Influence of Interferons on the Repair of UV-Damaged DNA

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Z. Naturforsch. **63 c**, 303–307 (2008); received June 29/October 2, 2007

The capacity for nucleotide excision repair of a normal (WISH) and three tumour (MCF-7, HeLa, Namalva) cell lines treated with human recombinant interferons (hrIFN-α and hrIFN-γ) was compared by the host cell reactivation assay. The cells were transfected with *in vitro* UV-damaged plasmid DNA (pEGFP-N1). The repair capacity was determined by measuring the fluorescence intensity of the expressed marker protein in total cell lysates. The correlation between the interferon-induced NO content and the suppressive effect of interferons on DNA repair was shown. The decrease of repair activity and NO induction by hrIFN-α were greatest in WISH, followed by MCF-7, Namalva and HeLa cells, whereas hrIFN-γ was the best NO inducer and inhibitor for the repair of Namalva, followed by WISH, MCF-7 and HeLa cells. Our data clearly show that the two types of interferon have a strong inhibitory effect on the repair of UV-damaged DNA and this effect is cell type-dependent.

Key words: Interferon, DNA Repair

Introduction

Nucleotide excision repair is the major repair pathway that recognizes and corrects DNA-distorting lesions caused by UV irradiation (cyclobutane pyrimidine dimers and 6–4 photoproducts) and some chemical agents (intrastrand cross-links and other bulky adducts) (Brooks, 2007). During nucleotide excision repair a small region surrounding the damage is removed from the affected strand of the DNA helix, the single-stranded gap is then filled in by a DNA polymerase and, finally, ligated by a DNA ligase. In addition to the activation of repair pathways in response to DNA damage, cells can invoke a complex signaling network, which halts cell cycle progression to allow sufficient time for DNA repair proteins to operate. This signaling pathway is called DNA damage checkpoint, and its timely activation is important for successful DNA repair. Failure to adequately initiate checkpoint response and repair DNA lesions can lead to increased mutagenesis, genomic instability or cell death, thus underscoring the central role of DNA repair mechanisms to enhance the ability of organisms to survive DNA insults.

Interferons (IFNs) are cytokines playing key roles in mediating antiviral (Isaacs and Lindenmann, 1957; Hurgin *et al*., 2007; Tsanev and Ivanov, 2002), antiproliferative (Tsanev and Ivanov, 2002; Damdinsuren *et al*., 2007a) and apoptotic responses (Oehadian *et al*., 2005; Buckley *et al*., 2007; Mullan *et al*., 2005; Seidelin and Nielsen, 2005). Due to these biological effects IFNs have emerged as promising antitumour agents (Tsanev and Ivanov, 2002; Buzaid and Legha, 1994; Damdinsuren *et al*., 2007b; Pestka *et al*., 2004). However, a straightforward evaluation of the real antitumour activity of the IFNs could be challenging. Previous reports have shown that the effect of IFNs may vary from clear-cut tumour throughout to symptomatic relief (Pestka *et al*., 2004). Moreover, the mechanism, by which IFNs exert their antitumour activity is still unclear. It has been suggested that their effect may range from protection from DNA damage to inhibition of DNA repair (Seidelin and Nielsen, 2005; Mertens *et al*., 1993; Jaiswal *et al*., 2000; Schmidberger *et al*., 2003). It has become clear that the capacity of IFNs to alter the DNA repair pathways depends on the interferon type and on the cell genotype (Makedonov *et al*., 1990; Lew *et al*., 1989).

The most recent methods for measuring DNA repair rates in individual living cells are based on studying single-cell gene expression (Gospodinov and Anachkova, 2004; Roguev and Russev, 2000). Quantitative measurement of fluorescent proteins

0939–5075/2008/0300–0303 $ 06.00

expressed in cells is complicated because of the ubiquitous presence of autofluorescence. The cellular autofluorescence originates from mitochondria and from lysosomes. The majority of fluorescence present in cells is due to aromatic amino acids, derivatives of pyridoxal, NADH and flavins (Andersson *et al.*, 1998), as well as to intermediates of various biochemical processes (Swain *et al.*, 2002; Elowitz *et al.*, 2002; Ramsey *et al.*, 2006; Bar-Even *et al.*, 2006; Croce *et al.*, 1999). There is a correlation between the autofluorescence noise and protein abundance, suggesting that the intrinsic noise dominates the gene expression noise (Ramsey *et al.*, 2006).

Materials and Methods

Cell cultures and culturing

Four cell lines were used in our experiments: WISH (adherent amnion epithelial cell line, ATCCCCL25), HeLa-Ohio (adherent human cervix carcinoma cell line, ATCCCCL2.2), MCF-7 (adherent epithelial human breast cancer cell line, ATCCHTB22) and Namalva (suspension human lymphoblastoid cell line, ATCCCRL1432). WISH cells were grown in minimum essential medium (MEM, Gibco, Invitrogen Corporation, Paisley, Scotland) supplemented with 10% fetal bovine serum (Biochrom KG, Berlin, Germany), and the HeLa and MCF-7 cells were grown in Dulbecco MEM/Nut Mix F-12(HAM) (Gibco) containing 10% fetal bovine serum. The suspension Namalva cells were cultivated in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum. The cells were cultivated in flasks (Corning, Lowell, MA, USA) at 37 °C in humidified air containing 5% CO_2.

