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European Journal of Cancer 39 (2003) 2103–2109

European Journal of Cancer

www.ejconline.com

Recombinant IFN-α2b treatment activates poly (ADPR) polymerase-1 (PARP-1) in KB cancer cells

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Received 16 December 2002; received in revised form 9 April 2003; accepted 9 May 2003

Abstract

In the present paper, we investigated the relationship between the growth inhibitory effects of recombinant interferon-α2b (rIFN-α2b) and poly (ADPR) polymerase-1 (PARP-1) activity in the human squamous KB cancer cell line. Growth inhibition of the KB cells mediated by 1000 IU/ml of rIFN-α2b was accompanied by a transient rise in PARP-1 specific activity 24 h after rIFN-α2b treatment, confirmed by both the increase of intracellular poly (ADP-ribose) content and the PARP-1 auto-modification level. At longer times of incubation, the onset of apoptosis accompanied KB cell growth inhibition, as demonstrated by both flow cytometry and western-blotting analysis showing an 89 kDa apoptotic fragment of PARP-1. Moreover, pretreatment of the cells with the PARP-1 inhibitor, 3-aminobenzamide (3-ABA), at non-cytotoxic concentrations (1 mM), reduced the cell-growth inhibition, cell-cycle perturbation and apoptosis caused by rIFN-α2b. Taken together, these results strongly suggest that PARP-1 may be directly involved in the effects of rIFN-α2b in the KB cancer cell line.

Keywords: PARP-1; IFN-α; 3-Aminobenzamide; KB cells; Apoptosis

1. Introduction

Interferon-α (IFN-α) belongs to a family of cytokines defined as Type I interferons (IFN-α and IFN-β), which are secreted by immune cells in response to various physiological stimuli. Type I interferons bind to specific receptors and activate a number of tyrosine kinases (JAK-1, JAK-2, tyk2). Such enzymes phosphorylate the transcription factors (STAT-1α, STAT-1β, STAT-2), that constitute the transcriptional factor 'ISGF-3' composed by the association of STAT-2 with STAT-1α or STAT-1β, or the STAT-1α/β dimer. The phosphorylated ISGF-3 complex is translocated to the nucleus and forms (with the addition of a fourth subunit, p48 or IRF-9) a DNA-binding complex specific for the IFN-stimulated response element (ISRE), and results in the activation of IFN-specific genes (ISGs) [1,2].

Several data demonstrate that IFN-α has an antiproliferative effect on different tumour cell lines, which are important for a potential application in cancer therapy. Treatment of cells with IFNs can induce many hundreds of genes [1,3] making it difficult to attribute their antiproliferative effects to particular gene products. However, several ISGs are involved in programmed cell death induction [3] and such induction represents an important pathway to inhibit cell proliferation of different tumour cell types. In this regard, Caraglia and colleagues [4] demonstrated that rIFN-α2b treatment inhibits cell growth and induces apoptotic cell death in the KB epidermoid carcinoma cell line.

This evidence prompted us to study poly (ADPR) polymerase 1 (PARP-1) involvement in the cytotoxic response to rIFN-α2b in KB cells. The 116-kDa nuclear enzyme PARP-1 acts as a DNA nick sensor and catalyses the synthesis of a polymer of ADP-ribose units (pADPR), utilising βNAD^+ as a substrate. The pADPR chain can be covalently linked to acceptor proteins, mainly PARP-1 itself, or can interact non-covalently

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doi:10.1016/S0959-8049(03)00433-7

with target proteins that are modified in their functional properties [5,6]. PARP-1 is involved in various cellular functions including DNA repair, DNA replication and genetic recombination [7–10]. PARP-1 is the best characterised member of the poly(ADPR)polymerase family of enzymes [10].

