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Apoptosis Induced by Interferon-α and Antagonized by EGF Is Regulated by Caspase-3-Mediated Cleavage of Gelsolin in Human Epidermoid Cancer Cells

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We have previously reported that interferon-α (IFNα) induces apoptosis and EGF can antagonize this effect in human epidermoid cancer KB cells. Since apoptosis occurs together with cytoskeleton reorganization we have evaluated if IFNα and EGF could modulate cell remodeling in our experimental conditions. We have found that 48 h 1,000 IU/ml IFNα induced structural reorganization of stress fibers and membrane delocalization and partial capping of the actin severing protein gelsolin. The transfection of KB cells with both a wild type (WT) or a C-terminal truncated form of gelsolin caused overexpression of the protein and an increase of both the spontaneous and IFNα-induced apoptosis and cell cytoskeletal modifications. In fact, after 48 h of treatment IFNα induced 45% of apoptotic cell death in parental cells while an approximately 80% of cell population was apoptotic in transfected cells. These effects occurred together with an increase of the expression and consequent degradation of gelsolin. Again the addition of EGF to IFNα-treated transfected cells caused a recovery of the apoptosis. Notably, IFNα and EGF did not modify the expression of other molecules associated to cytoskeleton such as focal adhesion kinase and vinculin. In the same experimental conditions IFNα induced also gelsolin cleavage that occurred together with caspase-3 activation and release of cytochrome c. All these effects were antagonized by the exposure of IFNα-treated KB to 10 nM EGF for the last 12 h. Moreover, the specific inhibition of caspase-3 with 20 μM DEVD completely abrogated apoptosis and gelsolin cleavage induced by IFNα. In conclusion, our data are the first demonstration that IFNα can induce morphological cell changes that are peculiar of apoptosis onset through the caspase-3-mediated cleavage of gelsolin. Furthermore, we have demonstrated that EGF is able to antagonize these effects through the inhibition of caspase-3 activation. J. Cell. Physiol. 201: 71–83, 2004.

Interferon-α (IFNα) is a biological agent widely used in the therapy of several neoplasms such as myeloma, renal cell carcinoma, epidermoid cervical and head and neck tumors, and melanoma (Gutterman, 1994; Vlock et al., 1996; Agarwala and Kirkwood, 1998). IFNα, the first cytokine to be produced by recombinant DNA technology, has emerged as an important regulator of cancer cell growth and differentiation, affecting cellular communication and signal transduction pathways, and is also produced during the immunological control of tumor growth (Gutterman, 1994).

The way by which tumor cell growth is suppressed by IFNα is not well known. A possibility is that cancer cells undergo apoptosis after the exposure to the cytokine. In fact, it is reported that IFNα induces apoptosis on human squamous cancer (Rodriguez-Villanueva and McDonnell, 1995; Caraglia et al., 1999), glioma (Roth et al., 1998; Tanaka et al., 1998), and virus-infected cells (Tilly et al., 1992). Therefore, it is likely that this cytokine acts, at least in part, through the triggering of programmed cell death.

On the other hand, EGF is able to protect eukaryotic cells from the onset of apoptosis (Tilly et al., 1992; Peng et al., 1998; Stoll et al., 1998; Sibilia et al., 2000).

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We have reported that IFNα increases the expression and function of the epidermal growth factor-receptor (EGF-R) at the surface of human epidermoid carcinoma cells (Budillon et al., 1991; Caraglia et al., 1995). Moreover, we have shown that IFNα induces apoptosis in these cells through the triggering of a stress response that leads to the activation of NH2-terminal Jun kinase-1 (JNK-1) (Caraglia et al., 1999). In the same experimental conditions, a specific hyperactivation of the EGF-mediated ras → Erk-1/2-dependent pathway occurs (Caraglia et al., 2003). We have, in fact, demonstrated that the inhibition of Ras activity induced by the transfection of the dominant negative Ras plasmid RASN17 and the inhibition of Mek-1 with PD098059 strongly potentiates the apoptosis induced by IFNα. Moreover, the selective inhibition of this pathway abrogates the counteracting effect of EGF on the IFNα-induced apoptosis (Caraglia et al., 2003). All these findings suggest that epidermoid tumor cells counteract the IFNα-induced apoptosis through a survival pathway which involves the hyperactivation of the EGF-dependent Ras → Erk signaling.

