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Inhibitor of apoptosis-1 (IAP-1) expression and apoptosis in non-small-cell lung cancer cells exposed to gemcitabine

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Abstract

Exposure of lung cancer cells to gemcitabine (2′,2′-difluorodeoxycytidine) arrests cells in S phase and induces secondary apoptotic cell death. Gemcitabine treatment decreased the expression of IκB-α protein and, concomitantly, increased the activity of nuclear factor-κB (NF-κB) transcription factor, a known inhibitor of the apoptotic response. This increase was accompanied by a similar increment in the expression of inhibitor of apoptosis-1 (IAP-1) protein and mRNA, a caspase inhibitor responsive to NF-κB. These changes were important to the final destiny of the cells, since overexpression of a dominant negative version of IκB-α, which suppresses NF-κB activation, blocks the increase of IAP-1 protein and potentiates the action of gemcitabine. Additionally, overexpression of IAP-1 protein in A549 cells expressing the IκB-α mutant restored the initial sensitivity to gemcitabine and demonstrated that this protein was responsible for the inhibitory effect of NF-κB. These results support the notion of IAP-1 as an important antiapoptotic protein mediating sensitivity to deoxynucleotides analogs in non-small-cell lung cancer cells.

Keywords: NF-κB; A549 cells; IAP-1; Gemcitabine; Apoptosis; Antineoplastic

1. Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine) is an antineoplastic agent with activity against solid tumors [1,2]. This drug is phosphorylated intracellularly to produce active nucleotide forms. The difluorodeoxycytidine triphosphate competitively inhibits DNA polymerase and terminates DNA-chain elongation [3,4]. The diphosphate form reduces the deoxynucleotide pools by inhibiting the ribonucleotide reductase. This decline allows a more rapid phosphorylation of gemcitabine, enabling a decrease of its metabolic clearance by deoxycytidine monophosphate deaminase [5].

Due to its synergistic effects, gemcitabine is an attractive drug to combine with a wide range of anticancer drugs [2,6]. More important, this drug is also a potent radiosensitizer of human tumor cells. [7]. *In vitro*, gemcitabine inhibits proliferation and induces apoptosis of HL60 [8], myeloma, plasma cell leukemia [9,10], and human T lymphoblastoid CEM cell lines [11]. There are no reports of this effect in solid tumor cell lines.

The IAP proteins belong to a gene family that protects diverse cells from programmed cell death in response to a variety of stimuli. These proteins bind to and potently inhibit specific cell death proteases (caspases) that function in the proteolytic cascades involved in apoptosis [12,13]. These proteins, in conjunction with the TNF-α receptor-associated proteins TRAF-1 and -2 provide a signal amplification loop that promotes cell survival. The transcriptional activity induced by this pathway is mediated by (NF-κB) [14,15]. In unstimulated cells, this factor resides in the cytoplasm in association with the inhibitory subunit IκB-α. Upon cellular stimulation, this factor is phosphorylated, ubiquitinated, and degraded by the proteasome. Subsequently, NF-κB translocates to the nucleus where it induces the transcription of target genes, such as IAP-1 and -2 [16]. Blocking NF-κB activation can, in some instances, release the resistance to TNF-α-induced apoptosis, or potentiate the

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Abbreviations: IAP, inhibitor of apoptosis; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and DTT, dithiothreitol.

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death caused by DNA-damaging agents, such as etoposide [17].

This study was performed to investigate the effect of gemcitabine (dFdC), a drug widely employed in lung cancer chemotherapy [18,19], in the small cell lung cancer line A549 and the role of IAP proteins as potential sensitivity factors to this antineoplastic drug.

2. Materials and methods

2.1. Cell culture

Human A549 cells were maintained as a monolayer in Dulbecco's Minimal Essential Medium containing 10% (v/v) fetal bovine serum and were incubated at 37° in a humidified atmosphere of 5% (v/v) CO_2 in air. Dulbecco's Minimal Essential Medium and fetal bovine serum were obtained from Gibco, BRL; gemcitabine was purchased from Lilly; and other chemicals were obtained from the Sigma Chemical Co., unless otherwise stated. Cells were transfected with plasmids using DOTAP (Boehringer Manheim) and selected in 800 μg/mL of G418 for 4 weeks. The plasmids used were: pRC-CMV eukaryotic expression vector (Invitrogen), as a control and pRC-CMV containing IκB-α double mutant, donated by Dr. P. A. Baeuerle [20].

