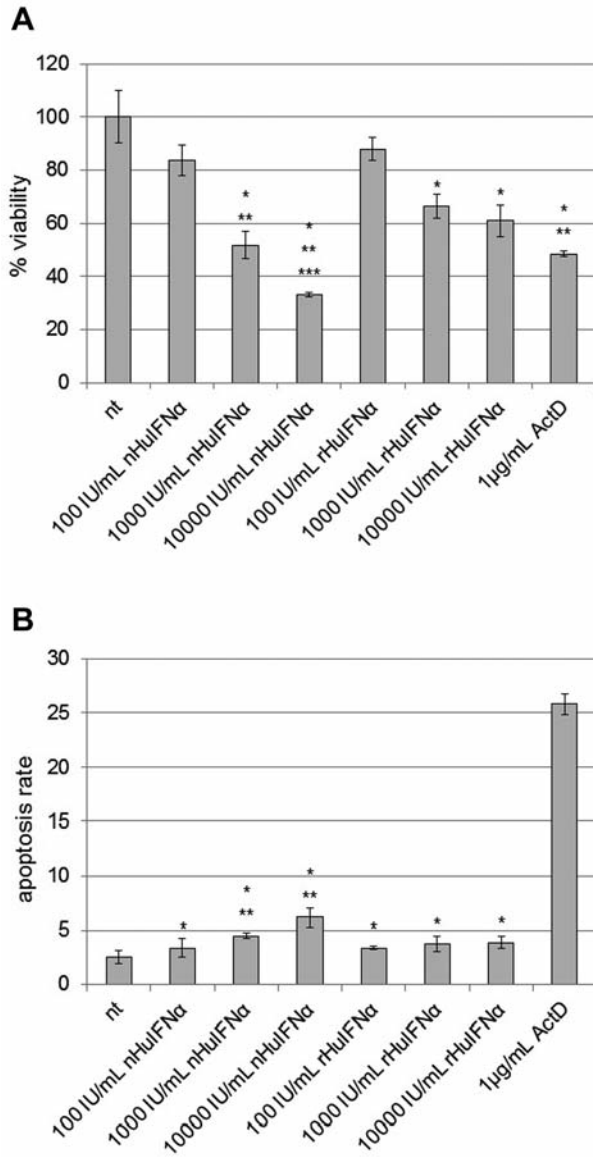


Figure 1. Effects of native human interferon- α (nHuIFN- α) and recombinant human interferon- α (rHuIFN- α) on the A549 cells viability and apoptosis. A549 cells were incubated with increasing concentrations (100, 1,000 or 10,000 IU/ml) of either nHuIFN- α or rHuIFN- α for 72 h. Cell viability was measured by the MTT test (A), and apoptotic rate was analyzed by flow cytometry (B). Cells treated with 1 μ g/ml Actinomycin D (ActD) served as a positive control. Non-treated A549 cells (nt) were used as negative control with cell viability set up to 100%. $p < 0.05$ compared with *non-treated cells, **cells treated with rHuIFN- α and ***cells treated with ActD.

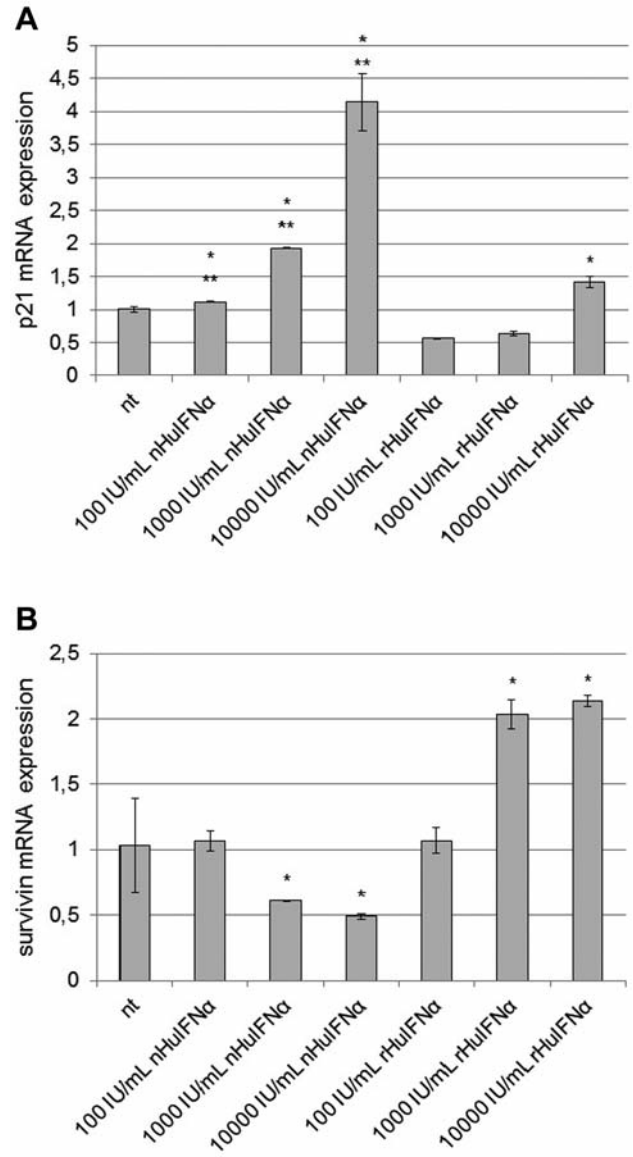


Figure 2. Effects of native human interferon- α (nHuIFN- α) and recombinant human interferon- α (rHuIFN- α) on p21 and survivin mRNA abundance in A549 cells. A549 were incubated for 24 h with the addition of 100, 1,000 or 10,000 IU/ml of either nHuIFN- α or rHuIFN- α . PCR was performed for p21 (A) and survivin (B) mRNA. The data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Fold-change relative to expression in non-treated A549 cells (nt) is shown. $p < 0.05$ compared with *non-treated cells; and **cells treated with rHuIFN- α .

apoptosis and reduction of A549 cell proliferation, compared to rHuIFN- α . It is most likely that the stronger nHuIFN- α effects on apoptosis and viability of A549 cells, compared to rHuIFN- α , at least in part, depend on higher induction of p21

and reduction of survivin expression. It should be interesting to explore whether cytokines in traces from nHuIFN- α have effects on the induction of p21 and survivin through interaction with IFN- α .

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