Recent studies have delineated two primary pathways leading to apoptosis. The first involves the interaction between cytokine signals, for example, tumor necrosis factor, and their receptors, which leads to activation of the initiator caspase-8 (Srinivasula et al., 1996). The second pathway is a stress response pathway in which release of mitochondrial cytochrome c leads to formation of the apoptosome complex in which caspase-9 is activated (Li et al., 1997). Both pathways then converge to activate the effector caspase-3, -2, and -7, which have multiple downstream targets, resulting in the morphological and nuclear changes of apoptosis (Kluck et al., 1997). Gelsolin was identified as a prominent substrate of caspase-3 in murine embryos in an in vitro translation assay system (Kothakota et al., 1997). Gelsolin is cleaved between residues Asp-352 and Gly-353, and the N-terminal gelsolin fragment contributes to the morphologic changes of apoptosis, demonstrated by the accelerated apoptosis of wild-type neutrophils in comparison with gelsolin null neutrophils. However, in Jurkat cells, a human T lymphocyte cell line, expression of intact gelsolin has been reported to inhibit apoptosis induced by any of several different stimuli, including both cytokine apoptosis inducers and stress response activators (Ohtsu et al., 1997). Moreover, the intact form of gelsolin inhibits apoptosis by blocking the mitochondrial membrane potential loss and cytochrome c release. On the other hand, its caspase-3-dependent fragmentation is coupled to apoptosis in vascular smooth muscle cells (Geng et al., 1998; Koya et al., 2000). Gelsolin is an 82-kD protein that has been shown to affect the length of actin polymers in vitro. Gelsolin severs actin filaments, nucleates actin polymerization, and blocks actin monomer exchange of the fast-growing (barbed) ends of actin filaments (Yin, 1987). Calcium ions, pH, and polyphosphoinositides regulate these activities in vitro (Janmey, 1994). Increased expression of gelsolin in fibroblasts, by stable transfection of the gelsolin cDNA, causes rapid cell movement in tissue culture (Cunningham et al., 1991). Moreover, adult skin fibroblasts from gelsolin null mice move slower and exhibit reduced membrane ruffling compared with wild-type fibroblasts (Witke et al., 1995). In addition, we demonstrated that truncation of the carboxyl-terminus, required for the regulation of gelsolin functions by the Ca^{2+} ion, resulted in the reduction of the suppressive effect of gelsolin on chemotaxis to fibronectin and metastasis (Fujita et al., 2001). This truncate served as a good control for evaluating the effects of full-length gelsolins because transfectants expressing C-del gelsolin metastasized to lung in mice similar to the neocontrols and parental B16-BL6 cells, even though they exhibited reduced colony-forming ability in soft agar assay. Furthermore, these results suggest that the C-terminus of gelsolin could have a critical role in the suppression of chemotactic cell migration and metastasis, but not for growth suppression and likely for apoptosis (Fujita et al., 2001).

In this article, we have investigated the molecular bases of the counteracting effects of IFNα and EGF on the apoptosis of human epidermoid cancer cells. In details, we have evaluated the involvement of caspase-3 activation and of the consequent gelsolin cleavage in the apoptosis induced by IFNα, and on the counteracting effect of EGF. Moreover, we have evaluated the cytoskeletal changes and the cellular distribution of gelsolin in human epidermoid cancer cells treated with EGF and/or IFNα. Finally, we have evaluated the effects of the overexpression of the carboxyl-terminus truncated (CTT) or wild type (WT) forms of gelsolin on the apoptosis and cytoskeleton changes induced by IFNα and antagonized by EGF in these tumor cells.

