IFN-α competes with TNF-α for STAT-1α; molecular basis for immune escape of human colon adenocarcinoma COLO 205 cells

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Abstract. The resistance of transformed colon epithelial cells to immune system-mediated extrinsic apoptosis allows the development of fast growing colon cancer. Several tactics have been shown to clarify how colon adenocarcinomas avoid cell deletion and remain viable. Regardless of the presence of active membrane receptors, colorectal cancer cells resist interferon-mediated cell death. Similarly, they are refractory to TNF-α-dependent apoptosis. In our studies, we assumed that IFN-R and TNF-R1 receptors compete for STAT-1α kinase. Western blot and immunoprecipitation analyses were used to evaluate the protein to protein interactions. Cell viability was measured by MTT assay. We observed that STAT-1α kinase is bound to TRADD protein in TNF-R1 signalosome irrespective of the TNF-R1 bound ligand. The amount of STAT-1α kinase associated with TRADD was diminished after pretreatment with IFNs. IFN-α stimulated the survival of COLO 205 cells rather than promoted cell death. The latter was accompanied by NF-κB activation, a fact known to promote anti-apoptosis. STAT-1α renders colon adenocarcinoma COLO 205 cells less susceptible to TNF-α-induced apoptosis but IFN-α further extends the immune escape.

Introduction

One group of cytokines used in anti-cancer therapy is represented by the family of interferons (IFNs) (type I and type II). IFNs, administrated alone or combined with anti-tumor drugs were reported to be successful in the treatment of chronic myelogenous leukemia, myeloma, hairy cell leukemia, Kaposi's sarcoma, lymphomas, melanomas, renal cell and bladder carcinomas (1-4). However, despite the beneficial effects of IFNs in certain types of malignant tumors, a substantial portion of patients fail to respond to IFNs. Thus, a better understanding of the mechanisms that underline IFN anti-tumor effect and the factors that are responsible for the lack of response to IFNs would probably improve the efficacy of cytokines used to treat neoplastic diseases. Hitherto, there are no available data concerning the molecular mechanisms of the IFN action in colon adeno-carcinoma cells.

The IFN family includes two main classes of related cytokines (type I and type II). There is a variety of type I interferons, all of which have a considerably similar structure. In humans, this group includes IFN-α, IFN-ß, IFN-ε, IFN-κ and IFN-ω (5,6). All type I IFNs assemble with a common cell surface receptor, which is known as the type I IFN receptor (IFNAR) (5). By contrast, there is only one type II interferon called IFN-γ that binds another cell surface receptor known as the type II IFN receptor (IFNGR) (6). The type I receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with the Janus activated kinase (JAK1) and tyrosine kinase 2 (TYK2), respectively. The type II receptor is also composed of two subunits: IFNGR1 and IFNGR2, which are in turn associated with JAK1 and JAK2, respectively (7,8). Upon JAK-mediated receptor protein tyrosine phosphorylation, the signal transducers and activators of transcription (STATs), (STAT-1α, STAT-2 and STAT-3) dimerize and translocate to the nucleus to activate transcription of IFN-stimulated genes (9).

It is apparent that the activation of JAK-STAT pathway alone is not sufficient for the generation of all of the biological activities of IFNs. There is accumulating evidence that several other IFN-regulated signaling elements and cascades are required for the generation of many cellular responses to IFNs. Some of these pathways cooperate with the JAK-STAT pathway, whereas others only with STATs. TNF-α signaling pathway is one of the postulated targets for IFNs. For the first time in 1997 Kumar *et al* (10) observed that the U3A STAT-1α-null cells were resistant to TNF-α-induced apoptosis. The authors did not explain the mechanism of STAT-1α cooperation with TNF-α signaling cascade. The possible scenario was described by Wang *et al* (11), who revealed that in HeLa cells STAT-1α is bound to