It has been shown that during the execution phase of apoptosis, PARP-1 is proteolysed by caspases into two fragments one of 89 kDa, containing the catalytic domain, and one of 24 kDa, consisting of the DNA binding domain [11]. The PARP-1 cleavage inactivates the enzyme and prevents its over-activation that would cause a depletion of the cellular pool of NAD^+ that is required for the execution of the apoptosis [12,13]. Interestingly, recent studies suggested that PARP-1 is directly involved in the regulation of gene transcription. In fact, PARP-1 has been identified as a partner of transcription factors such as p53 [6], YY1 [14], Oct-1 [15] and Nuclear Factor (NF)-κB [16,17]. Moreover, a role has been proposed for PARP-1 in the regulation of the transcription induced by the melanoma growth stimulatory activity (CXCL1) protein [18].

All these data suggest that PARP-1 could play a role in the modulation of the effects induced by IFNs. In this paper, we investigate the role of PARP-1 in the induction of the growth inhibitory effects caused by rIFN-α2b in the KB human oral squamous cancer cell line.

2. Materials and methods

2.1. Cell culture and reagents

The human epidermoid carcinoma KB cell line, obtained from the American Type Culture and Collection (ATCC), was maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) heat-inactivated foetal bovine serum (FBS), 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM L-glutamine and 1% (v/v) sodium pyruvate (all chemicals were from Flow Laboratories, Rockville, MD, USA). KB cells were incubated at 37 °C in a humidified atmosphere, *plus* 5% CO_2. Recombinant IFN-α2b was purchased by Schering-Plough (Schering-Plough, NJ, USA). 3-ABA was from Sigma (Sigma Chemical Co., St. Louis, MO, USA). [^{14}C]NAD^+ nicotinamide (U^{14}C adenine dinucleotide ammonium salt–250 mCi/mmol) and [^{32}P] NAD^+ (^{32}P-adenylate nicotinamide adenine dinucleotide di/triethylammonium salt–1000 Ci/nmol), were supplied by Amersham International, UK. Propidium iodide (PI) and RNAse for DNA-content analysis and apoptosis studies were from Sigma. Anti-Poly (ADP-ribose) rabbit polyclonal antibody (Biomol Research Laboratories Inc., USA) and goat F(ab')2 anti-rabbit IgG-fluorescein isothiocyanate (FITC) conjugate (Caltag Laboratories, USA) were used for the immunofluorescence studies. Rabbit anti-PARP-1 polyclonal antibody (clone H250, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used for the western-blotting analysis.

2.2. Cell-growth inhibition experiments

For cell-growth experiments, KB cells were seeded at 1×10^6 cells in 100 mm Petri dishes. After 24 h, growth medium was replaced with fresh medium containing rIFN-α2b (100, 500, 1000, 2000 IU/ml). At different time points (24, 48 and 72 h), cell-growth inhibition was assessed by a Trypan blue dye exclusion assay. KB cell growth inhibition induced by 1 mM of 3-ABA PARP inhibitor, was also determined at the same time points. Finally, to assess the effect of PARP-1 inhibition on the response to IFN treatment, KB cells were incubated with 3-ABA 1 mM 30 min before the addition of rIFN-α2b 1000 IU/ml and cell growth assessment was performed at 24, 48 and 72 h.

2.3. Immunofluorescence

Control and treated KB cells were grown on coverslips and fixed in a 3% buffered-paraformaldehyde solution at different time points. After 15 min at 4 °C, the cover slips were permeabilised by Triton X-100 0.2% in phosphate-buffered solution (PBS) for 5 min at 4 °C. Fixed/permeabilised KB cells were then incubated with the anti-pADPR rabbit polyclonal antibody (dilution: 1/300) as a primary antibody. After 1 h at 4 °C, the coverslips were rinsed three times in cold PBS and incubated with a goat anti-rabbit F (ab') 2 IgG-FITC conjugate (dilution: 1/100) for the same length of time, in the dark at 4 °C. Finally, the coverslips were rinsed four times in PBS and mounted on a drop of 90% (v/v) glycerol and examined with a Zeiss Axiovert fluorescence microscope.