MATERIALS AND METHODS

Cell culture

The human oropharyngeal epidermoid carcinoma KB cell line, obtained from the American Type Tissue Culture Collection, Rockville, MD, was 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 grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Expression vectors and DNA transfection by electroporation

The expression vectors carrying mouse WT and His321mutant full-length gelsolin cDNAs were described previously (Mullauer et al., 1993). The CTT cDNA was created by PCR-mediated mutagenesis as described previously (Fujita et al., 1997) and subcloned into multicloning sites of LK444. Cells were detached from confluent 100-mm dishes. 100×10^6 cells were incubated in appropriate electroporation vials with 800 μl of electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose) and 15 μg of the WT or CTT form of gelsolin DNA and/or 5 μg/ml β-galactosidase DNA in 20 mM HEPES. Then cells were electroporated at 250 V and at 975 μF for 6 sec. Approximately 90% of the total cell population was viable after electroporation as evaluated with trypan blue dye. The cells were incubated at 37°C with or without 1,000 IU/ml IFNα for 48 h and/or 10 nM EGF for 12 h. An aliquot of cells was subjected to FACS analysis for the determination of β-galactosidase expression and, consequently, of transfection efficiency. In details, 48 h

cultured cells were fixed in methanol and then incubated with an anti-β-galactosidase MAb for 1 h at 4°C and labeled with a FITC anti-mouse rabbit antibody for 1 h. FACS analysis was performed as described below. Approximately 85% of the total electroporated cell population was transfected. After the incubation the cells were processed for FACS analysis or immunocytochemical experiments as described below.

Western blot analysis

KB cells were grown for 48 h with or without IFNα and were thereafter exposed for the indicated times to 10 nM EGF at 37°C. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped, and centrifuged for 30 min at 4°C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 NaCl, 1 mM EDTA, pH 7.5, 10 mM Na_2HPO_4, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, 0.025 U/ml aprotinin). Equal amounts of cell proteins were separated by SDS–PAGE. The proteins on the gels were electrotransferred to nitrocellulose and reacted with the different MAbs.

Mitochondrial extraction protocol

For the determination of cytochrome c release the cells were harvested by centrifugation at 600*g* for 10 min at 4°C. The pellets were washed once with ice-cold PBS and resuspended with the following buffer: 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT e 0.1 mM PMSF, 250 mM sucrose.

The cells were homogenized with 75–100 strokes of a dounce homogenizer, and the homogenates were centrifuged twice at 750*g* for 10 min at 4°C. The supernatants were centrifuged at 10,000*g* for 15 min at 4°C, the resulting mitochondrial pellets were discarded and the supernatants were stored at −20°C.

Internucleosomal DNA fragmentation (Ladder)

For all apoptosis evaluation experiments (gel ladder and FACS analysis) both attached and suspended cells were collected prior the processing. DNA fragmentation was measured after extraction of low molecular weight DNA. Briefly, 10×10^6 cells were resuspended in 900 μl 1× Tris-EDTA buffer and lysed with 25 μl 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.

Evaluation of apoptosis by DNA-flow cytometry

Cells were centrifuged and directly stained in a propidium iodide (PI) solution (50 μg 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) interfaced with a Hewlett Packard computer (mod.310) for data analysis. To evaluate cell apoptosis, PI fluorescence was collected as FL2 (log scale) by the CellFIT software (Becton Dickinson). For the evaluation of intracellular DNA content at least 10,000 events for each point were analyzed in at least three different experiments giving a SD less than 5%.

Determination of caspase activity

Cells were seeded and treated with 1,000 IU/ml IFNα for different times and/or 10 nM EGF for 12 h. At the time of caspase determination cells were lysed. For each reaction 50 μl of cell lysate were added to 1 ml reaction mixture composed by reaction buffer, DTT 10 mM and the specific substrate peptide (DEVD for caspase-3) conjugated to 7-amino-4-trifluoromethylcoumarin (AFC). The mixtures were incubated for 1 h at 37°C. The levels of free AFC were measured using a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength range of 480–520 nm (peak at 505 nm).

Cell cytoskeleton studies

Cells were plated on 35 × 10 mm tissue culture dishes with DMEM medium for 12 h, fixed, washed two times with PBS containing sodium azide, permeabilized with 0.1% Triton X-100 in PBS, and stained for F-actin with 2 μg/ml fluorescein isothiocyanate (FITC)-conjugated phalloidin (F-PHD; Sigma, Milan, Italy) and for gelsolin with 2 μg/ml FITC-conjugated anti-gelsolin MAb (Santa Cruz, Santa Cruz Biotechnology, Santa Cruz, CA) after being stimulated with IFNα and/or EGF.