2.2. Cellular viability

Cells were seeded in 24-wells chamber dishes and treated with the stated concentrations of gemcitabine. At the times described, cells were fixed in 70% ethanol at −20°, washed in PBS, and stained in crystal violet (1% in water). After washing, the stain was solubilized in 33% acetic acid, and the absorbance was determined in an ELISA reader at 570 nm [21]. The analysis was performed in triplicate in five independent experiments.

2.3. Clonogenicity assays

Cells were exposed to the stated gemcitabine concentrations for 24 hr. The cells were detached and seeded in culture dishes at a density of 50 cells/cm^2. After 14 days, the dishes were rinsed, and the colonies were fixed with 70% ethanol, stained with crystal violet, and counted. The experiments were performed in triplicate five times.

2.4. Cell cycle analysis and mitotic index determination

After exposure to gemcitabine or vehicle, the cells were pelleted and washed twice with cold 5 mM EDTA in PBS. The cells were centrifuged at 1500 g for 5 min at 4°; the pellet was resuspended with 1 mL of 5 mM EDTA in PBS and fixed by slowly adding 1 mL of 100% ethanol while vortexing. After washing, the cells were incubated for 30 min with RNase A/T1. One milliliter of propidium iodide was added, and the cells were stored in the dark at 4°. The analysis was made by flow cytometry (FACS, Beckton Dickinson) in the linear mode with excitation at 488 nm using 620 nm filters for red fluorescence. To determine the mitotic index, logarithmic growing cells exposed to gemcitabine (1×10^{-5} M) for 24 hr were fixed and stained with propidium iodide. One thousand cells were counted and mitotic figures scored under a 40× objective in an epifluorescence Olympus microscope. The experiments were performed five times in triplicate in a blind manner.

2.5. Immunoblotting

The procedures were performed as described previously [22]. Briefly, monolayer cultures were washed twice with cold PBS, and cell extracts were prepared by lysis with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate buffer). Protein was quantified using a modified micro-Bradford procedure [23]. Equal amounts of protein were separated by 10% SDS-PAGE, transferred to PVDF membranes (Amersham), and, after blocking, incubated with the monospecific polyclonal antibodies anti-IκB-α, or anti-ICE (Santa Cruz Biotechnology), washed, and reincubated with anti-mouse or anti-rabbit IgG-horseradish peroxidase antibody (Amersham). The antibody binding was determined using enhanced chemiluminescence (Boehringer Mannheim), with X-Omat AR films (Kodak). As a protein load control, parallel gels were subjected to western blot using a β-actin antibody (Santa Cruz Biotechnology). For IAP-1 and IAP-2, extracts were prepared as described previously and incubated overnight with 1 μg/mL of polyclonal antibodies (Santa Cruz Biotechnology) preconjugated with protein A-agarose (Sigma). The complexes were then centrifuged, washed two times with RIPA buffer, boiled in SDS sample buffer, and subjected to immunoblotting as described. As a control, an aliquot of the unprecipitated extracts was subjected to western blot analysis using anti-β-actin antibody. The experiments were repeated four times.

2.6. RT PCR assays

RNA was isolated with TRIzol (Gibco, BRL) as recommended by the manufacturer. cDNA was synthesized using the thermoscript RT-PCR system (Gibco, BRL). Briefly, 10 μg of DNAse I-treated RNA was incubated with random hexamers, dNTPs, and reverse transcriptase at 50° for 1 hr in the supplied buffer, and the RNA template was removed with RNase H. To verify equal cDNA loading, primers 5′-CCCCTTCATTGACCTCAAC-3′ and 5′-TGTCATG-GATGACCTTGGC-3′ were used to amplify a small fragment of the house-keeping gene GAPDH using Advantage Taq polymerase (Clontech). IAP-1 cDNA was amplified using the primers: MIHBs 5′-GGG-TTG-TCA-TGT-TAA-AGT-GCT-TAT-AGG-G-3′ and MIHBa 5′-GCC-CTG-AAA-GGT-GGA-TGC-ACT-TC-3′. These primers amplify

a 229-bp nucleotide fragment from the 5′ end of IAP-1. PCR products were subjected to electrophoresis in agarose gels. Curves were constructed using the amplicons produced with different PCR cycles in order to obtain amplification in the log phase.