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TRADD protein in TNF-R1 signalosome and thus inhibits NF-κB activation and promotes apoptosis induction. The TRADD-STAT-1α association was detected only in immunoprecipitates from TNF-α-treated cells, whereas STAT-1α did not assemble with TRADD in untreated cells. The authors assumed, that STAT-1α plays a role in either stabilizing the death signaling complex (TRADD-FADD) or preventing TRADD-RIP complex formation. In cells lacking STAT-1α, TRADD-FADD complex formation was unaffected, whereas TRADD-RIP and TRADD-TRAF2 interactions in response to TNF-α treatment were enhanced and comparable to parental cells. Moreover, Wang *et al* (11) showed that TNF-α treatment caused Y701 phosphorylation of STAT-1α. In spite of constitutive S727 residue phosphorylation, which determines the transcriptional activity of STAT-1α, no DNA binding activity was detected. It was concluded, that STAT-1α recruited to TNF-R1 might function as a signal transducer rather than as an activator of transcription. The steps in the pathway through which TNF-α induces such a response are still not fully understood. Neither TNF-α receptor contains a protein tyrosine domain or any motif suggesting such biochemical activity (12,13). However, Guo *et al* (14) identified protein tyrosine kinases involved in TNF-α signal transduction. In 3T3-L1 adipocytes the TNF-α-induced tyrosine phosphorylation and activation of JAK1, JAK2 and TYK2, although the most evident was the JAK2 phosphorylation. Moreover, direct interaction between JAK2 and TNF-R1 was also demonstrated (14). In addition to promoting JAK kinase activity, TNF-α also induced tyrosine phosphorylation of a selected group of STAT proteins, among them also the STAT-1α. Similarly to Wang *et al* (11), in 3T3-L1 adipocytes, despite the tyrosine phosphorylation of STAT-1α, no DNA-binding activity was detected. In contrast, Wesemann *et al* (15) showed that TNF-α did not stimulate TRADD-STAT-1α complex formation in macrophages. This association was induced by IFN-γ treatment. Interestingly, the previous studies (15) described TNF-α-induced formation of TNF-R1-STAT-1α complexes. In both cases the authors concluded that the complexes formed do not require STAT-1α phosphorylation. In contrast to Guo *et al* (14), via coimmunoprecipitation assay the authors were unable to detect any TNF-R1-JAK1 or TNF-R1-JAK2 associations in the absence or presence of either TNF-α or IFN-γ. Finally, Wesemann *et al* (15), similarly to Wang *et al* (11) confirmed that STAT-1α is able to inhibit TNF-α-mediated NF-κB activation. Furthermore, the transcriptional action of TRADD-STAT-1α complexes and their involvement in IFN-γ-induced gene expression was also postulated (15). The other point of view was presented by Mukhopadhyay *et al* (16), who found that in U3A fibroblasts STAT-1α which had a minimal effect on TNF-α-induced NF-κB activation, was involved in IFN-α-induced suppression of TNF-α-mediated IκB degradation. It is also widely known that in colon cancer cells, STATs are often constitutively activated (17). According to our previous results, in COLO 205 cells regardless of the dose and time TNF-α did not induce NF-κB activation (18). Hitherto, there are no studies concerning the role of STAT-1α kinase in TNF-α signaling pathway in human colon adenocarcinoma cells. The aim of our study was to investigate if STAT-1α kinase is involved in TNF-α signaling pathway in COLO 205 cells stimulated by IFNs.

Materials and methods

Reagents. All reagents: dimethyl sulfoxide (DMSO), Tris, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), (2-amino-ethoxyethane)-N,N,N',N'-tetraacetic acid (EGTA), poly-oxyethylene sorbitan monolaurate (TWEEN-20), sodium chloride (NaCl), bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT), phenylmethylsulphonylfluoride (PMSF), dithiothreitol (DTT), 2-(4-morpholinyl)-8-phenylchromone (LY 294002), interferon-α (IFN-α), interferon-γ (IFN-γ) were cell culture tested, of high purity and unless otherwise stated they were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Reagents for experimental applications were prepared according to the manufacturer's instructions and if possible stored as stock solutions (1000-fold the highest working concentration). Primary polyclonal rabbit anti-NF-κB, anti-IκB, anti-FADD and goat polyclonal anti-TRADD, anti-ß-actin and mouse monoclonal anti-STAT-1α, anti-Y701p-STAT-1α IgG antibodies and secondary horseradish peroxidase (HRP) conjugated donkey anti-rabbit, donkey anti-goat and donkey anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other reagents were purchased as stated in the description of the respective methods. Sodium dodecyl sulphate (SDS) 100 g/l, sequi-blot polyvinylidene fluoride (PVDF) membrane 0.2 μm and all reagents for immunoblotting were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Sera, media and antibiotics were obtained from Gibco Life Technologies (Paisley, UK).

Cell culture. Human colon adenocarcinoma cell line COLO 205 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in the exponential phase of growth in growth medium [GM, 100 ml/l Fetal Bovine Serum (FBS)/Dulbecco's Modified Eagle Medium (DMEM) with Glutamax and antibiotic-antimycotic mixture (penicillin G sodium salt 50 IU/ml, streptomycin sulphate 50 μg/ml, gentamycin sulphate 20 μg/ml, fungizone - amphotericin B 1 μg/ml)]. The cells were grown at 37˚C, in a controlled, humidified 50 ml/l CO_2 atmosphere, on a multiwell (4) Lab-Tek Chamber Slide w/Cover (Permanox Slide Sterile, Nalge Nunc International, Naperville, IL, USA) or tissue culture Petri dishes (100-mm diameter, BD Biosciences, Franklin Lakes, NJ, USA).