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

Effects of IFNα and EGF on epidermoid cancer KB cell remodeling

We have previously reported that IFNα induces apoptosis and EGF can antagonize this effect in human epidermoid cancer KB cells (Caraglia et al., 1999; Caraglia et al., 2003). Apoptosis occurs together with cytoskeleton reorganization. Therefore, we have evaluated if IFNα could induce cell remodeling in our experimental conditions. We have studied actin cytoplasmic distribution with fluorescence microscopy after labeling of KB cells with a FITC-conjugated phalloidin. Moreover, we have again evaluated if EGF could antagonize such effects. The incubation of KB cells with 1,000 IU/ml IFNα for 48 h induced shape changes and modified the normal distribution of actin-containing stress fibers (Fig. 1B) (780 ± 60 vs. 35 ± 4, Table 1). After exposure of KB cells to IFNα cells showed a rounded shape and disruption of the actin cytoskeleton characterized by the depolymerization of actin fibers that assumed a peripheral and spotted cytoplasmic distribution that caused cell rounding but preserved attachment (Fig. 1B). Incubation with 10 nM EGF for 12 h did not cause relevant modifications (Fig. 1C, 38 ± 4, Table 1) but the concomitant exposure of KB cells to IFNα for 48 h and to EGF for the last 12 h prevented the changes in cell shape or cytoskeleton organization induced by IFNα