2.7. Analysis of low molecular weight DNA and nuclear staining

The procedures were performed as described previously [24] with minor modifications. Briefly, cells were scraped from culture dishes, pelleted by centrifugation for 1 min at 5000 g at 4°, resuspended in 0.25 mL of lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2% Triton X-100], and incubated for 60 min at 50° with 50 μg/mL of RNase followed by 60 min at 37° with 0.5 μg/mL of proteinase K. Two volumes of absolute ethanol at −20° were added, and high molecular weight DNA was eliminated with a pasteur pipette. The low molecular weight DNA was obtained by centrifugation at 15,000 g for 20 min at 4°. The pellet was resuspended in 10 mM Tris, 1 mM EDTA and analyzed by conventional agarose gel electrophoresis. Nuclear morphology analysis was performed by fixing the cells with 70% ethanol at −20°, incubating the slide with RNase A (10 μg/mL) for 1 hr at 37°, and staining with a 10 μg/mL solution of ethidium bromide.

2.8. Electrophoretic mobility shift assays

A549 cells were incubated with gemcitabine (1×10^{-5} M) at different times and washed twice with PBS. The nuclear extracts were prepared as described previously [25]. To visualize NF-κB-protein complexes, binding reactions were carried out in buffer B [20 mM HEPES (pH 7.9), 60 mM KCl, 20% glycerol, 0.25 mM EDTA, 0.125 mM EGTA, 1 mM DTT, 4 mM $MgCl_2$, 4 mM spermidine, 200 mM NaCl · 25 mg/mL of BSA, 2 μg poly-(dI-dC)] with 8 μg of nuclear extract on ice for 15 min. The reactions were incubated with 100 pg of end-labeled double-stranded oligonucleotide (approximately 100,000 cpm) for 30 min on ice. Protein-DNA complexes were resolved by electrophoresis on a 5% polyacrylamide gel in 0.5× TBE (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.3). Gels were vacuum dried and exposed to films at −80° for 2–12 hr and quantified by densitometry. Competition experiments to demonstrate specific binding were performed by adding 2.5, 25, and 250 molar excess of cold homologous oligonucleotide sequence or 250 molar excess heterologous (p53 consensus site) oligonucleotide to the binding reactions. The sequence of the κB oligonucleotide was: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The probes were end-labeled with [γ-^{32}P]-dATP (3000 Ci/mmol; Amersham) by T4 polynucleotide kinase (Boehringer Mannheim) using standard procedures.

2.9. In situ apoptotic assays

A549-v or A549-IκB-α cells were cotransfected with pEF-MIHB or pEF vector and the Green lantern plasmid in a 1:6 ratio using Lipofectamine-plus (Gibco, BRL) as recommended by the manufacturer. pEF-MIHB expresses IAP-1 under the control of the EF-1a eukaryotic promoter (donated by Dr. David Vaux [26]). The Green lantern plasmid contains the enhanced green fluorescent protein cDNA under the control of a cytomegalovirus minimal promoter (Gibco, BRL). The cells were exposed to gemcitabine (1×10^{-5} M) or vehicle for 24 hr, propidium iodide was added to a final concentration of 10 μg/mL, and the cells were observed under fluorescent light. Green cells and apoptotic green cells were scored in a blind manner. One thousand green cells were counted. The experiment was repeated three times with similar results.

3. Results

Exposure of A549 lung adenocarcinoma cells to gemcitabine decreased viability in a time-dependent manner (Fig. 1, left panel). The effect was evident at 1×10^{-6} M gemcitabine. When the more sensitive clonogenicity assay was used, we found that gemcitabine induced cell death at concentrations as low as 1×10^{-8} M (Fig. 1, right panel).

