Decreased DNA Repair Capacity of UV-Irradiated Cells Following Interferon Treatment

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The aim of this study was to examine the effect of interferons (IFNs) on the recovery of UV-damaged cells by means of measuring cell viability rates. The influence of the recombinant human interferons IFN-α, IFN-β and IFN-γ on the repair capacity of the UV-irradiated human cell lines WISH and HeLa was studied. The ability of cells to repair UV-induced damage was determined by the comet assay and both short- and long-term survival assays in proliferating cell cultures. We found that INFs negatively regulated DNA repair in cells damaged by UV light. One day after treatment, in both cell lines tested, IFN-α had a stronger inhibitory effect than IFN-γ. Combined treatment with different IFNs exhibited a stronger inhibitory effect on cell recovery than treatment with each of them. The protein kinase inhibitor wortmanin further aggravated the effect of IFNs on cell survival.

Key words: Interferon, DNA Repair

Introduction

DNA integrity of cells is threatened by damaging effects of chemical and physical agents that can affect their function. UV light provokes DNA lesions that interfere with replication, transcription and DNA repair. The replication of damaged DNA involves different cascades with increasing complexity but decreasing accuracy. The accurate mechanism uses low-fidelity DNA polymerases (Pol H and Pol I). Replicative bypass of DNA damage by these polymerases produces an accurately replicated, newly synthesized strand. The cells negative in Pol H adopt a proposed secondary bypass mechanism or a recombinational mode (Cleaver, 2002). The mechanism of the secondary bypass is unclear, but a number of experiments suggests roles for nucleotide excision repair (NER) to remove the damage ahead of replication forks. The NER pathway is characterized by three major steps: recognition of the damage; DNA incision and excision; and DNA re-synthesis and ligation (Cleaver, 1996; Sancar, 1996). NER possesses two major overlapping sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). Many studies reported an impairment of both sub-pathways in various cell lines (Zhu *et al.*, 2000). DNA repair efficiency is an important determinant of the cell response to DNA damaging agents, since the inability to eliminate the damage may affect the genomic stability and contribute to malignant transformation.

Important roles of the NO content in cellular damage and viability are being increasingly recognized. In mammalian cells, production of NO from the substrate L-arginine is catalyzed by NO synthases (Gao *et al.*, 1997). It is known, that the cytokines-provoked DNA damage relates to an increased production of fee radicals (Rosales *et al.*, 2004). NO can modify not only the enzymes directly involved in DNA repair but also critical regulators of the cell cycle (Jaiswal *et al.*, 2000).

Cytokines are potent biologically active proteins. Cells induced by different viral and bacterial stimuli produce interferons (IFNs). Biological responses to IFN are initiated by specific IFN-binding cell surface receptors and subsequent activation of cytoplasmic signal transduction pathways and manifested following expression of a number of IFN-inducible genes. The "IFN family" consists of different receptors for type I and type II IFNs, all species have been shown to exert a similar spectrum of *in vitro* biological activities in responsive cells (Meager, 2002). Type I IFNs are categorized into IFN-α, -β, -ω and -τ based on their immunogenic properties; they are generally acid-stable. Type II IFNs, which are acid-labile, are referred to as IFN-γ immune interferon, a product of activated T cells and natural killer cells. IFN-α and IFN-β compete for the same receptor,

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IFN-γ uses a separate receptor system. Recombinant human IFNs also significantly modulate cell growth and cell viability in addition to their effects on immune responses (Pestka *et al.*, 2004). Studies employing these cytokines for immune therapy offer new opportunities to treat many diseases that have been fairly intractable to therapy such as autoimmune diseases, viral diseases and cancer.

Materials and Methods

Cell cultures and culturing

Two human epithelial cell lines were used in our experiments: WISH (adherent amnion epithelial cells, ATCCCCL25) and HeLa-Ohio (adherent cervix carcinoma cells, ATCCCCL2.2). WISH cells were grown in Minimum essential medium (Gibco, Invitrogen Corp., Paisley, Scotland) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), and the HeLa cells were grown in Dulbecco MEM/Nut Mix F-12 (HAM) (Gibco) containing 10% fetal bovine serum. The cells were cultivated in flasks (Corning) at 37 °C in humidified air containing 5% CO_2.

Cytokines

IFN-α: Recombinant human IFN alpha (Heberon Alfa R, 5 000 000 IU, Heber Biotec, SA Havana, Cuba).