Experimental procedure. During propagation, the medium was changed every other day until cultures reached 100% confluence. One-day (24 h) prior to the experiment, confluent cells (cells of this same cell density fully covering the surface of the dish) were then switched to post-mitotic status to induce quiescence (withdrawal from cell cycle) by replacing GM with 20 g/l BSA/DMEM designated as a control medium (CTRL). In the above-mentioned conditions divisions of COLO 205 cell have been completed. During the study, freshly prepared media with or without experimental factors were changed according

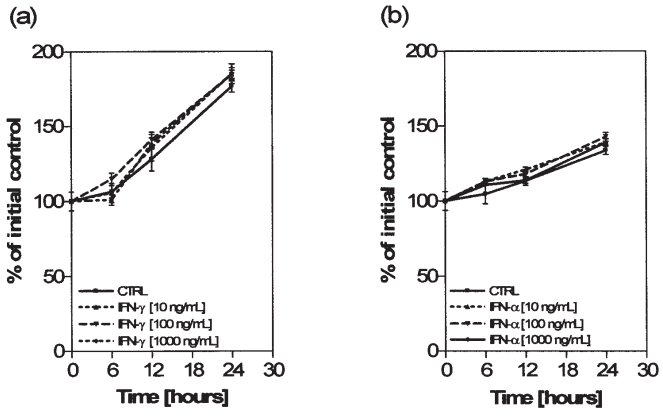

Figure 1. MTT assay of COLO 205 cells in the presence of (a) interferon-γ (IFN-γ) (10, 100, 1000 ng/ml), (b) interferon-α (IFN-α) (10, 100, 1000 ng/ml). Viability was calculated as a percentage of initial control value at time '0'. No significant differences between IFN-treated and untreated cells were observed (P>0.05).

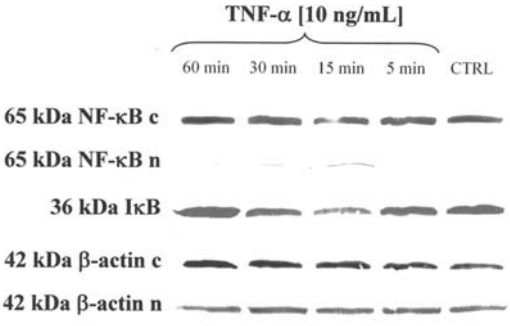

Figure 2. Western blot analysis of cytoplasmic (c) and nuclear (n) lysates of COLO 205 cells showing the expression and localization of NF-κB and IκB proteins after TNF-α (10 ng/ml, 5, 15, 30, 60 min) treatment. Equal protein loading was checked by ß-actin protein level.

to the experimental schedule. With regard to DMSO as solvent, the lowest DMSO concentration (0.1% v/v) was used in the control system, while for other treatments a fresh control medium became the control system (CTRL). Exactly 30 min prior to the application of water-soluble reagents, DMSO-dissolved reagents (LY 294002) were added.

Cell viability. Assessment of cell viability based on mitochondrial function was assayed by the ability of cells to convert soluble MTT into an insoluble purple formazan reaction product with minor modifications to protocol described by Jacobson *et al* (19). For this assay, during the last 1 h of incubation time the media were replaced by MTT solution (5 mg/ml in DMEM without phenol red, sterilized by filtration). MTT solution was then aspirated and formazan in cells was instantly dissolved by the addition of 100 μl DMSO. Cells were examined under phase-contrast microscopy before application of MTT to visually assess the degree of cell death. The absorbance was measured at 570/630 nm with ELISA reader type Infinite 200 (TECAN, Grodig, Austria). Percentage cell viability (MTT conversion into purple formazan in comparison with CTRL (20 g/l BSA/ DMEM) or 1 ml/l DMSO in control medium indicates cell viability (mitochondrial respiration or activity of mitochondrial dehydrogenases).

Preparation of whole-cell lysates. Cells were grown on 100-mm diameter culture Petri dishes. To obtain whole-cell lysates a 1 ml aliquot of ice-cold Phosphate-Buffered Saline Ca^{2+} and Mg^{2+} (PBS) was added and cells were immediately scraped from the plastics and collected by centrifugation (10000 x g for 10 min, 4˚C). An aliquot (1.0 ml) of RIPA buffer (1x PBS, 10 ml/l Igepal CA-630, 5 g/l sodium deoxycholate, 1 g/l SDS) supplemented with 0.4 mM PMSF, 10 μg/ml of aprotinin and 10 μg/ml of sodium orthovanadate (Sigma-Aldrich Chemicals Co.) was added to lyse the cell pellet and cells were broken up by repetitive trituration with the syringe with an attached needle (0.6-mm diameter). Cell suspension was then left on ice (4˚C) for 30 min, then centrifuged for another 5 min (4˚C, 10000 x g). Next, viscous solution was divided into smaller volumes and transferred to fresh Eppendorf tubes and stored at -80˚C until used.