To obtain insight into the mechanisms of this viability decrease, we performed cytofluorometric DNA and mitotic index analyses. As expected, a decrease in the mitotic index from 5 (±0.7) to 0.4 (±0.2%) was found in cells exposed to gemcitabine for 24 hr. As shown in Fig. 2, this reduction was due to an arrest in S phase, as previously reported for other cancer cell lines [27]. Interestingly, the analysis also showed progressive disappearance of the S phase population with a concomitant increase of an hypodiploid population. This is a hallmark of apoptotic cells, as described elsewhere [28].

As these results suggested that the cytotoxicity induced by gemcitabine was due to apoptotic cell death, we analyzed morphological features following exposure of A549 cells to the drug. As shown in the upper panel of Fig. 3, cells exposed for 24 hr to gemcitabine presented typical apoptotic morphology, with nuclear condensation and the formation of apoptotic bodies. Also, exposure to gemcitabine produced DNA degradation into nucleosomal-size fragments (Fig. 3, lower panel). This fragmentation was detected mainly after 24 hr of drug exposure.

Since several commonly used antineoplastic drugs activate NF-κB, and this factor acts as an antiapoptotic signal in some cells [29], we analyzed the relevance of this factor in the gemcitabine effect. The DNA-binding activity of NF-κB increased 112 ± 8.4% after 12 hr of exposure to the drug, as assessed by electrophoretic mobility shift assays (Fig. 4, upper panel). As expected, this increase correlated with a concomitant decrease of the IκB-α protein, the principal NF-κB activity

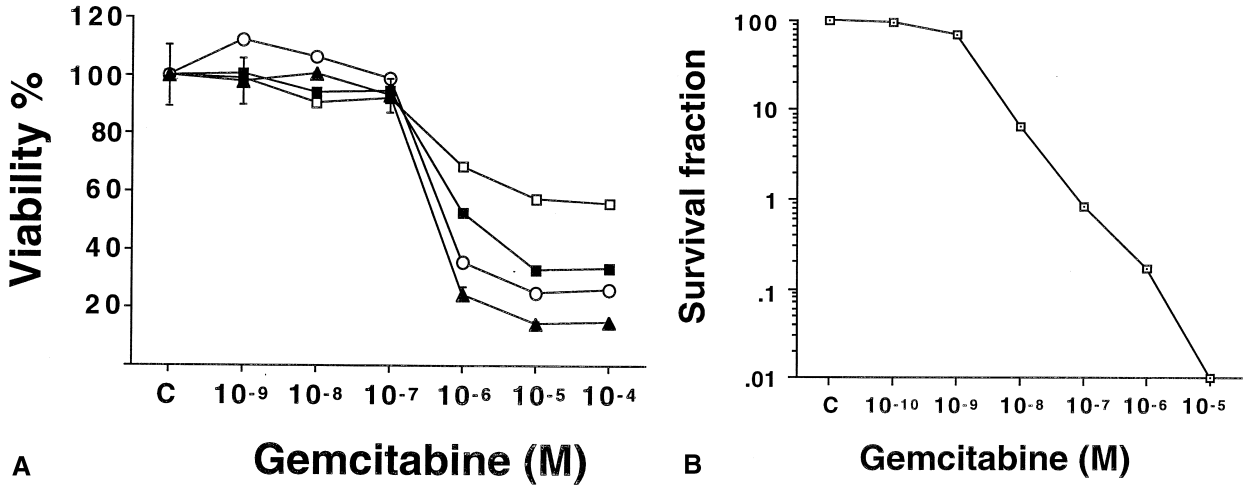

Fig. 1. Left panel: Viability of A549 cells exposed to different concentrations of gemcitabine for: (□) 24 hr, (■) 36 hr, (○) 48 hr, and (▲) 60 hr. Cells (5×10^4) were seeded in 24-well chamber dishes, and viability was determined with crystal violet assays as stated in "Materials and methods." Values (means ± SD) were derived from five independent experiments performed in triplicate. Right panel: Clonogenicity assay of A549 cells exposed to different concentrations of gemcitabine for 24 hr, as stated in "Materials and methods." The results shown are representative of five independent experiments.

inhibitor (Fig. 4, lower panel). To further demonstrate that this factor is implicated in the final response to gemcitabine, we overexpressed a mutant IκB-α in which the serines 32 and 36 are substituted by alanines [20]. These changes preclude the phosphorylation of the residues, and, by this, the ubiquitination and eventual degradation of the protein. Thus, the IκB-α mutant functions as a dominant negative protein and inhibits NF-κB activity. As shown in Fig. 5, cells overexpressing the double mutant had increased sensitivity to gemcitabine toxicity, compared with cells transfected with vector alone.