IFN-β: Recombinant human IFN beta (Betaferon, 250 μg/ml, Schering AG, Berlin, Germany).

IFN-γ: Recombinant human IFN gamma ($1.5 \cdot 10^7$ IU/mg, Lab. Regulation of Gene Expression, Institute of Molecular Biology, Bulgarian Academy of Science).

Protein kinase inhibitor

Wortmanin (150 nM) (Debiopharm, Switzerland)

UV irradiation

A commercial germicide 15 W lamp (Tungsram, Hungary) was used as a source of UV radiation. The culture medium from a 96-multiwell plate with adherent cells was replaced with 50 μl/well PBS (Dulbecco's phosphate buffered saline) into ice. After irradiation for 2, 4, 8 s (corresponding to 22, 45 and 90 J/m^2) the cultures were placed in new complete medium containing (400 IU/ml) IFN-α, IFN-β or IFN-γ (separate or in combination) and incubated further. UV irradiation of the cells treated with 150 nM wortmanin and IFNs was performed at a dose rate of 5, 11, 22, 45 and 90 J/m^2.

Comet assay

The comet assay was performed by a modified protocol of Sastre *et al.* (2001). Standard microscope slides were used on which a layer of 0.5% normal melting agarose had been pre-dried. Cell suspension was spun down in a microcentrifuge at $1200 \times g$ for 5 min. The supernatant was discarded. The pellet of the cells containing approximately $5 \cdot 10^4$ cells was resuspended in 500 μl PBS and added to 500 μl 1.4% low-melting agarose (Appli-Chem) solution. This mixure was heated and added onto a coated microscope slide, and a cover slip was spread the agarose. The slides were placed in lysis buffer [2.5 M NaCl, 10 mM Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol), pH 7.5, 0.1 M EDTA, 1% Triton X-100 and 10% dimethyl sulfoxide, pH 10.0] and incubated at 4 °C for 1 h. After lysis slides were washed in distilled H_2O and then placed in a submarine-gel electrophoresis chamber filled with alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA) for 30 min, after which electrophoresis was performed at a constant voltage of 12 V, 185 mA, for 60 min. The slides were neutralized by three 5 min rinses in 0.4 M Tris, pH 7.5, and dehydrated by a 10 min rinse in cold 95% ethanol. They were dried at 27 °C and stained with 40 μl ethidium bromide (20 μg/ml). The slides were examined under a microscope with an epifluorescent set (excitation filter, 510–560 nm green light; barrier filter, 590 nm).

MTT assay

The assay, first described by Mosmann (1983), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to reduce tetrazolium salts, in particular MTT bromide [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. MTT is cleaved to formazan by the 'succinate-tetrazoliun reductase' system, which belongs to the mitochondrial respiratory chain and is active only in viable cells. The number of surviving cells is directly proportional to the level of formazan obtained.

The cells were cultivated in 96-well plates with 100 μl suspension of $2 \cdot 10^5$ viable cells in culture medium for 24 h. The growth medium was re-

moved and 100 μl fresh medium containing IFN-α or -γ (400 U/ml) were added onto the cell monolayer. Following 24 h incubation at 37 °C, 10 μl of MTT solution (5 mg/ml MTT dissolved in PBS and filter-sterilized) were added. Following additional incubation for 1 h at 37 °C in a CO_2 incubator, acidic isopropanol (100 μl of 0.04 M HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After standing a few minutes at room temperature to ensure that all crystals were dissolved, the reaction intensity was quantitatively measured by a multiwell scanning spectrophotometer (ELISA reader, Ultrospec1000, Amersham Pharmacia Biotech) at 570 nm.

Survival assay

For short- and long-term survival assays $1 \cdot 10^5$ cells/ml, non-irradiated and UV-irradiated with various doses, were tested. Cells were placed in a 24-well plate with 1 ml culture medium, completing 400 IU/ml IFNs. After 24 h, 3 and 8 d, the cultures were analyzed further and stained with neutral red for texting the cytotoxicity by a modified protocol as described by Bulychev *et al.* (1978). Briefly, the medium was replaced and after incubation for additional 2 h to allow for uptake of the dye (0.05% neutral red in 0.9% NaCl) the cells were rapidly washed with PBS. The dye was then extracted from the intact, viable cells with a solution of 0.1 M NaH_2PO_4 in 96% ethanol (v/v 1:1). The plate was left to stand at room temperature for 10–15 min, and then was measured on a Bio-Tek microplate reader (at 492 nm). The experiment was repeated three times.

