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The Eukaryotic Initiation Factor 5A Is Involved in the Regulation of Proliferation and Apoptosis Induced by Interferon-α and EGF in Human Cancer Cells

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Interferon-α (IFNα) can induce apoptosis, a process regulated by a complex network of cell factors. Among these, eukaryotic initiation factor-5A (eIF-5A) is peculiar because its activity is modulated by the post-translational formation of the amino acid hypusine. Here we report the effects of IFNα and epidermal growth factor (EGF) on apoptosis and eIF-5A activity in human epidermoid oropharyngeal KB and lung H1355 cancer cells. We found that 48-h exposure to 1,000 and 2,000 IU/ml IFNα induced about 50% growth inhibition and apoptosis in H1355 and KB cells, respectively, and the addition of EGF completely antagonized this effect. When IFNα induced apoptosis, a hyperactivation of MEK-1 and ERK signalling and a decrease of the hypusine-containing form and, thus, of eIF-5A activity were recorded. The latter effect was again antagonized by the addition of EGF to IFNα-pretreated cells, probably through the activation of the EGF→ERK–dependent pathway, since the addition of the specific MEK-1 inhibitor PD098059 abrogated the recovery of intracellular hypusine content induced by EGF in IFNα-pretreated cancer cells. Subsequently, we evaluated if the hypusine synthesis inhibitor (and eIF-5A inactivator) *N*1-guanyl-1,7-diaminoheptane (GC7) synergized with IFNα in the induction of cell growth inhibition and apoptosis. The analysis of the isobologram of IFNα and GC7 demonstrated a strong synergism between the two drugs in inducing cell growth inhibition. We also found that GC7 and IFNα had a synergistic effect on apoptosis. These data suggest that the apoptosis induced by IFNα could be regulated by eIF-5A that, therefore, could represent a useful target for the potentiation of IFNα antitumor activity.

Key words: apoptosis, eIF-5A, epidermal growth factor, GC7, interferon α.

Abbreviations: IFNα, Interferon-α; eIF-5A, eukaryotic initiation factor 5A; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; *N*1-guanyl-1,7-diaminoheptane, GC7; mitogen activated protein kinase, MAPK; extracellular signal regulated kinase, ERK; SDS-PAGE, sodium-dodecylsulphate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; IC_{50}, 50% growth inhibitory concentration; IC_{75}, 75% growth inhibitory concentration; IC_{25}, 25% growth inhibitory concentration; DRI, dose reduction index; TG, transglutaminase; PBS, phosphate-buffered saline; tTG, tissue transglutaminase.

There is presently a growing body of evidence which suggests the involvement of the initiation phase of protein synthesis and its translational factors in eukaryotic cell survival and in the regulation of apoptosis (*1*). Among these, the eukaryotic initiation factor 5A (eIF5A) is peculiar because its activity is modulated by a series of post-translational modifications that culminate in the formation of the unusual amino acid hypusine. Hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] is formed by the transfer of the butylamine portion from spermidine to the ε-amino group of a specific lysine residue of eIF-5A precursor (*2*) and by the subsequent hydroxylation at carbon 2 of the incoming 4-aminobutyl moiety (*3*, *4*). eIF-5A probably acts in the final stage of the initiation phase of protein synthesis by promoting the formation of the first peptide bond (*5*). Hypusine, plays a key role in the regulation of eIF-5A function because its precursors, which do not contain hypusine do not have activity (*6*). These biochemical correlates make eIF-5A, peculiar. In fact, only the hypusine-containing eIF-5A form is active and, consequently, the dosage of intracellular hypusine content measures also the activity of eIF-5A since hypusine is contained only in this factor. The correlation between hypusine, and thus eIF-5A activity, and cell proliferation (*7*, *8*) suggests that activated eIF-5A might play a role in cell growth and differentiation (*9*). More recently, a correlation has been found between the polyamine-dependent modification of eIF-5A and the triggering of apoptosis in tumour cells (*10*). In fact, excess putrescine accumulation in hepatoma tissue culture DH23A/b cells induces apoptosis and suppresses the formation of hypusine-containing eIF-5A (*10*). Furthermore, we have evidenced an *in vitro* post-translational modification of eIF-5A catalyzed by tissue transglutaminase (tTG) (*11*) that, if stably transfected in Balb-C 3T3 cells, strongly reduces hypusine levels. These effects occurred together with a significant reduction of cell proliferation and apoptosis (*11*).

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Interferon-α (IFNα) is a common cytokine widely used in the therapy of human cancers (*12–14*). However, its mechanism of action is still unclear even if one way by which it inhibits cell proliferation is the induction of apoptosis (*15–17*).

We have demonstrated that IFNα induces apoptosis and increases the function and the expression of the receptor for EGF in human head and neck cancer KB cells (*18*, *19*). This effect is paralleled by the activation of a stress pathway that has as terminal enzymes JNK-1 and p38 kinase and is antagonized by EGF, which can be postulated as a protective factor against apoptosis induced by IFNα (*16*). EGF is also able to protect eukaryotic cells from the onset of apoptosis (*20–23*). This factor acts through the binding to its specific receptor, EGF-R, a transmembrane protein with a cytoplasmic tyrosine kinase domain (*24*, *25*). The phosphorylation of the intracytoplasmic tail allows the interaction of EGF-R with cytoplasmic factors, which can induce Ras activation and the subsequent stimulation of the mitogen activated protein kinase (MAPK) cascade (*24*, *25*). Raf-1 (MAPKKK), stimulated after steric interaction with Ras, phosphorylates and activates a MKK whose main component is MEK-1. Finally, MEK-1 phosphorylates the extracellular signal regulated kinase (ERK) 1 and 2, MAPKs that translocate to the nucleus and phosphorylate gene transactivators that are involved in the regulation of cell proliferation (*24*, *25*).