2.4. Incubation of permeabilised KB cells with [^{32}P] NAD and analysis of [^{32}P] pADPR acceptor proteins

Control and treated KB cells were harvested and permeabilised (5×10^6 cells/ml) in 40 mM Tris–HCl buffer pH 7.8, containing 0.6 mM ethylene diamine tetra acetic acid (EDTA), 1 mM β-mercaptoethanol, 30 mM $MgCl_2$, 0.05% Triton X-100, 0.1 mM phenyl methyl sulphonyl fluoride (PMSF), a protease inhibitors cocktail solution (Roche Diagnostics, USA), for 30 min at 4 °C. Subsequently, permeabilised cells were incubated for 30 min at 30 °C with 200 μM [^{32}P] NAD^+ (100 000 counts per minute (cpm)/nmol). The reaction was stopped by the addition of 20% ice-cold trichloroacetic acid (TCA). TCA-insoluble material was obtained by centrifugation at 3000 rotations per minute (rpm) for 15 min. The resulting pellet was washed twice with ethanol

and twice with diethyl ether. [^{32}P] pADPR acceptor proteins present in the TCA insoluble fraction were resuspended in 10 mM Tris–HCl buffer, pH 6.8, 14 mM β-mercaptoethanol, 1% sodium dodecyl sulphate (SDS), 10% glycerol and analysed by autoradiography after SDS 5–18% polyacrylamide gradient gel electrophoresis [19]. The protein concentration was determined using the Bradford protein assay reagent (Bio-Rad, USA), with bovine serum albumin (BSA) as a standard.

2.5. Poly (ADPR) polymerase assay

The PARP-1 activity assay was performed by using an aliquot of permeabilised KB cells corresponding to 50 μg of proteins as an enzyme source. The reaction mixture (final volume 50 μl), was composed of 40 mM Tris–HCl (pH 7.8), 1 mM β-mercaptoethanol, 30 mM $MgCl_2$, 0.6 mM EDTA, 0.05% Triton X-100, 0.2 mM [^{14}C]NAD^+ (10 000 cpm/nmol). After 15 min incubation at 30 °C, the reaction was stopped by the addition of ice-cold TCA 30% (v/v) and the radioactivity associated to the acid-insoluble material counted on a Beckman LS8100 liquid scintillation spectrometer. One mU is defined as the amount of enzyme activity catalysing the incorporation per min of 1 nmol of [^{14}C] pADPR moieties into acid-insoluble material.

2.6. Western-blotting analysis

PARP-1 protein expression was analysed in either adherent and non-adherent (floating) KB cells after 48 h of culture, with and without 1000 UI/ml of rIFN-α2b. Floating cells were recovered from the culture medium by centrifugation at 1200 rpm for 15 min at 4 °C; adherent cells were detached by enzymatic treatment (Trypsin/EDTA 0.02%), washed in $PBS^{w/o\ Ca++-Mg++}$ and harvested by centrifugation at 1200 rpm for 10 min at 4 °C. Briefly, 50 μg of proteins were resolved by SDS 5–18% polyacrylamide gradient gels and transferred to a polyvinylidenefluoride (PVDF) membrane using an electroblotting apparatus. The PVDF membrane was blocked with 5% non-fat milk in 20 mM Tris–HCl pH 8, 150 mM NaCl and 0.5% Tween 20 (TBST) overnight and subsequently incubated with H-250 anti-PARP-1 polyclonal antibody (dilution: 1/2000) for 2 h at room temperature. Unbound antibody was washed with TBST. The membrane was incubated with the anti-rabbit secondary antibody horse radish peroxidase (HRP)-conjugate) (dilution: 1/2000) for 1.5 h at room temperature, washed and developed using a chemiluminescent detection system (Bio-Rad USA).

2.7. Cell-cycle analysis and determination of apoptosis

Control and treated KB cells were harvested as described above and fixed in 70% ethanol for 24 h at 4 °C. After a washing in $PBS^{w/o\ Ca++-Mg++}$, KB cells were stained in 2 ml of PI staining solution (50 μg/ml of PI, 1 mg/ml of RNAse in $PBS^{w/o\ Ca++-Mg++}$, pH 7.4) overnight at 4 °C. DNA-flow cytometry was performed in duplicate by a fluorescent activated cell sorting (FACS)can flow cytometer (Becton Dickinson, San Josè, CA, USA) coupled with a CICERO signals interface module (Cytomation, Fort Collins, CO, USA). Cell-cycle analysis was performed by the ModFit LT software (Verity Software House Inc., MA, USA). FL2 area versus FL2 width gating was done to exclude doublets from the G_2M region. For each sample, 15 000 events were stored in the list mode file.