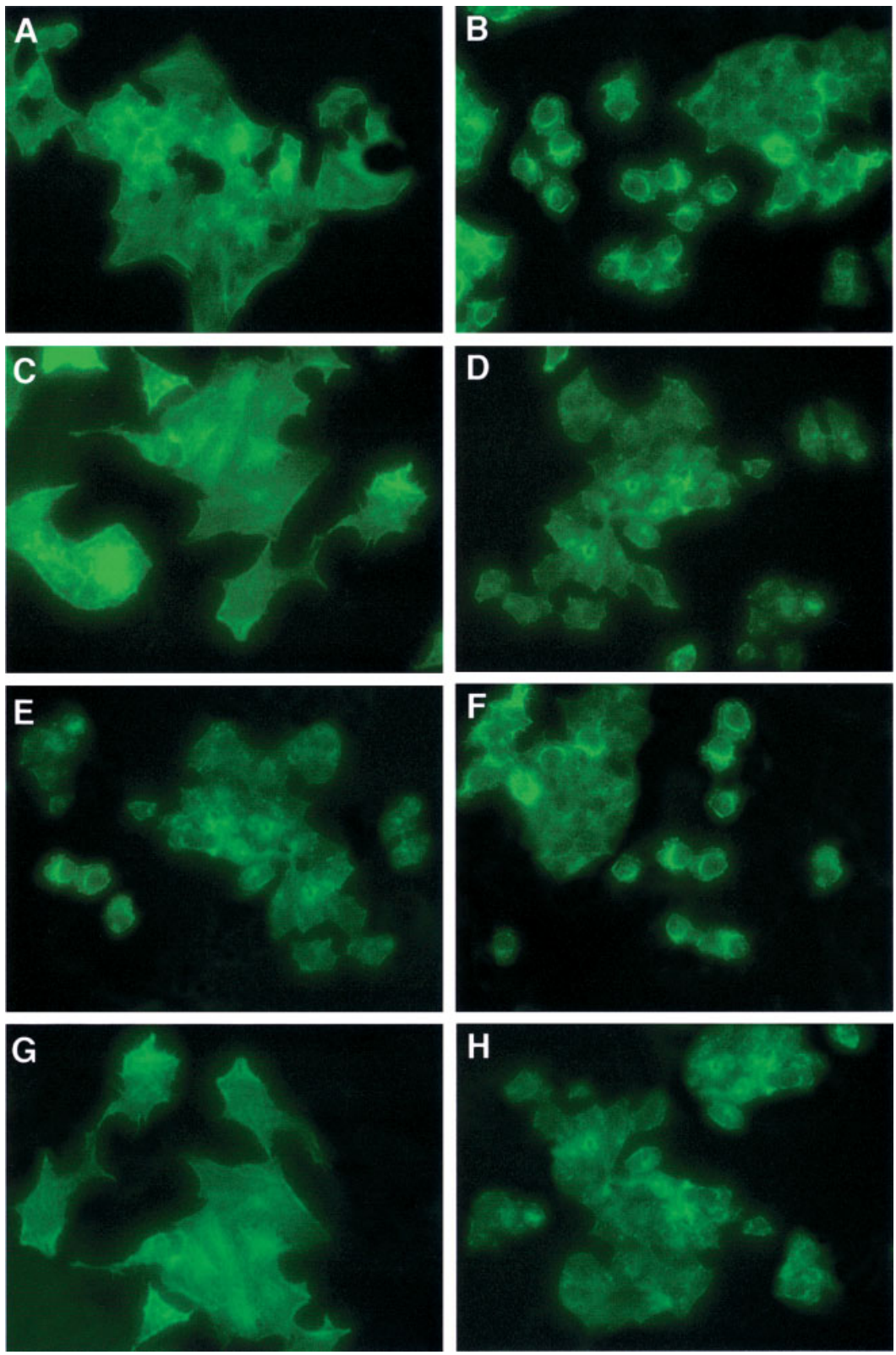

Fig. 1. Effects of IFNα and EGF on intracellular actin fibers distribution. KB cells were treated with IFNα and EGF and actin distribution was visualized with fluorescence microscope as described in "Materials and Methods." **A**: Untreated parental KB cells; (**B**) 48 h 1,000 IU/ml IFNα-treated parental KB cells; (**C**) 12 h 10 nM EGF-treated parental KB cells; (**D**) 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated parental KB cells (**E**) Untreated WT gelsolin transfected KB cells; (**F**) 48 h 1,000 IU/ml IFNα-treated WT gelsolin transfected KB cells; (**G**) 12 h 10 nM EGF-treated WT gelsolin transfected KB cells; (**H**) 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated WT gelsolin transfected KB cells. The experiment is representative of at least three different experiments that gave always similar results.

TABLE 1. Number of KB cells showing modification of cell shape and gelsolin distribution*

	Parental cells				WT gelsolin transfected cells				CTT gelsolin transfected cells			
Treatment	CTR	IFNα	EGF	IFNα + EGF	CTR	IFNα	EGF	IFNα + EGF	CTR	IFNα	EGF	IFNα + EGF
Cell shape change	35 ± 4	780 ± 60	38 ± 4	220 ± 21	590 ± 45	930 ± 78	206 ± 19	370 ± 39	480 ± 40	890 ± 78	195 ± 18	348 ± 36
Gelsolin polarization	15 ± 2	820 ± 77	20 ± 3	420 ± 38	500 ± 37	900 ± 85	192 ± 23	410 ± 45	480 ± 30	850 ± 80	200 ± 23	390 ± 42

*Data are means ± SD from three independent experiments (1,000 cells for each experiment were examined).
CTR, untreated cells; IFNα, 1,000 IU/ml 48 h; EGF, 10 nM 12 h; IFNα + EGF, 48 h 1,000 IU/ml IFNα; and 12 h 10 nM EGF.

(Fig. 1D, 220 ± 21, Table 1). The transfection of KB cells with WT gelsolin determined per se the reorganization of actin fibers that was enhanced by the treatment of transfected cells with 1,000 IU/ml IFNα for 48 h (Fig. 1E and F, respectively, 590 ± 45 and 930 ± 78). EGF was again able to antagonize the effects induced by the transfection and the treatment with the cytokine (Fig. 1G and H, respectively, 206 ± 19 and 370 ± 39). Similar results were obtained with the transfection of KB cells with the CTC form of gelsolin (data not shown, for a quantification of the data see Table 1). Therefore, IFNα induced cell shape remodeling through actin fiber reorganization and EGF was able to antagonize this effect. Moreover, the overexpression of either the WT or CTT form of gelsolin was able to induce per se cytoskeletal reorganization and to enhance that one induced by IFNα not appearing dependent from the C-terminal domain of gelsolin.