Determination of nitric oxide (NO) content

The NO content in cell culture media (supernatants) was measured colorimetrically using the Griess reagent (1% sulfanilamide dissolved in 5% phosphoric acid and 0.1% naphthylethylenediamine dissolved in H_2O). The cells were grown in 50 ml flasks and incubated in the presence of IFN-α or IFN-γ (400 IU/ml) for 48 h. The cells were removed by centrifugation and the supernatants were mixed with equal volumes of Griess reagent and incubated at room temperature for 10 min. The absorbance of the sample was measured at 550 nm against that of the blank, and the NO content was calculated based on a standard calibration curve covering a range from 0.5 to 2.5 μM.

Results and Discussion

The alkaline version of the comet assay (single-cell gel electrophoresis) was employed to study UV-induced nuclear DNA damages. Using the same assay, others have detected DNA damages induced by cytokines (Delaney *et al.*, 1993). Based on that earlier report, the appropriate dose range for UV irradiation was chosen. WISH and HeLa cells were irradiated with different doses and then incubated in the presence of IFNs. As expected, we observed an irradiation-dose-dependent increase of DNA damage (increase of the tail section of the comet) (data not shown).

No significant changes on the mitochondrial activity of the cells after UV irradiation with lower doses were observed. The data are presented in the Fig. 1. We thus have chosen 90 J/m^2 as irradiation dose in most experiments.

It is known, that cytokines induce NO in the cells and that NO inhibits repair of several types of DNA damage (Jaiswal *et al.*, 2000). In our previous experiments the cell lines were tested by the Griess reaction for the evidence of IFN-induced NO formation. The data revealed that the NO induction by IFN-α was stronger in WISH than in HeLa cells. Similarly, IFN-γ is a better NO inducer in WISH compared to HeLa cells (Fig. 2).

We next investigated the effect of IFNs on UV-irradiated and non-irradiated (control) cell cultures by short- and long-term survival assays. WISH and HeLa cells were UV-irradiated with three increasing doses of UV light – 22.5, 45 and 90 J/m^2. Immediately after irradiation medium containing IFN (IFN-α, IFN-β, or IFN-γ) was added and cells

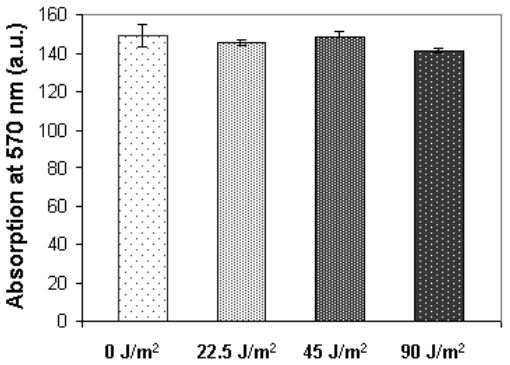

Fig. 1. MTT assay of WISH cells. The cells monolayer was UV-irradiated with different doses as indicated. The cell suspension was irradiated and after 24 h the MTT assay was carried out.

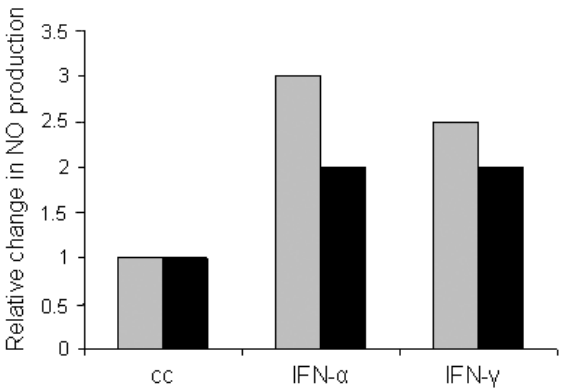

Fig. 2. Determination of NO contents in the culture medium by the Griess reagent. The cells were treated with 400 IU/ml IFN-α or IFN-γ for 24 h. Grey, WISH cells; black, HeLa cells. The results were averaged from 5 independent experiments.

were incubated for an appropriate time period – 1 or 8 days. In this case the cell viability was measured by neutral red uptake from live cells. The data for cells treated with 90 J/m^2 is presented in Fig. 3.