In this study, we evaluated the role of eIF-5A on cell growth inhibition and apoptosis induced by IFNα and antagonized by EGF in human epidermoid cancer H1355 and KB cells. We also studied if the MAPK-dependent pathway could be involved in the regulation of eIF-5A activity. Finally, we determined whether a specific hypusine synthesis inhibitor, *N*1-guanyl-1,7-diaminoheptane (GC7), was able to potentiate cell growth inhibition and apoptosis induced by IFNα in these experimental models (*26*).

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assays—The human lung epidermoid carcinoma H1355 and KB cell line, obtained from the American Type Tissue Culture Collection, Rockville, MD, were grown in DMEM supplemented with heat inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate. The cells were cultured in a humidified atmosphere of 95% air/5% CO_2 at 37°C. For cell proliferation experiments 1.5×10^5 control or IFNα-treated cells were seeded in 6-multiwell plates and incubated at 37°C. At the selected times the cell number was determined with a haemocytometric count after trypan blue dye.

Internucleosomal DNA Fragmentation (Ladder)—DNA fragmentation was measured after extraction of low molecular weight DNA. Briefly, 10×10^6 cells were resuspended in 900 μl of 1 × Tris-EDTA buffer and lysed with 25 μl of 20% SDS. DNA was precipitated in ethanol for 6 h in the presence of 5 M NaCl. The high molecular weight fraction was sedimented by high speed centrifugation, and the fragmented DNA was extracted from the aqueous phase with phenol and chloroform and then precipitated with ethanol. After resuspension in water, DNA was electrophoresed using 1.5% agarose gel and visualized by ultraviolet light following ethidium bromide staining.

TUNEL Technique—For TUNEL assay, after washing in PBS supplemented in 0.1% BSA, cells were treated with *in situ* detection kit, according to the manufacturers (Boehringer Mannheim Biochemicals). Nuclei with fragmented DNA were visualized with a fluorescence microscope.

Evaluation of Apoptosis by DNA-Flow Cytometry—Cells were centrifuged and directly stained in a propidium iodide (PI) solution (50 mg PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) overnight at 4°C in the dark. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) interfaced with a Hewlett Packard computer (mod.310) for data analysis. To evaluate cell apoptosis, PI fluorescence was collected as FL2 (log scale) by use of the CellFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least 10,000 events for each point were analysed in at least three different experiments giving a SD less than 5%.

Immunodetection of eIF-5A—For eIF-5A immunodetection, cells growing in complete alpha DME medium (80% confluent) were washed three times with phosphate-buffered saline (PBS). Five micrograms of total homogenate protein were subjected to sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel. Proteins were then electroblotted to type HA 0.45-mm pore nitrocellulose paper (Bio-Rad, Richmond, CA). The primary antibody, a rabbit anti–eIF-5A antibody kindly donated by Dr. K. Igarashi (Faculty of Pharmaceutical Sciences, Chiba University, Chiba) or the primary antibody to TGase (mouse monoclonal antibody; Dako), was diluted 1:2000 with a blocking solution containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% FBS. The secondary antibody, mouse anti-rabbit (Sigma, St. Louis, MO) or anti-goat IgG antiserum (Bio-Rad) conjugated with peroxidase was diluted 1:3000 with the blocking solution previously described. The intensities of the bands associated to eIF-5A or tTGase were determined by laser scanning using a common software (Gel-Pro Analyzer, Media Cybernetics, Silver Spring, MD).

Isolation, Purification and Identification of Hypusine—H1355 cells were seeded in 100-mm dishes and treated with IFNα and/or EGF and/or PD098059. At 24 h before processing, 8 μl of [terminal methylenes ^{3}H]spermidine 3 HCl (15 Ci/mmol) was added to each dish. Cell lysates were prepared using cells from 10 dishes (0.1-ml volume of washed cells) by suspending the cells in 4 ml of PBS, sonicating (10s at 70 watts), and finally centrifuging for 30 min at 25,000 ×*g*. The lysates were treated with solid ammonium sulphate (40–80% cut), and the precipitate was hydrolyzed in 6 N HCl at 110°C for 18 h. The hydrolysates were applied to 0.5 × 4 cm columns of AG 50 × 2 (H^+ form, 200–400 mesh) and eluted with 30 ml of 1 N HCl, 20 ml 3 N HCl and 30 ml 6 N HCl. Hypusine, contained in the 3 N HCl fraction, was determinated by using a reversed-phase high performance liquid chromatography (HPLC) method following a published procedure (*27*).

Gel Retardation Assay—KB cells were grown as described above. Then the cells were rapidly washed with ice-cold PBS without Ca^{2+} and Mg^{2+}. A hot SDS total cell extract was prepared by immediately scraping the cells into boiling SDS-polyacrylamide gel electrophoresis sample buffer and drawing through a 23-gauge needle 10 times to shear DNA. Then Western blot analysis was performed as described above using rabbit antisera raised against MEK-1 12-B, ERK-1 K-23, and ERK-2 MAb C-14 purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

MAPK Assay—KB cells were cultured and treated as described above. Then the cells were washed twice with ice-cold PBS, scraped and lysed for 1 h at 4°C in the following buffer: 10 mM TRIS, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% glycerol. Equalized amount of proteins from lysates of KB cells (50 μl) were incubated for 30 min at 30°C with 5 μl of a solution containing 1.2 mM Mg^{2+} ATP with 200 μCi/ml of ATPγ^{32}P and 15 μl of a solution containing a peptide that is specifically phosphorylated by MAPK (Amersham, Biotrak, Milan, Italy) (for reference, see Ref. *28*). The reaction was stopped by the addition of 2.94% (w/v) orthophosphoric acid and red carmosin solution and then microfuged for 15 sec. The reaction mixtures were spotted onto phosphocellulose filters (Whatman P81) and washed 3 times in 1% acetic acid. Filters were air-dried and then counted by liquid scintillation using Omnifluor/toluene (DuPont–New England Nuclear, Boston, MA).