Cytokines

hrIFN-α: Heberon Alfa R, 5 000 000 IU (Heber Biotec, SA Havana, Cuba).

hrIFN-γ: Human recombinant IFN-γ, 1.5. 107 IU/ml (Lab. Regulation of Gene Expression, Institute of Molecular Biology, Bulgarian Academy of Science, Sofia, Bulgaria).

All cell lines were treated with 400 IU/ml of IFN. The dose was non-toxic for cells determined by the neutral red test.

Expression plasmids

The expression plasmid, pEGFP-N1 (Clontech, Palo Alto, CA, USA), was used to express the green fluorescence protein. Plasmid was amplified in *E. coli* strain HB101 and isolated as described earlier (Todorova *et al.*, 2002).

UV irradiation

Plasmid DNA was dissolved in sterile TE buffer [10 mM Tris (trishydroxymethylaminomethane)-HCl, pH 7.5, 1 mM EDTA], pH 7.4, to a final concentration of 100 μg/ml. 20 μl of plasmid DNA were transferred into a 96-well plate on ice and UV-irradiated from a distance of 20 cm with a germicidal mercury lamp (emission maximum at 254 nm). Irradiation was carried out for different time intervals: 10, 20, 30 and 40 min, which, under our experimental conditions, corresponded to irradiation doses of 2.5, 5, 7.5 and 10 kJ m^{-2}, respectively.

To assess the extent of thymine dimer formation, the irradiated plasmid DNA was treated with endonuclease enzyme T4EndoV, followed by alkaline agarose gel electrophoresis (Lloyd *et al.*, 1980). To this end 25 μg irradiated and non-irradiated control plasmid DNA were mixed with T4EndoV (5–20 μg) in T4Endo buffer (50 mM NaH_2PO_4, 10 mM EDTA, 10 mM NaCl, 1 mM β-mercaptoethanol) and incubated for 3 h at 37 °C in a humidified atmosphere. The two samples were applied on denaturing (alkaline) agarose gel (Pospisilova and Kypr, 1997), and after electrophoresis the gel was rinsed in distilled water, neutralized in cold TCE buffer (100 mM Tris-HCl, 2 mM EDTA, pH 7.4) for 45 min and stained with ethidium bromide (1 μg/ml). The DNA in the gel was visualized under UV illumination and photographed.

Transfection

The spontaneous plasmid intake with irradiated or control plasmid DNA was carried out as described earlier (Tsoncheva *et al.*, 2005). Briefly, the cells were grown in 50 mm dishes to 80% confluence and then were trypsinized and collected in a small volume of culture medium. Non-irradiated or UV-irradiated (with a dose of 2.5 kJ m^{-2}) DNA (5 μg/ml) was added to the cell suspension, and the cells were incubated at 37 °C for 30 min. Finally, the suspension was diluted to 2.5 ml with culture medium (without phenol red) containing 10% fetal bovine serum.

In case of suspension cell cultures, the plasmid DNA was added directly to the cell suspension.

Fluorescence measurement

Quantitative fluorescence measurements were carried out 24 h after transfection. The adherent cells were washed twice with PBS, scraped off using a rubber rod and resuspended in 1 ml double distilled water. The cell suspension was freeze-thawed three times, and cell debris was removed by centrifugation at $250 \times g$ for 15 min at 4 °C. The fluorescence of clear cell lysates was measured on a Shimadzu RF-5000 fluorescence spectrofluorometer in 0.5 mm cuvettes. The fluorescence was measured at λ_{ex} = 470 nm and λ_{em} = 510 nm. The autofluorescence background of non-transfected cells was subtracted from the fluorescence intensity of transfected cells. The fluorescence intensity (in arbitrary units, a. u.) was referred to 1 mg/ml protein. The protein concentration was determinated by absorption at 280 nm (Tsoncheva *et al.*, 2005).

Determination of NO concentration

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). Cells were grown in 50 ml flasks and incubated in the presence of hrIFN-α or hrIFN-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 DNA repair was studied by means of the host cell reactivation assay, using UV-irradiated plasmid DNA containing a reporter gene encoding for green fluorescent protein (GFP) under the control of eukaryotic promoters. The DNA lesions in the promoter and coding regions of reporter genes (*gfp*) would preclude transcription resulting in a decreased protein expression and, hence, a drop in fluorescence intensity.

To standardize the DNA-damaging conditions, plasmid DNA was UV-irradiated for different time intervals, and the extent of damages was evaluated by agarose gel electrophoresis following a treatment with T4 endonuclease V, which cuts next to thymine dimers. For further experiments, we chose to expose the plasmid DNA to 5 kJ m^{-2} UV light before transfection.