It has been shown that the antiapoptotic effects of NF-κB are due, at least in part, to enhanced production of mammalian homologues of baculovirus inhibitor of apoptosis (IAPs) [14, 30]. These proteins inhibit the activity of diverse caspases involved in the apoptotic signaling cascade [12,13,31]. In particular, it has been shown that IAP-2 by itself, or in combination with IAP-1, suppresses etoposide-induced apoptosis by an NF-κB-dependent mechanism [14,15]. To test this hypothesis, IAP-1 and -2 protein levels were measured after exposure to gemcitabine. Under our experimental conditions, we could not detect basal or induced expression of IAP-2 in these cells (results not shown). Nevertheless, as shown in Fig. 6, IAP-1 mRNA and protein levels were increased in A549 cells after exposure to gemcitabine. NF-κB activation was responsible for the increase of IAP-1 levels, since expression of the IκB-α mutant abolished this up-regulation. To demonstrate that the

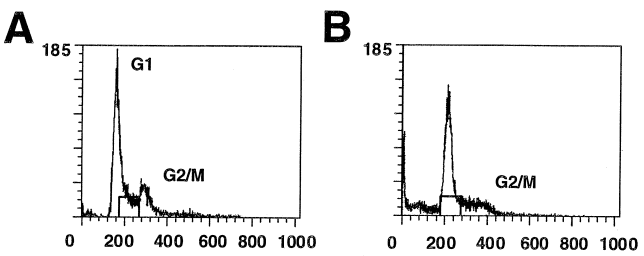

Fig. 2. Cytofluorometric DNA analysis of A549 cells exposed to gemcitabine (1×10^{-5} M). (A) control cells; (B) cells exposed to the drug for 24 hr. X-axis, fluorescence intensity in arbitrary units; y-axis, number of events (nuclei). The box indicates cells in S phase.

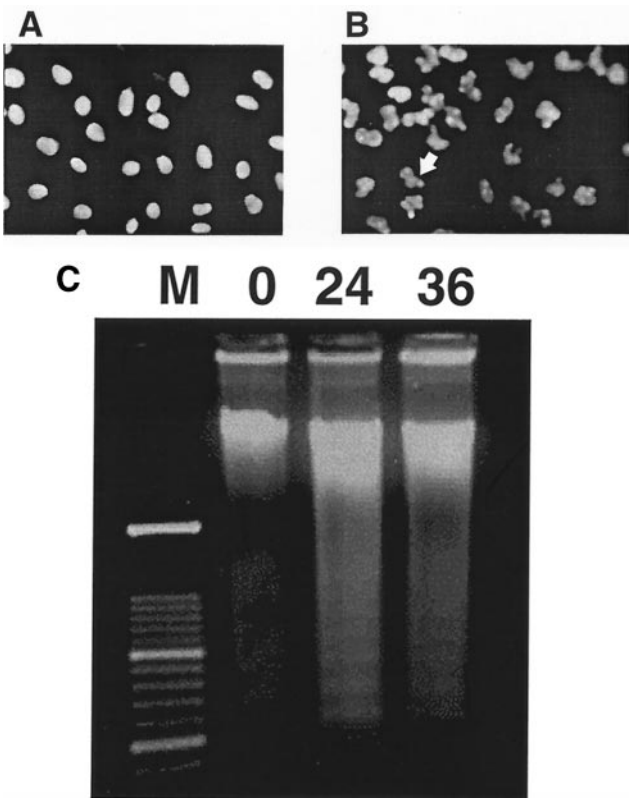

Fig. 3. Upper panel: Nuclear staining of A549 cells exposed to vehicle (A) or 10^{-5} M gemcitabine (B) for 24 hr; the arrow indicates a condensed and fragmented apoptotic nucleus. Lower panel: DNA fragmentation of A549 cells exposed to gemcitabine (1×10^{-5} M) for the times (hr) shown. M: 100 bp DNA marker.