Preparation of cytoplasmic and nuclear lysates. Cells were grown on 100-mm diameter culture Petri dishes. Following each experiment the cells were washed twice with PBS, scraped off in PBS and centrifuged (4˚C, 10000 x g, 5 min). Cell pellets were stored at -80˚C to the end of the experiment. Cell pellets were resuspended in 400 μl of ice-cold buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF), were then incubated on ice for 15 min, after which 25 μl of a 100 ml/l solution of Igepal CA-630 was added. After centrifugation (20˚C, 1000 x g, 30 sec), supernatants containing cytoplasm were transferred to fresh tubes and were stored at -20˚C. Nuclear pellets were resuspended in 200 μl RIPA buffer as described for whole-cell lysates. PMSF (0.1 mg/ml) was added and cells were incubated 30 min on ice. After centrifugation (4˚C, 10000 x g, 5 min) nuclear lysates were stored at -80˚C until analysis. Soluble protein concentrations in the whole-cell, the cytoplasmic and the nuclear fractions were determined by a protein-dye-binding method (20) with commercial reagent (Bio-Rad Laboratories).

Immunoprecipitation and Western blotting. Whole-cell lysates containing 900 μg of protein were incubated overnight at 4˚C with 1.5 μg (7.5 μl) rabbit polyclonal anti-FADD or mouse monoclonal anti-STAT-1α and for an additional 3 h with 30 μl protein A/G bead slurry (Santa Cruz, CA, USA). Beads were then washed four times with cold RIPA buffer, boiled in sample buffer (2 x Laemmli buffer, Sigma-Aldrich Chemical Co.) for 3 to 5 min, separated by 10% SDS-PAGE, transferred to a PVDF membrane and probed with a mouse monoclonal anti-STAT-1α or a goat polyclonal anti-TRADD IgG antibody (1 μg/ml) for detection of endogenous protein associations. For detection of activated proteins RIPA lysates from these same collections were separated by SDS-PAGE, transferred to PVDF membrane (0.2 μm) and probed with an appropriate antiserum. Finally, probing with primary antibody against immunoprecipitation antibody and subsequent species-specific secondary antibody were used to verify equal protein loading. The enhanced chemiluminescence (ECL or ECL Plus)

method was used for protein detection (Amersham International, Aylesbury, UK).

Electrophoresis and immunoblotting. Equal amounts of sample protein (either 50 μg or 30 μg) isolated from the treated or untreated COLO 205 cells were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Electrotransfer of proteins to PVDF membranes (0.2 μm) was performed for 1.5 h at 100 V and followed by overnight blocking (4˚C) in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) supplemented with 50 g/l non-fat powdered milk. Membranes were stained with *Poinceau S*. dye to check equal and homogeneous loading. After washing in TBST (TBS containing 0.5 ml/Tween-20), the membranes were immunostained by standard methods provided by the manufacturer (Santa Cruz, CA, USA). They were probed with a primary antibody (rabbit polyclonal anti-NF-κB or anti-IκB) (Santa Cruz, CA, USA) for 1 h at 20˚C or overnight at 4˚C, washed three times in TBST and were further incubated with the secondary donkey anti-rabbit or donkey anti-goat antibodies conjugated with HRP (see Reagent's section). Membranes were also probed with goat polyclonal anti-ß-actin antibody to normalize proteins level in whole-cell, cytoplasmic and in nuclear lysates (21). The blots were developed using the enhanced chemiluminescence (ECL or ECL Plus) detection system (Amersham International) according to the manufacturer's instructions. After exposure, photographs were taken with a Kodak DC 290 zoom digital camera and were scanned and analyzed using the Kodak EDAS 290/Kodak 1D 3.5 system (Eastman Kodak Company, Rochester, NY, USA).

Statistical analysis. Each treatment was carried out in triplicate and each experiment was repeated at least twice. The results were statistically evaluated with one-way ANOVA and Tukey's multiple range test when compared to control treatments, or by two-way ANOVA with Benferroni post-test to compare replicate means between the treatments. These analyses were performed using GraphPad Prism™ version 4.03 software (GraphPad Software Inc., San Diego, CA, USA). In order to show the quantitative differences, percentage of initial control value set arbitrary as 100% (experimental value/initial control value x 100) at each time point were used. Statistical differences were interpreted as significant at $P<0.05$ and highly significant at $P<0.01$.