Flow cytometric analysis of apoptotic cell death was performed on a cell pellet fixed in 70% ethanol, washed in $PBS^{w/o\ Ca++-Mg++}$, and stained in a PI staining solution as previously reported in Ref. [20]. The percentage of apoptotic cells was calculated on the sub-diploid region of the DNA content histogram, registered as FL3 signals in a log scale. To avoid cell debris contamination due to necrotic cell death, only living KB cells were selected by side-scatter (SSC) versus forward-scatter (FSC) gating.

3. Results

We first evaluated the effect of different concentrations of rIFN-α2b on KB cell growth and observed a growth inhibitory effect that was time- and dose-dependent (Fig. 1). The concentration of rIFN-α2b inducing the highest inhibition of cell proliferation was 1000 IU/ml, which led to approximately 30% of growth inhibition after 72 h of treatment.

Therefore, we evaluated the PARP-1 activity in KB cells treated with 1000 IU/ml rIFN-α2b, at different

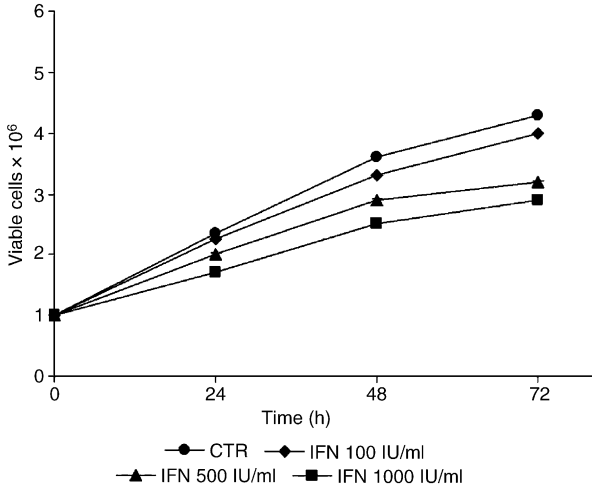

Fig. 1. KB cells grown in the presence of different concentrations of rIFN-α2b. Data refer to one of three independent experiments that gave giving similar results. CTR, control.

time points (6, 12, 24 and 48 h). To better understand the relationship between rIFN-α2b effects and PARP-1 activity, we measured the PARP-1 specific activity values by the incorporation of [^{14}C] pADPR into the acid-insoluble protein fraction and we analysed the formation of pADPR moiety by an immunofluorescence technique. Moreover, we identified the [^{32}P] pADPR acceptor proteins by incubating KB cells with [^{32}P] NAD.

As shown in Fig. 2a, rIFN-α2b treatment of KB cells stimulated PARP-1 activity; that increased after 6 h to reach the maximum at 24 h (150% of activity compared with control cells) and decreased thereafter to almost reach the control levels after 48 h. The immunofluorescence signal, due to the pADPR synthesis (Fig. 2B), increased after 12–24 h of treatment with rIFN-α2b and declined thereafter, resembling the kinetics of the PARP-1 enzyme activity. Fig. 2C shows the [^{32}P] pADPR acceptor proteins pattern obtained by autoradiography after SDS 5–18% polyacrylamide gel electrophoresis. The main labelled band corresponding to the PARP-1 molecular weight (>116 kDa) can be identified as the auto-modified form of the enzyme. Moreover, Fig. 2 shows that 24 h rIFN-α2b treatment led to an increase of the labelling for a comparable amount of protein on the gel (see the Coomassie-stained gel).