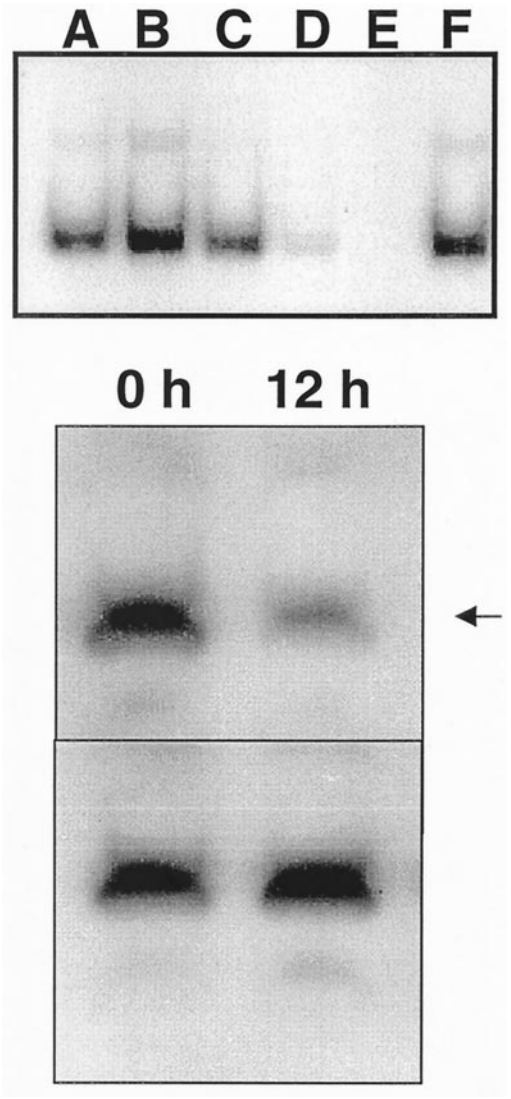

Fig. 4. Upper panel: NF-κB activity of A549 cells exposed to 1×10^{-5} M gemcitabine for 12 hr, measured with an electrophoretic mobility shift assay. (A) Control cells. (B) Cells exposed to gemcitabine for 12 hr. To verify the specificity of the assay, excess cold oligonucleotide was added at a (C) 2.5 molar excess, (D) 25 molar excess, and (E) 250 molar excess. As an additional control, heterologous oligonucleotide (p53 consensus site) was added in (F). Lower panel: Western blot of IκB-α protein in A549 cells exposed to 1×10^{-5} M gemcitabine for 12 hr. To demonstrate equal loading, the blot was stripped and reincubated with a β-actin antibody (bottom). Procedures were performed as stated in "Materials and methods."

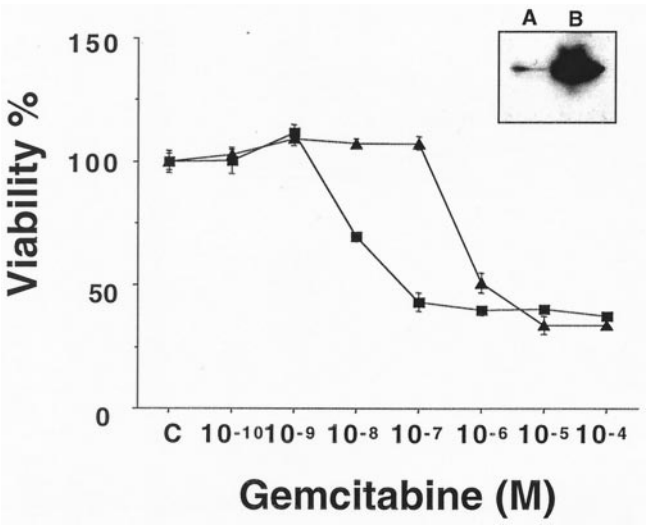

Fig. 5. Viability of A549 cells transfected with the IκB-α double mutant (■) or with vector alone (▲). Cells (5×10^4) were seeded in 24-well chamber dishes and exposed to the indicated concentrations of gemcitabine for 24 hr. Results are expressed as a percentage of vehicle-treated cells (control). Inset: Western blot of IκB-α protein in cells transfected with vector alone (A) or with the IκB-α double mutant (B). Experiments were performed in triplicate in five independent experiments as stated in "Materials and methods."