Results

COLO 205 cells are resistant to cytotoxic effect of interferons (IFN-α and IFN-γ). To examine whether interferons disturb vital cellular processes in COLO 205 cells, a series of viability tests (MTT assay) was carried out. Experimental values were compared with the control value from the hour '0', which indicates the viability of cells measured after the end of 24-h preincubation with 20 g/l BSA/DMEM (% of initial control) and is convenient to show the dynamics of changes. The reduction of MTT into insoluble formazan was not significantly different in IFN-γ (Fig. 1A) or IFN-α-treated (Fig. 1B) COLO 205 cells in comparison to untreated ones

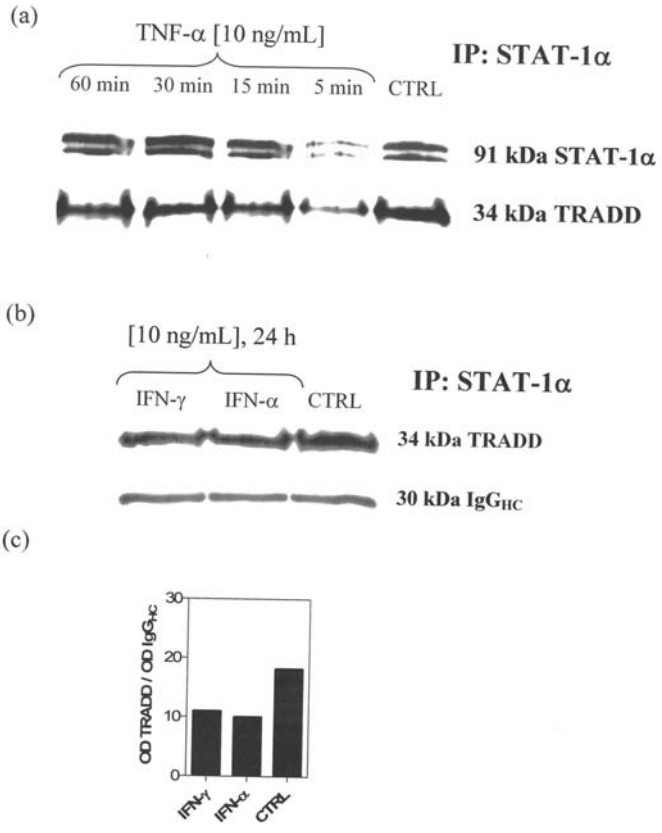

Figure 3. The analysis of protein-protein interactions by immunoprecipitation. The whole-cell lysates from: (a) TNF-α- (10 ng/ml, 5, 15, 30, 60 min) or (b) IFN-α- or IFN-γ-treated (10 ng/ml, 24 h) and untreated cells (CTRL) were immunoprecipitated with anti-STAT-1α antibody on A/G agarose beads. After PAGE the TRADD proteins were detected by immunoblotting. (c) Bar charts represent relative integrated optical density values.

($P>0.05$). Neither IFN-γ nor IFN-α if used individually can reduce COLO 205 cell viability, irrespective of dose (10, 100 and 1000 ng/ml) and time of incubation (6, 12 and 24 h).

IFN-α and IFN-γ diminish the STAT-1α kinase level in TNF-R1 signalosome. According to our previous observations TNF-α does not activate the NF-κB signaling pathway in COLO 205 cells as demonstrated by immunocytochemistry studies (22). These same results were obtained from Western blot analysis, which have shown ultimately cytoplasmic localization of NF-κB and the lack of IκB degradation (Fig. 2). We hypothesized, that the presence of additional protein(s) in TNF-α-TNF-R1 complex is in charge of inhibiting signal transduction. We performed the immunoprecipitation study according to Wang *et al* (11), to verify whether the STAT-1α kinase is bound to TRADD protein. The STAT-1α immunoprecipitate both from untreated control and TNF-α-treated COLO 205 cells (10 ng/ml, 5, 15, 30 and 60 min) demonstrated TRADD protein was bound to STAT-1α kinase, however, the concentration of the ligand (TNF-α) acting on its cognate receptor (TNF-R1) or the time of incubation were irrelevent to the level of associated protein (Fig. 3A). The immunoprecipitate of TRADD protein and subsequent immunoblotting of STAT-1α kinase revealed similar results (data not shown). The presence of TRADD in TRADD-STAT-1α complexes in control cells suggests, that STAT-1α could be preferentially