The cells lines were transfected with UV-irradiated and non-irradiated (control) plasmids as described (Tsoncheva *et al.*, 2005). The efficiency of transfection was almost invariable throughout the experiments and did not depend on the cell line used. An expression of green fluorescence protein in the cell cytoplasm was detected 24 h after transfection by fluorescent microscopy (data not presented). By measuring the fluorescence intensity of total cell lysates from the transfected cells allowed to evaluate the efficiency of DNA repair not at individual cell level (by observation under a fluorescence microscope) but in the whole population of transfected cells. The green fluorescence was monitored at 510 nm upon excitation at 470 nm. Our results showed that green fluorescence increased up to 24 h following the transfection and, therefore, we chose to examine the fluorescence intensity of cell lysates at 24 h after transfection. We also noted that the autofluorescence of the four host cell lines was different. We therefore normalized the fluorescence intensity data by subtracting the background fluorescence of the corresponding cell line.

The comparison of the capacity of the four human cell lines to repair UV-damaged DNA revealed that the most repair-proficient were Na-

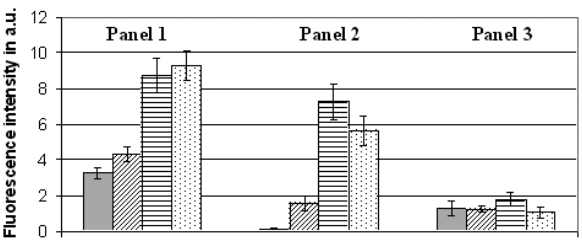

Fig. 1. Fluorescence in clarified lysates obtained from cells transfected with UV-irradiated plasmid DNA (pEGFP-N1). The presented fluorescence intensity in a.u. is measured after subtraction of the autofluorescence background. Grey, WISH; slashed, MCF-7; dashed, HeLa; doted, Namalva cells. Panel 1, control cells without IFN; panel 2, cells treated with hrIFN-α; panel 3, cells treated with hrIFN-γ.

malva cells, followed by HeLa, MCF-7 and WISH cells (Fig. 1, panel 1).

Next we studied the effect of human interferons on DNA repair in these cell lines. Cells transfected with UV-irradiated plasmid DNA were grown for 24 h in the presence of hrIFN-α or hrIFN-γ (test samples, the same concentration for all cell lines) and in the absence (control samples). Two approaches were taken to assess the DNA repair activity of the cells grown under these two conditions. First, the fluorescence intensity was measured in clarified whole cell extracts. Second, the number of fluorescing cells at 24 h post transfection was determined in the presence or absence of IFN.

The results presented in Fig. 1 show that both types of interferon have inhibiting effects on DNA repair in all cell lines tested. When comparing the two interferons, we conclude that DNA repair suppression by hrIFN-γ (panel 3) is much more pronounced than that caused by hrIFN-α (panel 2). Our findings can explain the synergistic effect of interferons and radiotherapy in cancer treatment.

Interferons are cytokines displaying multiple biological effects through modulating diverse biochemical pathways. Although some of these effects (antiviral, antiproliferative, immunomodulating, etc.) are common for both type I and type II interferons, they recognize different receptors and trigger separate signal transduction pathways (Lew *et al*., 1989). It is well known that hrIFN-γ exerts its intracellular antiparasitic effect by inducing the synthesis of nitric oxide (NO) (Fujigaki *et al*., 2003). The latter itself is a DNA-damaging agent (mutagen) modifying aminogroups in adenine, guanine and cytosine (Burney *et al*., 1999). Given the mutagenic properties of NO, one could surmise that NO may contribute either to accumulation of additional DNA damage in the already damaged (by the UV light) reporter gene or to prevention of DNA from repair. Therefore, we wanted to determine the NO content of medium obtained from cell cultures cultivated in the presence of hrIFN-α or hrIFN-γ. The results, presented in Table I, show a clear cut correlation between interferon-induced NO content and the suppressive effect of interferon on DNA repair. These data also reveal that the NO induction by hrIFN-α was greatest in WISH, followed by MCF-7, Namalva and HeLa cells, whereas hrIFN-γ was the best NO inducer for Namalva, followed by WISH, MCF-7 and HeLa cells. The results obtained here do not allow to make a reasonable conclusion about the exact molecular mechanism of inhibition of DNA repair by hrIFN-α and hrIFN-γ; and this will be subject of further studies. Our data, however, clearly show that the two types of interferons have a strong inhibitory effect on the repair of UV-damaged DNA, and this effect is cell type-dependent. These results provide an explanation of the synergistic effect of human interferons in cancer radiotherapy and highlight a new property of interferons that has to be taken into consideration when designing an appropriate scheme for cancer treatment.

Table I. Correlation between NO levels and IFN effect on DNA repair capacity of various cell lines.

Cell line	Suppression of pDNA reparation (%)		Increase of NO production (fold)	
	hrIFN-α	hrIFN-γ	hrIFN-α	hrIFN-γ
WISH	90	70	3.0	2.5
MCF-7	70	50	2.5	1.8
HeLa	28	24	1.0	1.0
Namalva	35	80	1.5	3.0

Acknowledgements

We are grateful to Prof. T. Deligeorgiev, Faculty of Chemistry, University of Sofia for providing us with the reagents for the Griess reaction and for the T4EndoV. This work was supported by Bulgarian NSF grant MU-B-1501/2005.

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