antiapoptotic effect of NF-κB was indeed due to the increase in IAP-1, we overexpressed this protein in A549-IκB-α cells. As shown in Fig. 7, the initial sensitivity to gemcitabine was restored when IAP-1 protein was overexpressed, with a decrease of roughly 20% in the death of cells at the time analyzed.

4. Discussion

In this paper, we show that gemcitabine arrests lung adenocarcinoma cells in the S phase of the cell cycle and induces apoptosis, as demonstrated by the typical apoptotic morphology, and nucleosomal-size DNA fragmentation. The concentration required for this effect was similar to the serum levels achieved in clinical trials [1]. Although a previous report [32] showed that apoptosis induced by interferon-γ in A549 cells could be related to new synthesis and activation of caspase I (ICE), we did not find changes in the expression or the activity of ICE (results not shown).

Recently, it has been shown that several antineoplastic drugs, including paclitaxel [33], cisplatin [25], etoposide [34], daunorubicin [17], and gemcitabine (this paper), can activate the NF-κB transcription factor. It seems that NF-κB activation could be a general response to antineoplastic drug exposure, perhaps by one or, at most, a few pathways. Although it has been proposed that the signal(s) for this activation could be newly synthesized cytokines or growth factors [35], intracellular activators, such as protein kinase C or DNA-PK [33] are more likely the main activators after antineoplastic drug exposure. Even though it has not been established, NF-κB activity or IAP family members could function as potential drug resistance factors. Indeed, in the present paper, we show that the cytotoxicity of gemcitabine was greatly enhanced when an NF-κB negative dominant was expressed in the cells, allowing lower concentrations of the drug to be effective. This is a cell-specific response as not all the cancer cell lines studied thus far present this phenomenon [36].

Among the possible antiapoptotic targets of NF-κB, the IAPs, a group of proteins that activate the NF-κB signaling pathway, directly inhibit effector caspases [12,13,31]. In the present report, we show that IAP-1 protein levels are in-

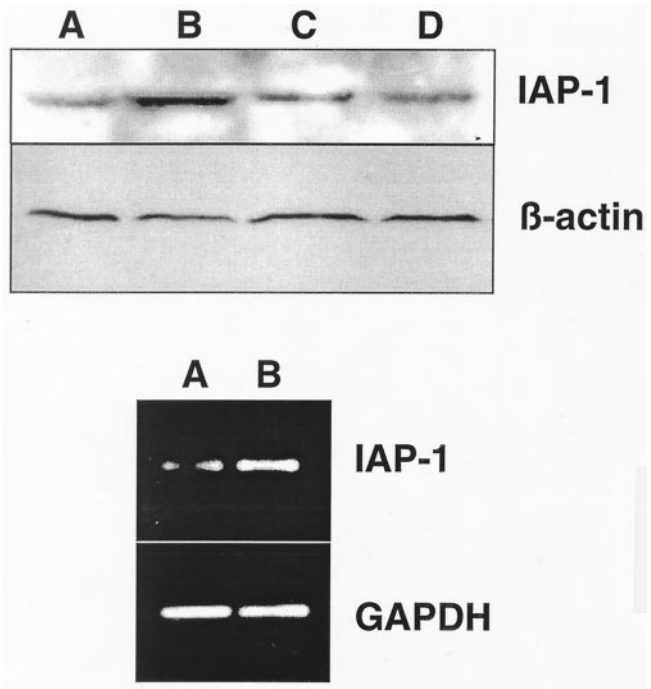

Fig. 6. IAP-1 expression. Upper panel: immunoprecipitation/western blot assays. A549-v cells were exposed to (A) vehicle or (B) gemcitabine for 18 hr. A549-IκB-α cells were exposed to (C) vehicle or (D) gemcitabine for 18 hr. To demonstrate equal loading, an aliquot of the extracts was subjected to western blot using a β-actin antibody. Lower panel: RT-PCR analysis in cells exposed to (A) vehicle or (B) gemcitabine. Procedures were performed as stated in "Materials and methods." These are representative blots from three independent experiments.