As can be seen from the results of Fig. 3a, the viability of WISH cells treated with IFNs (α, β and γ) at the first as well at the eighth day after irradiation was reduced. This may directly reflect an inhibition of global repair processes in these cells. At day 1, the inhibition of cell viability was stronger with IFN-α and weaker with IFN-γ and IFN-β. After 8 days, the repair was weakest in the case of IFN-γ, following the cells treated with IFN-α. IFN-γ exhibited a stronger inhibitory effect on the repair of damaged DNA. When IFN-β was added in the cells, no difference compared to UV-irradiated control cells was detected. As could be seen from Fig. 3b, at the early period post irradiation (1 day) and treatment with IFN of HeLa cells, only IFN-α inhibited the DNA repair. The effect of IFN-α was different at the early and late period after UV irradiation. After 8 days the opposite results were marked. The inhibitory effect of IFN-α was lost and an inhibition of repair was detected after IFN-γ or IFN-β treatment. Thus, these data demonstrates that the inhibition of DNA repair in the presence of INFs reduces the HeLa cell viability.

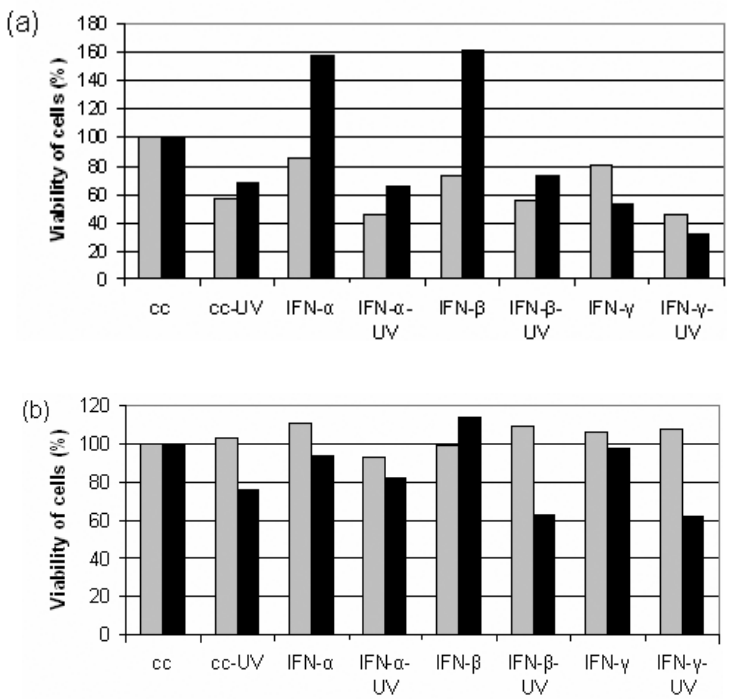

Fig. 3. Viability of UV-irradiated (a) WISH cells and (b) HeLa cells. The cells monolayer was irradiated with 90 J/m^2. Following irradiation, INF-containing medium (400 IU/ml) was added to the cells. The cells viability was determined by the neutral red uptake after 1 day (grey) and after 8 days (black). The data represent the percentage of viable cells relative to control untreated cells ($p < 0.05$).