Drug Combination Studies—For the study of the synergism between IFNα and GC7 on cell growth inhibition of H1355 and KB, the cells were seeded in 96-multiwell plates at the density of 5×10^3 cells/well. After 24 h of incubation at 37°C, the cells were treated with different concentrations of GC7 and IFNα. Drug combination studies were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells versus drug concentration (*29*) after 72 h of treatment. To explore the relative contribution of each agent to the synergism, three combinations with different GC7/IFNα molar ratios were tested for each schedule: equiactive doses of the two agents (IC_{50}), higher relative doses of GC7 (IC_{75} of GC7/IC_{25} of IFNα) and higher relative doses of IFNα (IC_{25} of GC7/IC_{75} of IFNα). Assessment of synergy was performed by quantitating drug interaction with the Calcusyn computer program (Biosoft, Ferguson, MO). Combination index (CI) values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively (*30*).

Statistical Analysis—All data are expressed as mean ± SD. Statistical analysis was performed by analysis of variance (ANOVA) with Neumann-Keul's multiple comparison test or Kolmogorov-Smirnov where appropriate.

RESULTS

IFNα Inhibited Proliferation and Induced Apoptosis in Human Epidermoid Lung H1355 and Oropharyngeal KB Cells—The effects of IFNα on cell growth of epidermoid lung H1355 and oropharyngeal KB cancer cells were studied. H1355 and KB cells were exposed to increasing concentrations of IFNα (data not shown), and concentrations of 1,000 and 2,000 IU/ml IFNα, respectively, were found to induce 50% growth inhibition after 48 h of treatment without cytotoxicity (Fig. 1, upper). On the other hand, higher concentrations (5,000 IU/ml) were toxic as determined by the count of floating cells and by trypan blue assay on both KB and H1355 cells (data not shown). The addition for 48 h of 10 nM EGF alone to both cell lines stimulated growth by about 20%. and the concomitant treatment with IFNα and EGF of epidermoid cancer cells caused an increase of about 35% in cell proliferation (Fig. 1, upper). Therefore, the addition of EGF to IFNα-treated cancer cells resulted in a more pronounced proliferation than in tumour cells exposed to EGF alone, suggesting a hyperactivation of the EGF-dependent signalling in IFNα-treated cells.

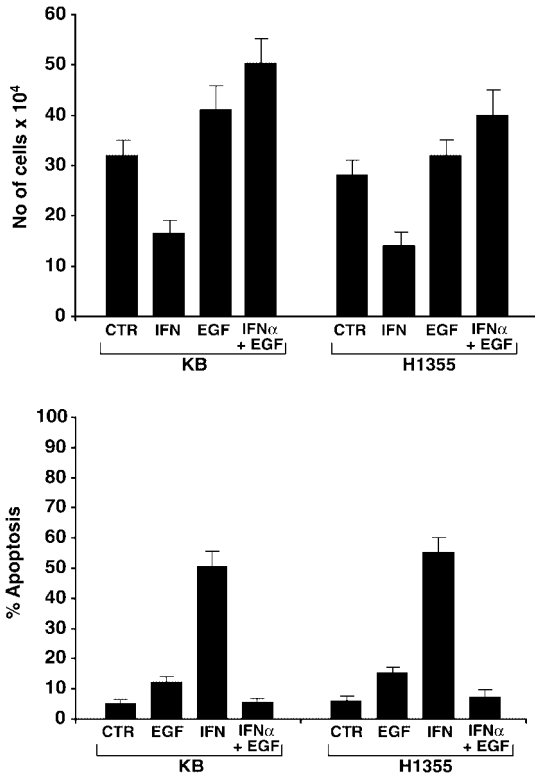


Fig. 1. **Effects of IFNα and EGF on cell proliferation and apoptosis of KB and H1355 cells.** Upper: KB and H1355 cells were treated with 1,000 and 2,000 IU/ml IFNα, respectively, and/or 10 nM EGF as described in "MATERIALS AND METHODS." At 48 h from the beginning of the treatment, the cells were counted with a haemocytometer after labelling with Trypan Blue. The values were expressed as Number of cells × 10^4. Columns, means of three different experiments. Bars, SDs. Lower: KB and H1355 cells were treated with 1,000 and 2,000 IU/ml IFNα, respectively, and/or 10 nM EGF as described in "MATERIALS AND METHODS." The percentage of apoptotic cells was evaluated by FACS analysis at 48 h from the beginning of the treatment, as described in "MATERIALS AND METHODS," and shown as columns. Columns, means of three different experiments. Bars, SDs.