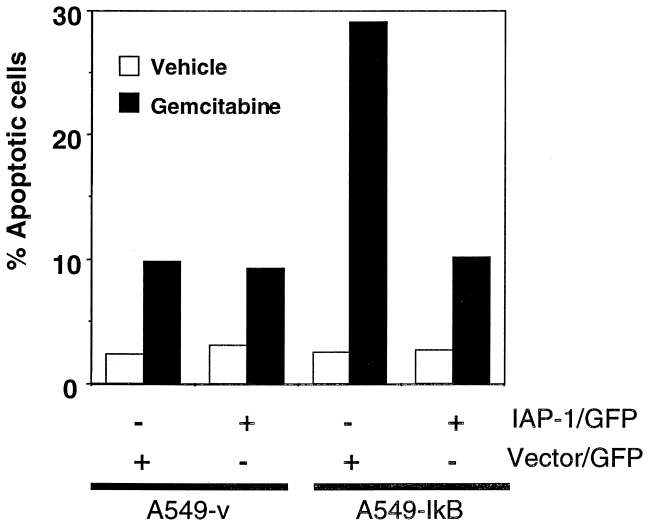

Fig. 7. Effect of the overexpression of IAP-1 protein on gemcitabine-induced apoptosis. A549 cells expressing the IκB-α dominant negative protein (A549-IκB-α) or control cells (A549-v) were cotransfected with plasmids containing IAP-1 cDNA or vector alone and a green fluorescent protein construct. Cells (5×10^4) were seeded on slides, and exposed to gemcitabine for 24 hr, and the percentages of green apoptotic cells/-total green cells were scored. Procedures were performed as stated in "Materials and methods." These are representative results of three independent experiments.

creased after exposure to gemcitabine, a result that is consistent with the activation of NF-κB. This increase was not detected when this activation was abolished by the expression of a dominant negative form of the inhibitor IκB-α, perhaps accounting for the differential sensitivity to gemcitabine. This explanation was further supported with the inhibition of cell death when IAP-1 was overexpressed independently of NF-κB regulation. Since the basal levels of IAP-1 were not modified by the mutant IκB-α expression, another transcriptional mechanism(s) must account for it. It is noteworthy that, at the times analyzed, the overexpression of IAP-1 rescued completely the cells sensitized to apoptosis by the IκB-α mutant, with no need of IAP-2, TRAF-1 or -2 overexpression, as in the case with TNF-induced apoptosis [15]. These results also differ from those presented by Wang, *et al*. [15], in which IAP-1 only partially suppressed the etoposide-induced apoptosis. This could be due to different expression levels obtained in their stable transfectants or to a cell-specific phenomenon.

Conceivably, antineoplastic drugs induce signaling cascades both for apoptosis and protection against it, with the final outcome depending on the fine balance between them. Activation of NF-κB and, in particular, overexpression of IAP-1 during antineoplastic treatment could be, at least in this cell type, the primary mechanism that counterbalances apoptotic signaling. Nevertheless, other signaling pathways and survival factors such as PI-3 kinase-Akt or stress-activated-protein-kinase (SAPK) should not be excluded. Current experiments in our laboratory are exploring these possibilities.

The present report shows that gemcitabine induces apoptotic cell death secondary to S phase arrest in non-small-cell lung cancer cells. In an attempt to counterbalance the cytotoxic stimuli, the cells simultaneously up-regulates IAP-1 protein by an NF-κB-dependent mechanism. Noteworthy, in our assays, the sole requirement for the antiapoptotic effect of NF-κB was IAP-1 expression. These results point toward the notion that IAP-1 could be an important sensitivity or primary resistance factor to gemcitabine and other antineoplastic drugs in lung cancer cells. Further analysis on clinical material would help to clarify this point.

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