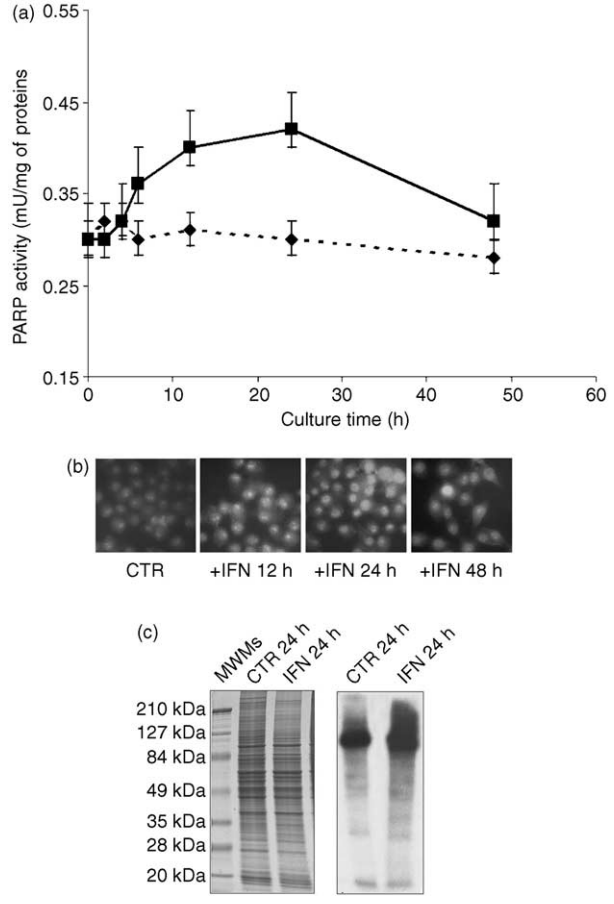

Fig. 2. PARP activity in KB cells at different times of rIFN-α2b treatment. (a) KB cells treated (—■—) or not (—◆—) with 1000 IU/ml of rIFN-α2b were harvested at the different time points (2, 4, 6, 12, 24 and 48 h) to measure the PARP activity as described in the Materials and methods. The bars indicate the standard error (SE) calculated for three different experiments done in duplicate. (b) Immunofluorescence analysis of poly(ADP-ribose) synthesis in KB cells treated with 1000 UI/ml of rIFN-α2b. Magnification: 40×. (c) Coomassie staining and autoradiogram of [^{32}P]poly(ADPribose) acceptor proteins of KB cells treated or not with rIFN-α2b and incubated with [^{32}P]NAD. The samples were separated by SDS 5–18% polyacrylamide gel electrophoresis. MWMs, molecular weight markers.

To determine whether the inhibition of PARP-1 activity modulated the growth inhibitory effects of rIFN-α2b, KB cells were treated with the PARP inhibitor 3-ABA 30 min before adding rIFN-α2b. As shown in Fig. 3a, the addition to the KB cells culture medium of 1 mM 3-ABA led to the inhibition of PARP activity by 86% after 24 h, and by approximately 60–50% after 48 and 72 h, respectively. The same dose of 3-ABA by itself was unable to affect the growth of the KB cell line, but reduced the growth inhibition induced by 1000 IU/ml of rIFN-α2b at 24, 48 and 72 h (Fig. 3b).