To determine whether apoptosis could be found in these experimental conditions, H1355 and KB cells were subjected to flow cytometric analysis after DNA labelling with propidium iodide. Exposure to 1,000 (KB cells) or 2,000 (H1355 cells) IU/ml IFNα for 48 h was found to cause about 40% of apoptosis (Fig. 1, lower). Before performing apoptosis detection assays, both attached and

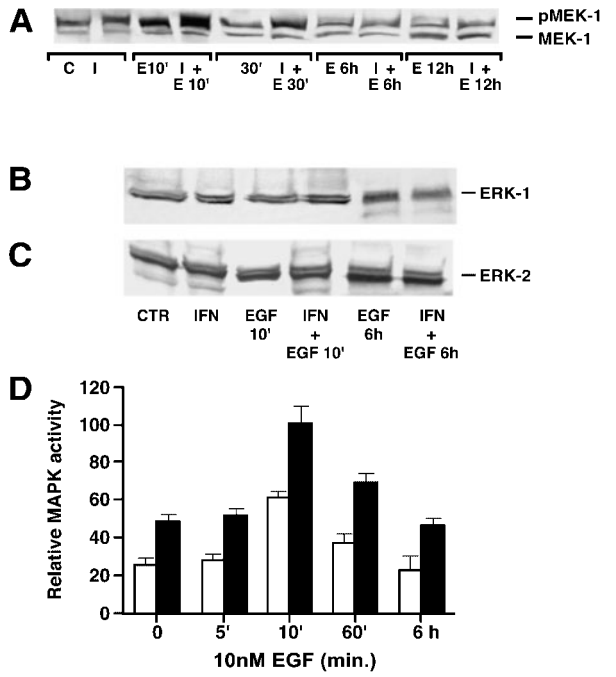


Fig. 2. **Effects of IFNα on the EGF-dependent MAPK pathway.** (A) KB cells were cultured for 48 h in the absence or presence of 1,000 IU/ml IFNα with or without the addition of 10 nM EGF for different times. Then the cells were processed for the determination of the phosphorylated isoform of MEK-1 with a gel retardation assay as described in "MATERIALS AND METHODS." C, untreated; I, IFNα; E 10′, 10 min. EGF; I + E 10′, IFNα- and 10 min. EGF; E 30′, 30 min. EGF; I + E 30′, IFNα- and 30 min. EGF; E 6h, 6 h EGF; I + E 6 h, IFNα- and 6 h EGF; E 12h, 12 h EGF; I + E 12h, IFNα- and 12 h EGF. The experiments were performed at least three times and the results were always similar. (B and C) KB cells were cultured for 48 h in the absence or presence of 1,000 IU/ml IFNα with or without the addition of 10 nM EGF for different times. Then the cells were processed for the determination of the phosphorylated isoform of (B) ERK-1 and (C) ERK-2 with a gel retardation assay as described in "MATERIALS AND METHODS." Upper bands show the phosphorylated isoforms. CTR, untreated; IFN, 48 h 1,000 IU/ml IFNα; EGF 10′, 10 min. 10 nM EGF; IFN + EGF 10′, 48 h 1,000 IU/ml IFNα- and 10 min. 10 nM EGF; EGF 6 h, 6h 10 nM EGF; IFN + EGF 6 h, 48 h 1,000 IU/ml IFNα and 6 h 10 nM EGF. The experiments were performed at least three times and the results were always similar. (D) KB cells were cultured for 48 h in the absence or presence of 1,000 IU/ml IFNα with or without the addition of 10 nM EGF for different times. Then a MAP kinase assay was performed as described in "MATERIALS AND METHODS." MAPK activity is expressed as relative activity compared to that of control cells. Untreated KB cells (open bars); IFNα-treated KB cells (solid bars). The experiment was performed three times and SDs were always less than 5%. *Bars*, SD.

suspended cells were collected. In these experimental conditions only about 10% of cells treated for 48 h with IFNα and exposed for 12 h to 10 nM EGF underwent to apoptosis (Fig. 1, lower). Interestingly, exposure to 10 nM EGF alone for 12 h also caused apoptosis in about 15% of the cell population (Fig. 1 lower). Such effect was not surprising, since apoptosis and cell proliferation are coupled processes. Therefore, the counteracting effects of EGF were also reproduced on the apoptosis induced by IFNα.

Effects of IFNα and EGF on MEK-1 Phosphorylation and ERK-1 and ERK-2 Phosphorylation and Activity—On the basis of the previous results, we evaluated the status of the EGF-dependent MAPK activation in IFNα-treated KB cells. The effects of IFNα and EGF on MEK-1 activation were detected by gel retardation assay, and the phosphorylated slow migrating isoform of MEK-1 was found to be increased almost 1.5-fold by treatment with IFNα (Fig. 2A). The phosphorylation of MEK-1 in control cells was maximal after 10 min of treatment with 10 nM EGF and resumed almost basal levels after 30 min, while it was increased 2-fold and the effect was protracted until 30 min of exposure to EGF in IFNα-pretreated KB cells (Fig. 2A). On the basis of the IFNα-induced MEK-1 hyperactivation, we evaluated the effects of this cytokine on the activity of ERK-1 and 2, the downstream enzymatic targets of MEK-1. The kinetics of ERK-1 and 2 activation was evaluated by EGF in untreated and IFNα-treated KB cells. Again, an increase of about 2-fold in ERK activity was found after 48 h in 1,000 IU/ml IFNα-treated cells. In both untreated and 48-h 1,000 IU/ml IFNα-treated KB cells, the maximal activation of ERK occurred after 10 min of exposure to 10 nM EGF, as evaluated with an enzymatic assay (Fig. 2D). However, the exposure to IFNα caused an almost 2-fold increase of the maximal activation of ERK induced by EGF (Fig. 2D). Similar data were obtained with the study of the phosphorylative status of ERK-1 and 2 performed with a gel shift assay as described in Materials and Methods (Fig. 2, B and C). In fact, the phosphorylation of the two proteins was increased by IFNα treatment and further potentiated by the exposure of IFNα-treated KB cells to 10 nM EGF for 10 min (Fig. 2, B and C). These experiments demonstrated that an increase of basal and EGF-induced enzymatic activity of MEK-1 and ERK-1/2 could be recorded in IFNα-treated KB cells.