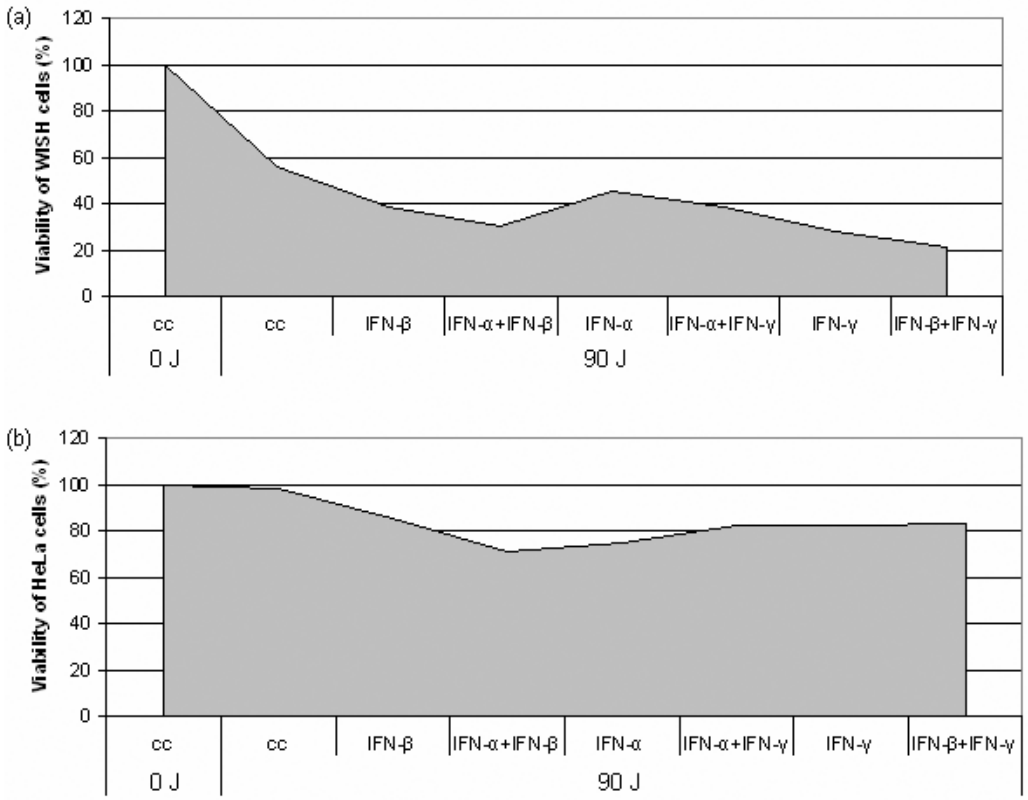

Fig. 4. Viability of (a) WISH cells and (b) HeLa cells treated with a cocktail of IFNs after UV irradiation. The cells monolayer was irradiated with 90 J/m^2. Following irradiation, the cells were incubated in medium containing IFN (400 IU/ml). After 24 h, the cells viability was determined by the neutral red uptake. The data represent the percentage of viable cells relative to control unirradiated cells.

To investigate the effect of combination treatment with two different IFNs, after self-one UV damage dose of 90 J/m^2 irradiation, a cocktail of IFNs was added to the culture medium for 24 h. The IFNs supplemented in the medium were combined as follows: IFN-α and IFN-β (IFNs of type I only); IFN-α and IFN-γ or IFN-β and IFN-γ (IFNs of type I and type II). The results are presented in Fig. 4.

The effect of IFN combination on cell viability was compared with the effect of a single IFN treatment. Comparing these treatments suggests that the decrease in the viability of WISH as well as HeLa cells after UV irradiation was more pronounced after treatment with the cocktail of type I IFNs (IFN-α and IFN-β). The combination effect of type II (IFN-γ) with some of type I (IFN-α or IFN-β) IFNs was different in WISH cells. The combination of INF-γ with IFN-α showed a better inhibitory activity than with IFN-β. In the same treatment, the divergence in the viability of HeLa cells was missed. In both cases the combination of IFNs showed a better inhibitory effect than each of them individually.

It has been established that wortmanin (WN), an inhibitor of the DNA-dependent protein kinase (DNA-PK), abolishes an important step of DNA repair process (Mitra *et al.*, 2005). For that reason, we tested the influence of WN on the IFNs inhibitory effect in DNA repair. Since proliferating cells are metabolically more active than non-proliferating cells, the assay is suitable not only for the determination of cell viability and factor-mediated cytotoxicity, but also for the determination of cell activation and proliferation (Jabbar *et al.*, 1989). The cells were irradiated with two different doses of UV light, and IFN plus WN were added to the medium. As a control, the irradiated cells were treated only with IFN or WN. The results are presented in Fig. 5.