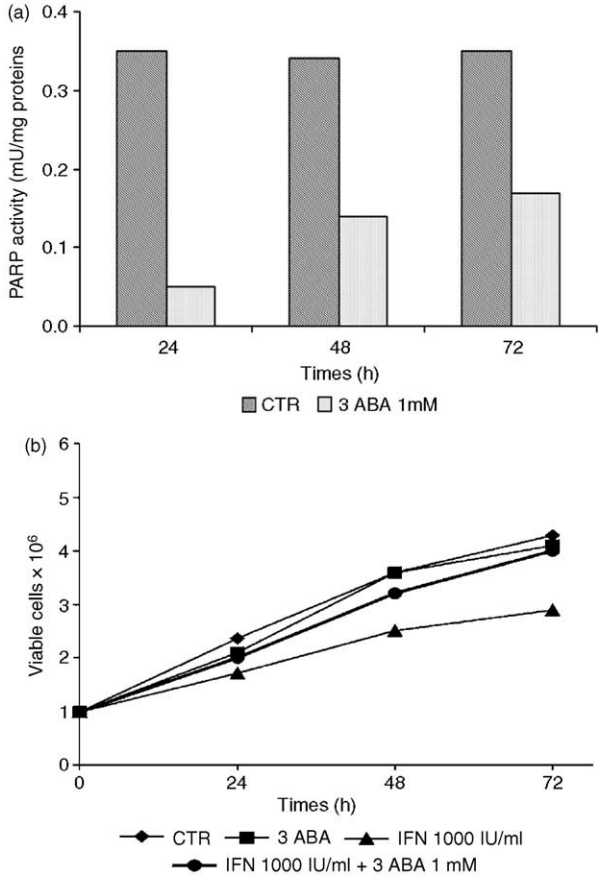

Fig. 3. (a) Inhibition of PARP activity by 3-ABA in KB cells. KB cells were treated with 1 mM 3-ABA, harvested at the different time points to perform the PARP activity assay as described in the Materials and methods. (b) KB cells grown treated with 1000 IU/ml of rIFN-α2b, 1 mM 3-ABA or a combination of both compounds. Data refer to one of three independent experiments that gave similar results.

The next step was to determine whether the growth inhibitory effect of rIFN-α2b was accompanied by a cell-cycle perturbation and/or apoptosis and whether the inhibition of PARP activity by 3-ABA modulated such effects. Table 1 reports the cell cycle analysis of KB cells 24, 48 and 72 h after rIFN-α2b treatment, with or without 1 mM 3-ABA. DNA-flow cytometry revealed the accumulation of KB cells mainly in the S phase of the cell cycle after 48 h of rIFN-α2b treatment (S-phase fraction 30% treated versus 21% control), which was abolished by 3-ABA.

Cell-growth inhibition and cell-cycle perturbation of KB cells were also accompanied by an induction of apoptosis, as shown by the presence of a sub-diploid population in 48 h rIFN-α2b treated cells (33% treated versus 3% control) (Fig. 4a). Furthermore, we analysed the presence of the apoptotic fragment of PARP-1 by Western-blotting analysis using a polyclonal antibody raised against the catalytic domain of the protein. When

Table 1
DNA flow cytometry of KB cells

Treatment	Cell-cycle phases		
	%G0/G1	%S	%G2/M
Control 24 h	53.0	28.5	18.5
3-ABA 24 h	51.0	28.3	20.7
rIFN-α2b 24 h	45.0	32.1	22.9
rIFN-α2b 24 h + 3-ABA	47.0	31.0	22.0
Control 48 h	67.0	21.0	12.0
3-ABA 48 h	65.0	22.0	13.0
rIFN-α2b 48 h	59.0	30.0	11.0
rIFN-α2b 48 h + 3-ABA	66.0	21.0	13.0
Control 72 h	66.0	23.0	11.0
3-ABA 72 h	67.0	23.0	10.0
rIFN-α2b 72 h	62.0	33.3	4.7
rIFN-α2b 72 h + 3-ABA	68.0	24.0	8.0

rIFN-α2b: 1000 IU/ml; 3-ABA: 1 mM.

Data refer to one of three independent experiments that gave similar results.