IFNα Reduced eIF-5A Activity in Epidermoid Cancer Cells, and EGF Antagonized This Effect—A correlation between the polyamine-dependent modification of eIF-5A and the modulation of cell proliferation and triggering of apoptosis has been previously described in tumour cells (*7–11*). On the basis of these data, we evaluated if IFNα and EGF could also modulate the intracellular content of the hypusine-containing eIF-5A form (post-translationally modified and, thus, active form). Firstly, we evaluated the effects of IFNα on the expression of eIF-5A with a Western blot assay. We found that IFNα increased eIF-5A expression, which reached the peak after 48 h of treatment, when the expression was enhanced by about 40 and 30% in H1355 and KB cells, respectively (Fig. 3, A and B, respectively). On the other hand, EGF alone or in combination with IFNα had almost no effect on eIF-5A protein expression on both cell lines (data not shown). However, the addition of IFNα alone for 48 h induced a reduction of about 50% in intracellular hypusine content in both cell lines (Table 1). Again the addition of 10 nM EGF for the last 12 h was able to antagonize this effect, while the addition of 10 nM EGF alone to both cell lines had poor effects on hypusine expression (Table 1). Interestingly, the addition for the last 12 h of 50 μM MEK-1 specific inhibitor PD098059 to cells treated concomitantly with IFNα and EGF antagonized the restoring effect of EGF on hypusine synthesis in both cell lines (Table 1). The addition of 50 μM PD098059 alone for 12 h to both cell lines was also able to reduce hypusine synthesis and enhanced the decrease of hypusine synthesis

Table 1. **Effects of IFNα, EGF, and PD098059 on intracellular hypusine levels.**

Treatment	Hypusine synthesis (pmoles/mg of protein ± SD)	
	H1355 cells	KB cells
None (control)	0.51 ± 0.015	2.61 ± 0.101
IFNα	0.20 ± 0.006	0.74 ± 0.062
EGF	0.91 ± 0.025	3.15 ± 0.130
IFNα + EGF	0.49 ± 0.020	2.01 ± 0.170
IFNα + EGF + PD098059	0.19 ± 0.009	0.81 ± 0.063
PD098059	0.25 ± 0.012	0.91 ± 0.089
PD098059 + IFNα	0.05 ± 0.002	0.25 ± 0.015

The values are the mean of 4 experiments and are expressed as pmol/mg of proteins ± SDs.

induced by IFNα, suggesting a role of the EGF→ERK-dependent pathway in the regulation of eIF-5A activity, since hypusine levels represent a marker of eIF-5A activation (Table 1). Interestingly, the addition of PD098059 alone for 12 h also induced a 30% apoptotic onset in KB cells and, in these experimental conditions, was also able to completely abrogate ERK activity (*10*). Moreover, the specific MEK-1 inhibitor U0126 was also able to induce similar results (data not shown). These data suggest that the growth inhibition and apoptosis induced by IFNα were paralleled by a strong decrease of the active form of eIF-5A, which was, in turn, completely antagonized by the antiapoptotic and growth-restoring EGF-dependent MAPK signalling. Moreover, the potentiation of reduction of hypusine synthesis induced by PD098059 demonstrates that the activation of the MEK→ERK pathway induced by IFNα could be a protective response against the apoptotic stimuli induced by the cytokine. In fact, the addition of the MEK-1 inhibitor abolishes the counteracting effects of the ERK-dependent pathway on apoptosis and hypusine expression.

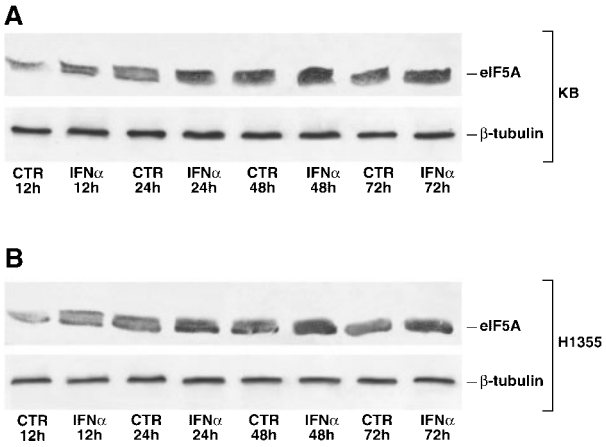


Fig. 3. **Effects of IFNα on the expression of total eIF-5A protein in KB and H1355 cells.** KB (A) and H1355 (B) cells were seeded and treated with 1,000 IU/ml and 2,000 IU/ml IFNα, respectively, for the indicated times. After the different times of treatment, cells were processed for the immunodetection of eIF-5A or β-tubulin (as house-heeping protein) with Western blotting as described in "MATERIALS AND METHODS." The experiments were performed at least three different times and the results were always similar. Hypusine levels in total cell extracts are represented in Table 1.