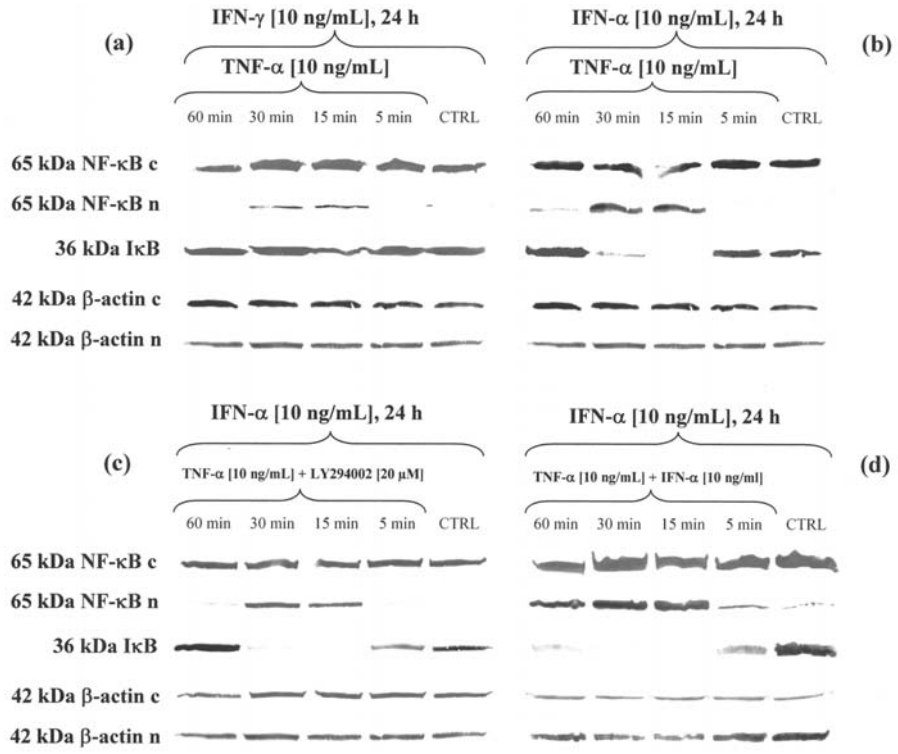

Figure 4. Western blot analysis of cytoplasmic (c) and nuclear (n) lysates of COLO 205 cells showing the expression and localization of NF-κB and IκB proteins after: (a) 24-hour preincubation with IFN-γ (10 ng/ml) and subsequent TNF-α treatment, (b) 24-hour preincubation with IFN-α (10 ng/ml) and subsequent TNF-α treatment, (c) 24-hour preincubation with IFN-α and LY 294002 (20 μM, 30 min) and subsequent TNF-α and LY 294002 treatment, (d) 24-h preincubation with IFN-α and subsequent TNF-α and IFN-α treatment. Equal protein loading was checked by ß-actin protein level.

linked to TNF-R1 irrespective to IFN-R in COLO 205 cells. Actually, the levels of TRADD protein in STAT-1α immunoprecipitates from IFN-α- or IFN-γ-stimulated cells (10 ng/ml, 24 h) were also lower in comparison to control cells (Fig. 3B).

IFN-α pretreatment allows TNF-α to induce NF-κB in COLO 205 cells. Keeping in mind the hypothesis of Wang *et al* (11) concerning the inhibitory role of STAT-1α in TNF-R1 signalosome, we decided to determine whether IFNs affect TNF-α-dependent NF-κB activation. Prior to TNF-α administration, COLO 205 cells were pre-treated with IFN-α or IFN-γ (10 ng/ml) for 24 h. Following pretreatment, the TNF-α (10 ng/ml) was added for 5, 15, 30 and 60 min and NF-κB intracellular location was examined. Surprisingly, if assessed by the nuclear position of NF-κB only IFN-α pretreatment allowed TNF-α to activate NF-κB. After 15 and 30 min of TNF-α administration the Western blot revealed a higher level of the NF-κB in the nucleus and a diminished level of cytoplasmic IκB (Fig. 4B). In spite of IFN-γ-mediated decrease in the level of STAT-1α-TRADD complexes (Fig. 3B) and regardless of the time of TNF-α treatment, NF-κB still remained in the cytoplasm (lack of NF-κB in nucleus and the presence of IκB in the cytoplasm, Fig. 4A). It is known, that IFN-α is able to support cell survival via other signaling pathways, such as through the phosphatidylinositol 3-kinase/protein kinase B-Akt (PI3-K/PKB-Akt). To find out, whether the IFN-α-mediates TNF-α-dependent NF-κB nuclear translocation through the PI3-K/Akt signaling pathway we performed an experiment with LY 294002 (20 μM), a highly specific PI3-K inhibitor (the concentration of LY 294002 was evaluated previously). We found that during TNF-α treatment, the PI3-K inhibitor did not affect the TNF-α-induced NF-κB activation (Fig. 4C). Similarly to IFN-α pretreated cells, the NF-κB shifted to the nucleus at least 15 min after TNF-α addition. At this same time the level of IκB dropped. NF-κB activation, after IFN-α preincubation with the subsequent TNF-α administration evoked a nuclear signal that lasted for at least 30 min (after 60 min of TNF-α action NF-κB was detected only in the cytoplasm). Probably, it suggests the existence of dynamic competition for STAT-1α between TNF-R1 and IFNAR. We observed extended NF-κB nuclear signal ultimately in cells pretreated with IFN-α and subsequently co-treated with TNF-α (10 ng/ml) and IFN-α (10 ng/ml). It appeared, that concomitant stimulation with IFN-α and TNF-α resulted in accelerated NF-κB nuclear translocation. We assume that the presence of IFN-α was crucial for NF-κB activation, because the combined treatment with TNF-α and IFN-α can keep NF-κB in the nucleus for at least 60 min as shown in Fig. 4D. Taken together, these results provide strong evidence for crosstalk between IFN-α and TNF-α signaling pathways.