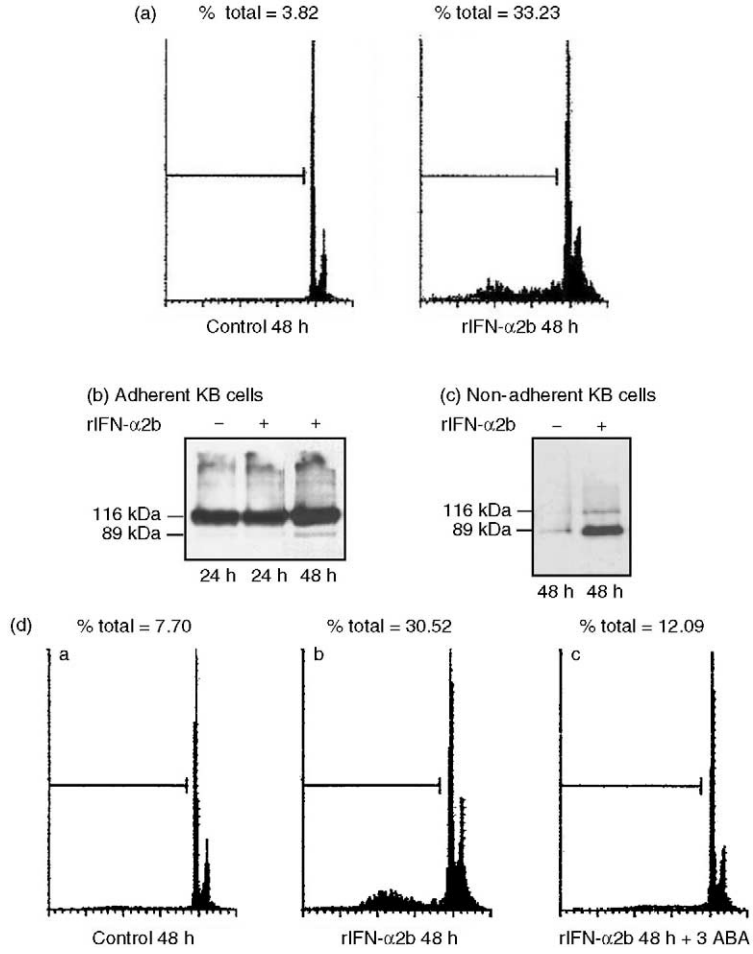

Fig. 4. Induction of apoptosis in KB cells. (a) Flow cytometric analysis of nuclear DNA content of KB cells by PI staining after 1000 IU/ml of rIFNα2b treatment. Cells gating was perform to select the hypodiploid sub-population representing apoptotic KB cells. (b) Western blotting analysis of PARP-1 status in adherent KB cells after 24 and 48 h of 1000 IU/ml rIFN-α2b treatment. (c) Western blotting analysis of PARP-1 status in non-adherent KB cells after 48 h of 1000 IU/ml rIFN-α2b treatment. (d) Flow cytometric analysis of nuclear DNA content of KB cells by PI staining after 48 h of rIFNα2b treatment, *with* or *without* 1 mM 3-ABA. Cells gating was perform to select the hypodiploid sub-population representing apoptotic KB cells: (a) control KB cells; (b) 1000 IU/ml rIFN-α2b; (c) 1000 IU/ml rIFN-α2b + 1 mM 3-ABA.

analysing the presence of the PARP-1 89 kDa apoptotic fragment, we distinguished between adherent and non-adherent (floating) KB cells, which were dead cells by Trypan blue dye exclusion test. As shown in Fig. 4b, the presence of 116 kDa full-length PARP-1 protein was observed in adherent KB cells, both in control and treated KB cells; only in rIFN-α2b-treated KB cells, a slight PARP-1 89 kDa apoptotic fragment was evident after 48 h. Indeed, the PARP-1 89 kDa apoptotic fragment was the most representative band in the non-adherent KB cells, evident after 48 h rIFN-α2b treatment of the cells (Fig. 4c).

In conclusion, both flow-cytometry and western-blot analysis confirmed that rIFN-α2b treatment induced programmed cell death in KB cells. Moreover, we observed that the onset of apoptosis after rIFN-α2b treatment was inhibited by 3-ABA. In fact, DNA-flow cytometric analysis showed that the percentage of sub-diploid cell population induced by 48 h treatment of rIFN-α2b was reduced by 3-ABA from 30 to 12% (Fig. 4D).

4. Discussion

Different types of tumour cell lines show cell growth inhibition and apoptosis induction after IFN-α treatment. At present, the mechanisms which mediate the biological and cytostatic effects of IFN-α remain to be defined, as do the cellular mechanisms of the anti-tumour action. Both IFN-α and IFN-β can affect all phases of the cell cycle. Prolongation of the G_1-phase, a reduced rate of entry into the S-phase and a lengthening of the S-and the G_2-phases have been previously described Ref. [21]. The effects of these cell cycle perturbations by IFNs in both normal and tumour cells result in cytostasis, an increase in cell size, and apoptosis [22].