Deoxyhypusine Synthase Inhibitor GC7 Potentiated Growth Inhibition Induced by IFNα—To demonstrate the key role of eIF-5A in the protection of human epidermoid cancer cells from the apoptosis induced by IFNα, we used the specific hypusine synthesis inhibitor GC7 in combination with IFNα. The treatment with different combinations of IFNα and GC7 was found to be strongly synergistic in inducing cell growth inhibition in both KB and H1355 cells, as evaluated with the dedicated software from Chou and Talalay (Fig. 4 and Materials and Methods). In fact, a combination index lower than 1.0 was

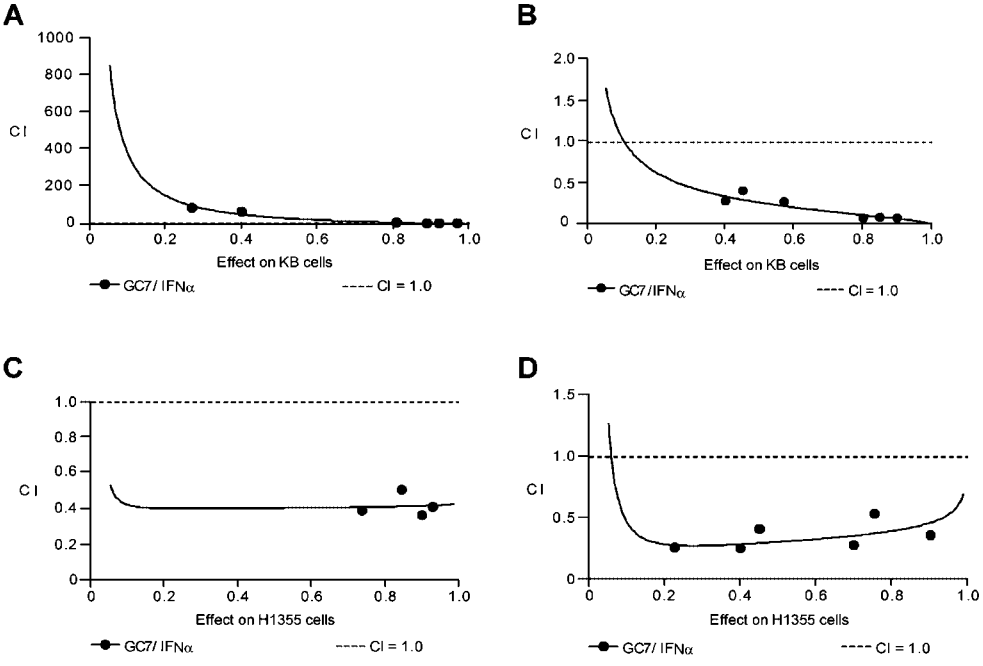


Fig. 4. **Isobologram analysis of the interaction between GC7 and IFNα on cell growth in KB and H1355 cells.** KB (A and B) or H1355 cells (C and D) were treated as described above with equiactive doses of the two drugs (IC_{50}) (A and C) or with higher relative doses of GC7 (IC_{75} of GC7/IC_{25} of IFNα) (B and D) for 72 h. Assessment of synergy was performed by quantitating drug interaction with the Calcusyn computer program (see "MATERIALS AND METHODS"). CI, combination index.

Table 2. **Combination index* (CI) and dose reduction index** (DRI) values for GC7 and IFNα combination.**

Cell lines	GC7 ED_{50} (μM)	IFNα ED_{50} (IU/ml)	Combination ratio (GC7/IFNα)	CI_{50}	DRI_{50}	I.tion
KB	0.09	270	1:30	0.26	GC7: 3.8 IFNα: 100.9	Strong synergism
H1355	9.2	460	1:50	0.30	GC7: 3.2 IFNα: 265.2	Strong synergism

* CI_{50} was calculated for 50% cell survival (ED_{50}) by isobologram analyses performed with CalcuSyn software. ** DRI represents the order of magnitude (fold) of dose reduction obtained for ED_{50} effect in combination setting as compared to each drug alone. I.tion = interpretation.

recorded when the two agents were used at equitoxic concentrations on both cell lines (Fig. 4, B and D). On the other hand, when higher doses of GC7 were used the synergism was lost in KB cells, but not in H1355 cells (Fig. 4, A and C). In detail, the CI_{50} at equitoxic concentrations [calculated for 50% cell survival (ED_{50}) by isobologram analyses performed with CalcuSyn software] was 0.26 and 0.30 in KB and H1355 cells respectively (Table 2). Moreover, the recorded synergism was mutually non-exclusive, and the dose reduction index [DRI, order of magnitude (fold) of dose reduction obtained for ED_{50} effect in combination setting as compared to each drug