IFN-α but not IFN-γ stimulates STAT-1α expression and Y701 phosphorylation. STAT-1α is the major transducer of IFN signals. To explain the resistance of COLO 205 cells to the cytotoxic action of IFNs (Fig. 1) we performed Western blot analysis. To examine the level of Y701 phosphorylated form of STAT-1α (p-Y701-STAT-1α) cells were initially incubated with IFN-α or IFN-γ (10 ng/ml) for 24 h. It was

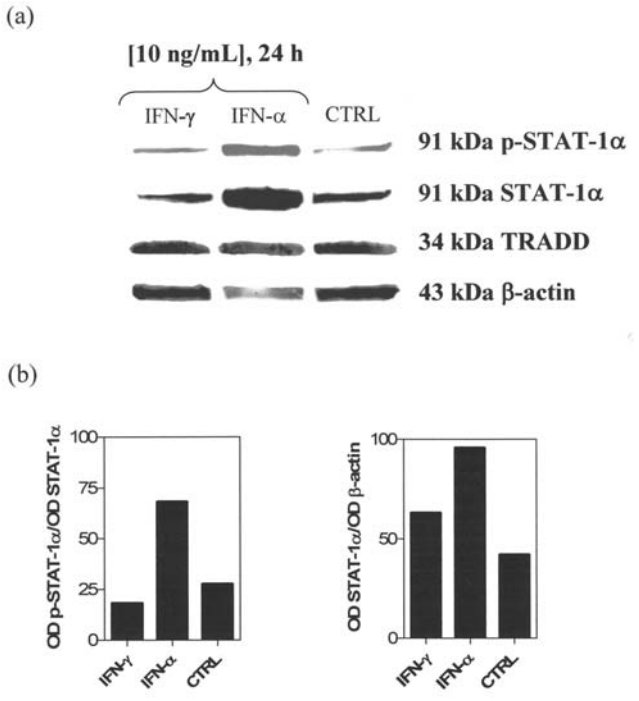

Figure 5. (a) Western blot analysis of whole-cell lysates of COLO 205 cells showing the expression of p-Y701-STAT-1α, STAT-1α and TRADD proteins after IFN-α or IFN-γ (10 ng/ml, 24 h) treatment. (b) Bar charts represent relative integrated optical density values.

found that the p-Y701-STAT-1α level was higher in IFN-α- than in IFN-γ-stimulated cells (Fig. 5). Likewise, a higher expression of STAT-1α was found in IFN-α-treated cells.