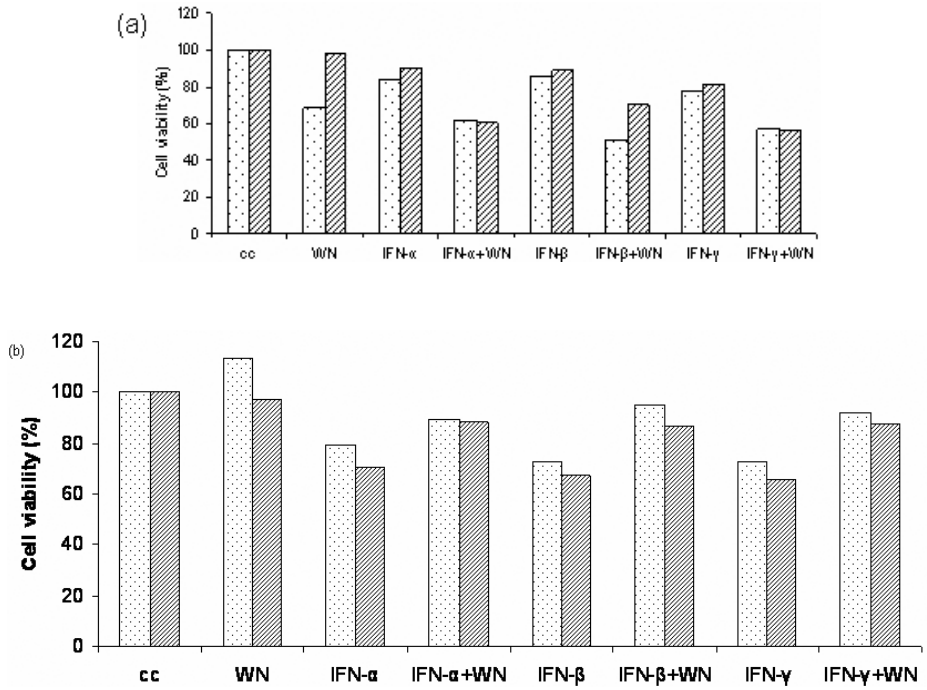

Fig. 5. Viability of (a) WISH and (b) HeLa cells determined by the MTT test. The cells were UV-irradiated with 45 J/m^2 (dotted columns) and 90 J/m^2 (slashed columns), and treated with IFN (400 IU/ml) and wortmanin (WN) (150 nM) ($p < 0.05$).

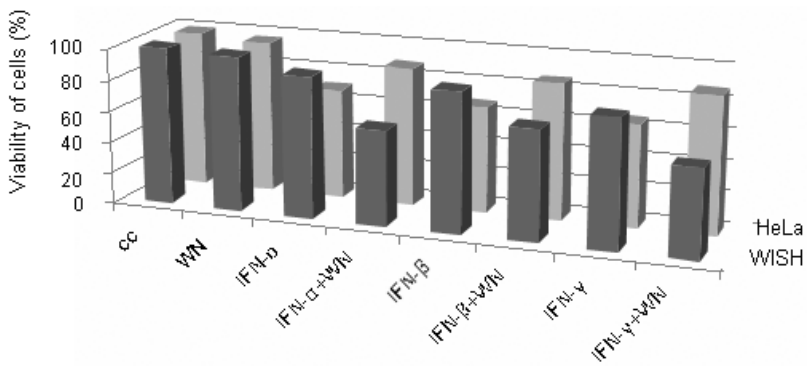

Fig. 6. Viability of WISH and HeLa cells determined by the MTT test. The cells were UV-irradiated with 90 J/m^2 and treated with IFN (400 IU/ml) and wortmanin (WN) (150 nM) ($p < 0.05$).

As can be seen from Figs. 5 and 6, the viability of irradiated cells was weakly decreased (about 2–6%) after WN treatment. WN increased the inhibitory effect of IFNs on the repair of UV-damaged WISH cells. As a result of the combined treatment the viability of cells was decreased. When a mixture of protein kinase inhibitor and IFN was added to HeLa cells, their viability was decreased in comparison with the addition of WN alone. But, the particular effect of IFNs was diminished.

The data presented here show that DNA repair in UV-damaged cells is inhibited by IFNs. One day after treatment, in both cell lines WISH and HeLA, IFN-α had a stronger inhibitory effect than IFN-γ. Our previous investigations are in accordance with these results. We conducted a study on

the repair proficiency of cells transfected plasmid DNA by *ex vivo* damaged by irradiation. The comparison of the capacity of human cell lines to repair UV-damaged DNA revealed that the most repair proficient were HeLa cells, followed by WISH cells. It was shown, that both types of interferon (IFN-α and IFN-γ) have an inhibiting effect on DNA repair in both cell lines tested. DNA repair suppression by IFN-α was much more pronounced than that caused by IFN-γ (Tsoncheva *et al.*, 2008). In summary, using two different test systems, we have now established that human interferons possess a strong inhibitory effect on repair of UV-induced damage of DNA.

Acknowledgements

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