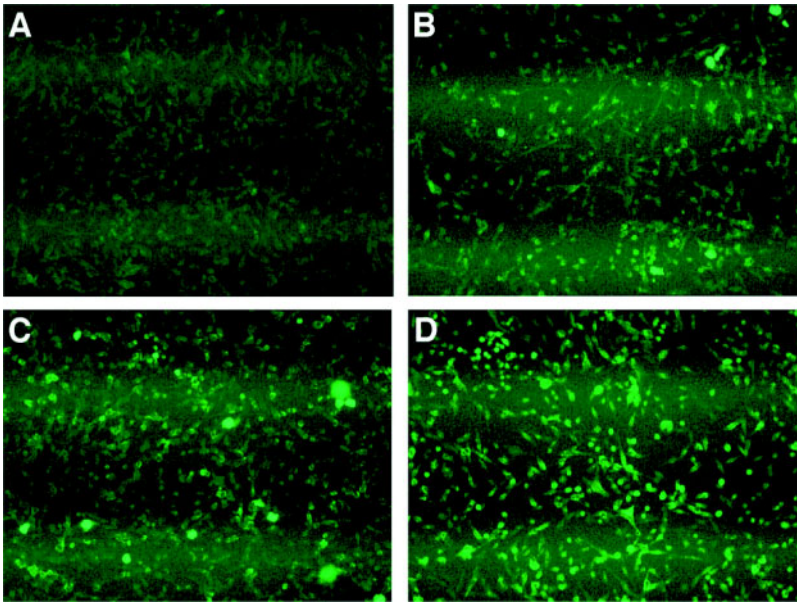
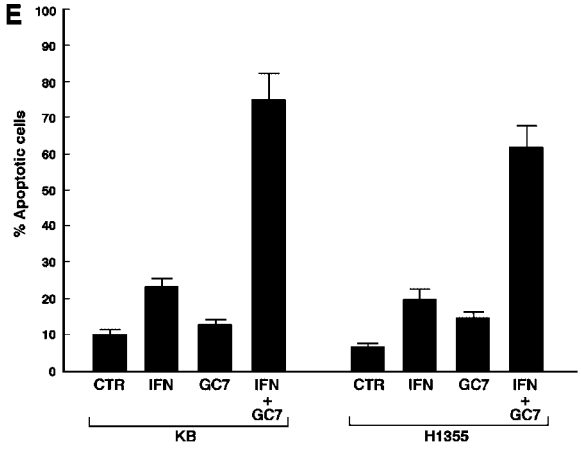
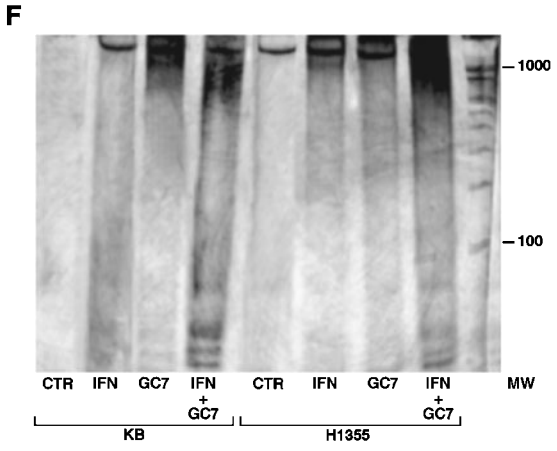


Fig. 5. **Synergistic effect of the combination between GC7 and IFNα on apoptosis of KB and H1355 cells.** (A–D) KB cells were seeded and treated with 150 IU/ml IFNα and/or 5 μM GC7. At the time of the experiment, cells were fixed and processed with TUNEL techniques as described in "MATERIALS AND METHODS." The experiments were performed at least three times and the results were always similar. (A) Control cells; (B) 48 h 150 IU/ml IFNα–treated cells; (C) 48 h 5 μM GC7-treated cells; (D) 48 h 150 IU/ml IFNα– and 5 μM GC7–treated cells. (E) The cells were seeded and treated with the different substances (150 IU/ml IFNα and 5 μg/ml GC7 for KB cells, and 500 IU/ml IFNα and 10 μg/ml GC7 for H1355 cells) as described above. At the time of the experiment, KB and H1355 cells were FACS analyzed after detachment and DNA labelling with propidium iodide as described in "MATERIALS AND METHODS." The experiments were performed at least three times and the SDs were always less than 5%. In each experimental point 10,000 events were analysed. Bars, SD. (CTR) Control cells; (IFN) 48 h IFNα–treated cells; (GC7) 48 h GC7–treated cells; (IFN + GC7) 48 h IFNα– and GC7–treated cells. (F) The internucleosomic DNA fragmentation was assessed in KB cells as described in "MATERIALS AND METHODS." (CTR) Control cells; (IFN) 48 h 150 IU/ml IFNα–treated cells; (GC7) 48 h 5 μg/ml GC7–treated cells; (IFN + GC7) 48 h 150 IU/ml IFNα– and 5 μg/ml GC7–treated cells; (MW) molecular weights.





alone] for IFNα was 100.9 and 265.2 in KB and H1355 cells, respectively (Table 2). On the other hand, the DRI for GC7 was 3.8 and 3.2 in KB and H1355 cells, respectively, suggesting that the combination is able to lower the growth inhibitory concentrations of IFNα and to sensitize tumour cells to antiproliferative activity of the cytokine (Table 2).

Interestingly, when IFNα:GC7 concentration ratio was high, the synergistic effect on cell growth inhibition was not recorded (data not shown). These data suggested a role of hypusine and, therefore, of the activated eIF-5A in the regulation of cell growth induced by IFNα.

Deoxyhypusine Synthase Inhibitor GC7 Potentiated Apoptosis Induced by IFNα—To evaluate the role of eIF-5A in the regulation of apoptosis induced by IFNα in epidermoid cancer cells we selected the following combinations of the two agents that were synergistic on growth inhibition: 150 IU/ml IFNα and 5 μM GC7 for KB cells, and 500 IU/ml IFNα and 10 μM GC 7 for H1355 cells. A Tunel assay and quantitation of apoptotic cells by FACS analysis were then performed after propidium iodide labelling (Fig. 5, A–E). The two drugs used in combination determined a potentiation of the number of green fluorescent KB cells as assessed with TUNEL assay (Fig. A–D). In detail, IFNα and GC7 alone caused 23 and 13% apoptosis, respectively, in KB cells while the two agents used together induced 60% apoptosis (Fig. 5, A–D and E). Similar results were obtained with H1355 cells, in which IFNα and GC7 alone induced 20 and 15% apoptosis, respectively, while the two agents together induced 62% apoptosis (Fig. 5E). Analogous results were obtained with the analysis of internucleosomal DNA fragmentation in KB cells (Fig. 5F). In fact, IFNα and GC7 alone caused poor DNA fragmentation, while considerable fragmentation was evident when the two agents were used in combination (Fig. 5F). These data suggest that eIF-5A regulated tumour cell proliferation through the control of the apoptotic program.