Discussion

In the present study, we provided direct evidence for crosstalk between TNF-α and IFN-α signaling pathways in human colon adenocarcinoma COLO 205 cells. Colon cancer cells are extremely resistant to the natural immune system that mediates apoptosis. Therefore, considerable attention has been paid to death ligands and interferons as therapeutic agents to treat human cancers. It was previously demonstrated that the combined treatment of IFN-γ together with TRAIL caused caspase-8 cleavage, the loss of mitochondrial potential, cytochrome *c* release and cell death (23). Similarly, Park *et al* (24) and Abadie *et al* (25) demonstrated that IFN-γ mediated the TRAIL-induced apoptosis through stimulation of IRF-1 (Interferon Regulatory Factor 1). Moreover, Abadie *et al* (25) reported on the exposure of mitochondria membranes to Apo2.7 antigen (antigen restricted to cells undergoing apoptosis) in IFN-γ or IFN-γ and IFN-α or IFN-γ and TNF-α-treated cells. No such effects were observed when cells were treated individually with IFN-α or TNF-α, even in high, non-physiological concentrations. Irrespective to numerous reports describing the anti-cancer activity of IFNs and their synergistic action with death ligands, our observations revealed that no such response occurred either in IFN-α- or in IFN-γ-treated COLO 205 cells, even when those cytokines were used in a very high concentration (1 μg/ml) (Fig. 1). Furthermore, the preincubation with either IFN-α or IFN-γ did not sensitize COLO 205 cells to the TNF-α apoptotic signal. Also, the simultaneous exposure to IFNs and TNF-α did not render cells reactive to apoptogenic stimuli (data not shown). Assumed lack of IFN-dependent signal transduction in colon cancer cells prompted us to verify if the resistance to IFNs resulted from the limited accessibility of STAT-1α kinase, the main transducer of IFN signals. Confronting Wang *et al* (11) and Wesemann *et al* (15,26) studies, the STAT-1α kinase might be timely and spatially restricted to TNF-R1-TRADD signalosome, where it inhibits NF-κB. As we previously demonstrated, in COLO 205 cells, TNF-α did not stimulate NF-κB nuclear translocation, irrespective to time of incubation (0.5, 1, 2, 4 h) and concentration (1, 10, 100 ng/ml) of TNF-α (18). In accordance with Wang *et al* (11) and Wesemann *et al* (26) we performed the immunoprecipitation analyses, which revealed STAT-1α complexed with TRADD protein (Fig. 3A). In contrast to Wang *et al* (11), however, we found that a non-phosphorylated form of STAT-1α was prevalent in complexes with TRADD and that TNF-α did not enhance the level of p-Y701-STAT-1α in COLO 205 (22). Noteworthy, our data indicate that IFN-Rs can dynamically compete with TNF-R1 for STAT-1α in COLO 205. Moreover, TNF-R1 is the main target for STAT-1α kinase since the IFN administration led to a decrease of STAT-1α level associated with TRADD protein in comparison to untreated cells (Fig. 3B). Therefore, the lack of STAT-1α phosphorylation at the IFN-γR but not IFN-αR might explain the resistance of COLO 205 cells to IFN-γ stimuli.

An intriguing question remains to be answered: what is the role of constitutive presence of STAT-1α-TRADD complexes in COLO 205 cells which are neither affected by IFNs nor TNF-α? In particular, it would be advantageous to know if the presence of STAT-1α affects the TRADD-FADD complex formation and if it contributes to the resistance to TNF-α-induced apoptosis. According to Wang *et al* (11) and Wesemann *et al* (26) STAT-1α plays the negative role in NF-κB activation. Based on our data, the reduced level of STAT-1α in TNF-R1 complex results in TNF-α-induced NF-κB activation. Thus, one can admit the pretreatment with IFNs and subsequent TNF-α administration as a stimulus to the NF-κB nuclear translocation and IκB degradation. In any case, the series of Western blot analyses clearly showed, that although STAT-1α is involved in both of IFN signaling pathways, it was IFN-α but not IFN-γ that led TNF-α to evoke NF-κB nuclear signals (Fig. 4A, B). IFN-α can also act on other targets, such as PI3-K/Akt signaling pathway which maintains cell viability using NF-κB. We therefore, used LY294002 to exclude the commitment of PI3-K to IFN-α/TNF-α-dependent NF-κB activation. For the first time, we provide evidence that IFN-α supports TNF-α-induced NF-κB activation via STAT-1α- but not PI3-K/Akt-dependent route. It is not clear why IFN-α but not IFN-γ exerted such an effect. The TNF-α-dependent NF-κB activation is determined by the phosphorylation status of STAT-1α kinase. In Fig. 5, it is shown that p-Y701-STAT-1α level was increased in IFN-α-treated cells. It was not the case for IFN-γ.

In conclusion, it is possible that ultimately non-phosphorylated STAT-1α kinase possesses the ability to ligate with TRADD, whereas once activated it is most likely released from TNF-R1 signalosome. Lack of p-Y701-STAT-1α

after IFN-γ treatment was previously reported by Boudny *et al* (27) and was indicative for malignant melanoma cells. It is possible that by immunoediting (28) - phenotypical changes, such as by turning off the IFN-γ/STAT-1α signaling pathway cancer cells acquire the ability for extensive growth.

The presented studies define an unexpected novel mechanism of immunoediting and provide new insight into the cooperation between IFN-α and TNF-α. Based on the presented results we conclude that the IFN-α, which is postulated in the widely accepted anti-cancer therapy, should be used with caution in certain types of malignant tumors. Human colon adenocarcinoma cells treated with IFN-α are unlikely to undergo apoptosis, moreover, they would probably gain a feature to progress in growth via NF-κB-dependent anti-apoptosis. Altogether, our findings show that the more the immune system is trying to eliminate cancer cells, the more resistant they become. To provide rationale for more specific and more suitable targets of anti-cancer immunotherapy better insight into the IFNs or death ligand actions on cancer cells is urgently needed.

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