In the present paper, we show a dose- and time-dependent growth inhibitory effect mediated by rIFN-α2b in KB cells; such growth inhibition is accompanied by a cell-cycle perturbation, mainly represented by the accumulation of cells in the S phase, and finally results in the activation of apoptotic cell death programme.

The main observation of this study is the stimulation of PARP-1 activity in KB cells occurring as an early event during rIFN-α2b treatment. Moreover, we show that PARP-1 activation by rIFN-α2b determines an increment of the pADPR endocellular content that modifies mainly PARP-1 itself. A transient burst of poly (ADP-ribosyl)ation was observed in human osteosarcoma cells undergoing spontaneous apoptosis, followed by caspase-3-mediated cleavage of PARP-1 [23] and in different transformed human cell lines, such as HL-60 and HeLa cell lines, after etoposide treatment [24]. PARP-1 activation was also observed in HL-60 and Jurkat cell lines undergoing FAS-mediated or camptothecin-induced apoptosis [8]. Our observation, for the first time, correlates PARP-1 activity to apoptosis commitment induced by rIFN-α2b in KB cells.

PARP-1 activation is usually induced by DNA damage, which has never been correlated to IFN's cytostatic effects. Nevertheless, some data show a relationship between treatment with IFNs and DNA-damage. Ghezzi and colleagues [25] demonstrated that IFN treatment is able to induce xanthine-oxidase and thus produces free oxygen radicals. Bertini and colleagues [26] showed that rIFN-α treatment induced a significant increase in 0^6-alkylguanine-DNA alkyltransferase (AT) in the rat liver. At present, no data are available regarding the levels of AT or of DNA-damaging compounds, such as nitric oxide or other reactive oxygen species in rIFN-α2b-treated KB cells.

Alternatively, PARP-1 could regulate the induction of IFN-responsive genes and thus it will be of interest to identify a possible link between PARP-1 activity and the rIFN-α2b signal transduction system, through the identification of a target protein of the polymers linked to the auto-modified enzyme. Interestingly, several transcription factors involved in IFN-dependent gene transcription, such as CBP/p300, Sp1, AP1, NF-kB, have been reported to be regulated by PARP-1 [2,27]. For instance, in PARP-1-null mice, a defective NF-kB activation has been observed that can be restored by the expression of PARP-1 in PARP-$1^{-/-}$cells [28]. Furthermore, NF-kB and PARP-1 can form a stable immunoprecipitable complex [17]. These observations suggest that PARP-1 itself could be a component of IFN gene transcription complexes, active in stimulating the loading of the complex to the cognate target sequence.

Further demonstration that PARP-1 is required for the induction of IFN-α action is based on the use of the PARP inhibitor 3-ABA. Indeed, the addition of 3-ABA to the cell cultures reversed the rIFN-α2b effects on cell growth, cell-cycle kinetics and induction of apoptosis. 3-ABA has also been used recently to demonstrate a role for PARP-1 in the regulation of the gene expression of the melanoma growth stimulatory activity/growth regulated protein (CXCL1), a chemokine that has been implicated in inflammation and tumorigenesis [18].

We believe that the definition of a role of PARP-1 in the induction of IFN-α antineoplastic action would be of some help in the design of new chemotherapeutic strategies. Indeed, chronic treatment with IFN-α, modulating the activity of PARP-1 enzyme, may act as resistance modifiers when used in conjunction with radiation or chemotherapeutic agents. Great effort should be made to ameliorate the efficacy of anticancer treatments, and to reverse intrinsic or acquired chemo- and radio-resistance in tumour cells, with the application of new adjuvant therapeutic strategies. Therefore, specific PARP-1 inhibitors could be used to define new

therapeutic strategies associating IFNs with chemotherapy and/or radiotherapy in different tumour models.

Acknowledgements

This study was supported by a grant of the "Associazione Italiana per la Ricerca sul Cancro" (A.I.R.C.).

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