DISCUSSION

IFNα is a cytokine that has shown well-defined but still limited activity against human tumors (*12–14*). The way in which tumour cell growth is suppressed by IFNα is not well known. One possibility is that cancer cells undergo apoptosis after exposure to the cytokine. In fact, it is reported that IFNα induces apoptosis in human squamous cancer cells (*15*, *16*). Therefore, it is likely that this cytokine acts, at least in part, through the triggering of programmed cell death. We have also demonstrated that the growth inhibition induced by IFNα is due to apoptosis triggered by a stress response leading to the activation of JNK-1 (*16*). These effects are parallelled by the increase of tissue transglutaminase (tTG) expression and activity mediated by the reduction of its proteasome-dependent degradation induced by the cytokine (*31*). Moreover, we have previously demonstrated that hypusine is an *in vitro* and *in vivo* substrate of tTG and the reduction of intracellular hypusine levels, and thus of eIF-5A activity, induced by tTG is paralleled by growth inhibition and apoptosis of eukaryotic cells (*11*). On the basis of these data, a possible mechanism of action of IFNα is the induction of apoptosis through the regulation of eIF-5A activity. In fact, we have already reported that IFNα induces growth inhibition and reduction of the activity of eIF-5A in human epidermoid cancer KB cells (*32*). The activity of eIF-5A was evaluated through the determination of hypusine levels, since this amino acid is essential for the function of this translational factor that is involved in the regulation of cell proliferation and transformation (*32*). The cell proliferation regulatory properties of eIF-5A could be correlated by its reported mRNA chaperon functions, since eIF-5A is involved in the transport of mRNAs from the nucleus to the cytoplasm (*33*). It has also been proposed that these mRNAs could encode for proteins involved in the regulation of cell proliferation (*1*). In the present study, we found that IFNα induced cell growth inhibition and apoptosis in human epidermoid cancer cells and that these effects were antagonized by EGF. We also found hyperactivation of the terminal enzymes of the EGF-dependent MAPK pathway, since increased activity of MEK-1 and ERK-1/2 was induced by IFNα in human epidermoid cancer cells. When EGF antagonized the growth inhibiton and apoptosis induced by IFNα, a further increase of MEK-1 and ERK-1/2 activity was recorded, suggesting a role of these enzymes in the protection of tumour cells from the antiproliferative stimuli caused by IFNα. In this regard, we have recently demonstrated that the abrogation of the EGF-mediated ras→ERK pathway enhances the apoptosis induced by IFNα in human epidermoid cancer cells (*34*). In the present study, we also showed that IFNα was able to induce strong inhibition of eIF-5A activity, since a reduction of hypusine synthesis was found with a parallel increase of eIF-5A protein expression that was recorded as early as 24 h after exposure to IFNα. This finding suggests a further reduction of the active fraction of eIF-5A (hypusine-containing eIF-5A:total eIF-5A ratio). On the other hand, when EGF antagonized the apoptosis induced by IFNα, a restoration of hypusine synthesis caused by the cytokine was recorded in cancer cells. In the same experimental conditions, we also found that PD098059, a specific inhibitor of MEK-1, reduced hypusine synthesis and enhanced the decrease of intracellular hypusine content caused by IFNα. Moreover, PD098059 was also able to antagonize the recovery of hypusine synthesis induced by EGF. Notably, PD098059 induced apoptosis and potentiated the programmed cell death induced by the cytokine (data not shown and *34*). The reduction of hypusine synthesis could be even higher if tumour cells treated with IFNα did not show an antiapoptotic response based on the hyperactivation of the MEK→ERK pathway. Therefore, the addition of PD098059 to IFNα-pretreated cells overcame this survival pathway, inducing a potentiation of both hypusine level reduction and apoptosis. On the other hand, the addition of EGF to IFNα-treated cells overstimulated this survival pathway, inducing a recovery of both hypusine levels and apoptosis. On the basis of these results, it could be hypothesized that the restoring effect on apoptosis and hypusine levels due to EGF could be mediated by the ERK-dependent pathway, even if the concurrence of other signalling molecules such as AKT/PKB can not be completely excluded. Interestingly, MEK-1 inhibitor potentiated the hypusine synthesis decrease induced by IFNα, suggesting again a protective role of the ERK-dependent pathway in the

reduction of hypusine synthesis induced by the cytokine. Moreover, these effects were parallelled by an enhancement of apoptosis in epidermoid cancer cells. On the basis of these results, we investigated if eIF-5A could be really critical for the biological effects induced by IFNα. We used the specific deoxyhypusine synthase inhibitor GC7, which prevents hypusine formation and thus blocks eIF-5A activity (*26*). This agent synergized with IFNα in inducing cell growth inhibition and apoptosis, suggesting a critical role for eIF-5A in the modulation of cell proliferation induced by IFNα in human epidermoid cancer cells. On the basis of these findings, we can hypothesize that the selective interference on eIF-5A activity could be a useful target in order to potentiate the antitumor efficacy of antineoplastic drugs. We have recently performed a computer-based prediction of the three-dimensional structure of eIF-5A in order to define the structure of the hypusine-containing site (*35*). We are now planning a pharmacological screening of drugs with potential eIF-5A-inhibiting properties.

The inhibition of eIF-5A either through the blocking of hypusine synthesis or the selective binding with the hypusine-containing site could represent a new scenario of intervention in anti-cancer therapy. Moreover, it must be considered that epidermoid cancer cells survey in host tissues through the activation of the EGF-dependent signalling (*36*). Therefore, the selective inhibition of a suspected target of this pathway could be an interesting strategy in the chemoprevention of human tumour.

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