Effects of IFNα and EGF on intracellular gelsolin distribution in epidermoid cancer KB cells

Gelsolin severs actin filaments, nucleates actin polymerization, and blocks actin monomer exchange of the fast-growing (barbed) ends of actin filaments and is involved in cytoskeletal organization. Therefore, we have investigated on the changes of intracellular gelsolin distribution induced by IFNα and EGF. We have found that 48 h 1,000 IU/ml IFNα induced polarization and increased localization of gelsolin on the cell membrane resulting in several capping features (Fig. 2B). These modifications are in agreement with the actin reorganization since this kind of distribution allows the disaggregation of actin polymers and favors the round cell shape. The exposure of KB cells to EGF did not cause significant changes (Fig. 2C), but again antagonize the effects induced by IFNα (Fig. 2D). The transfection of KB cells with the WT form of gelsolin induced per se an increase of the fluorescent signal (dependent from gelsolin overexpression) and the finding of membrane capping features that were furtherly increased by IFNα addition (Fig. 2E and F, respectively). The exposure of WT gelsolin transfected KB cells to EGF partially antagonized the morphological changes induced by both the transfection and IFNα treatment (Fig. 2G and H, respectively). Similar data were observed in CTC transfected KB cells exposed to IFNα and/EGF (Fig. 3A–D, for a quantification of the data see Table 1). These data suggest that both IFNα-induced apoptosis and cell cytoskeletal reorganization occur together with changes in intracellular gelsolin localization that did not depend upon C-terminal region of gelsolin.

Effects of IFNα and EGF on gelsolin cleavage in epidermoid cancer KB cells

Intact gelsolin has a protective role in the apoptotic events while the cleaved N-terminal fragment induces apoptosis in some experimental models. Therefore, we have evaluated in our experimental conditions whether apoptosis induced by IFNα occurred together with gelsolin cleavage and if EGF could overcome this effect. We have found that IFNα induced an about threefold increase of the 41 KDa gelsolin immuno-specific band with the consequent decrease of the intact form (Fig. 4A). EGF again did not modify the expression of gelsolin while it antagonized the cleavage of gelsolin induced by IFNα (Fig. 4A). We have, moreover, found that gelsolin cleavage was time-dependent reaching a peak after 72 h of exposure of KB cells to 1,000 IU/ml IFNα (Fig. 4B). In fact, at 72 h we have recorded an about fourfold increase of the fragmented gelsolin with a corresponding twofold decrease of the intact form. More prolonged exposure times induced a decrease of gelsolin cleavage accordingly to the timing of apoptosis occurrence in KB cells exposed to IFNα (data not shown). The decrease of the 41 kD fragment after 72 h of IFNα treatment is in agreement with our previous data on the timing of apoptosis in our experimental system (see Caraglia et al., 2003 and Esposito et al., 2003). In fact, apoptosis reaches a peak after 72 h of treatment with IFNα and then it decreases. Therefore, the maximal expression of the pro-apoptotic fragment occurs when the maximal apoptotic effect is recorded. The transfection of KB cells with both WT and CTT gelsolin induced an increase of gelsolin expression after 48 h from the transfection, while the treatment with IFNα induced a decrease of the intact form with a consequent increase of its fragmentation antagonized by exposure to EGF (Fig. 4C). Interestingly, the exposure of transfected cells to EGF alone did not induce any change in the gelsolin expression while treatment with IFNα + EGF reduced the amount of 41 kD fragment below the background level observed in the control lanes (Fig. 4C). The effects of EGF on the turn-over of gelsolin (in WT or CTT gelsolin overexpressing cells) in IFNα-pretreated cells are not known. Therefore, we cannot exclude that EGF increases the degradation of the N-terminal fragment of gelsolin only in IFNα-treated cells and not in control cells accounting for the reduced levels of the fragment only in IFNα-pretreated KB cells. In fact, it is known the IFNα can differentially affect the ubiquitination and the consequent degradation of intracellular proteins, thus regulating cell proliferation (Esposito et al., 2003). As a consequence, it is not surprising that the 41 kD

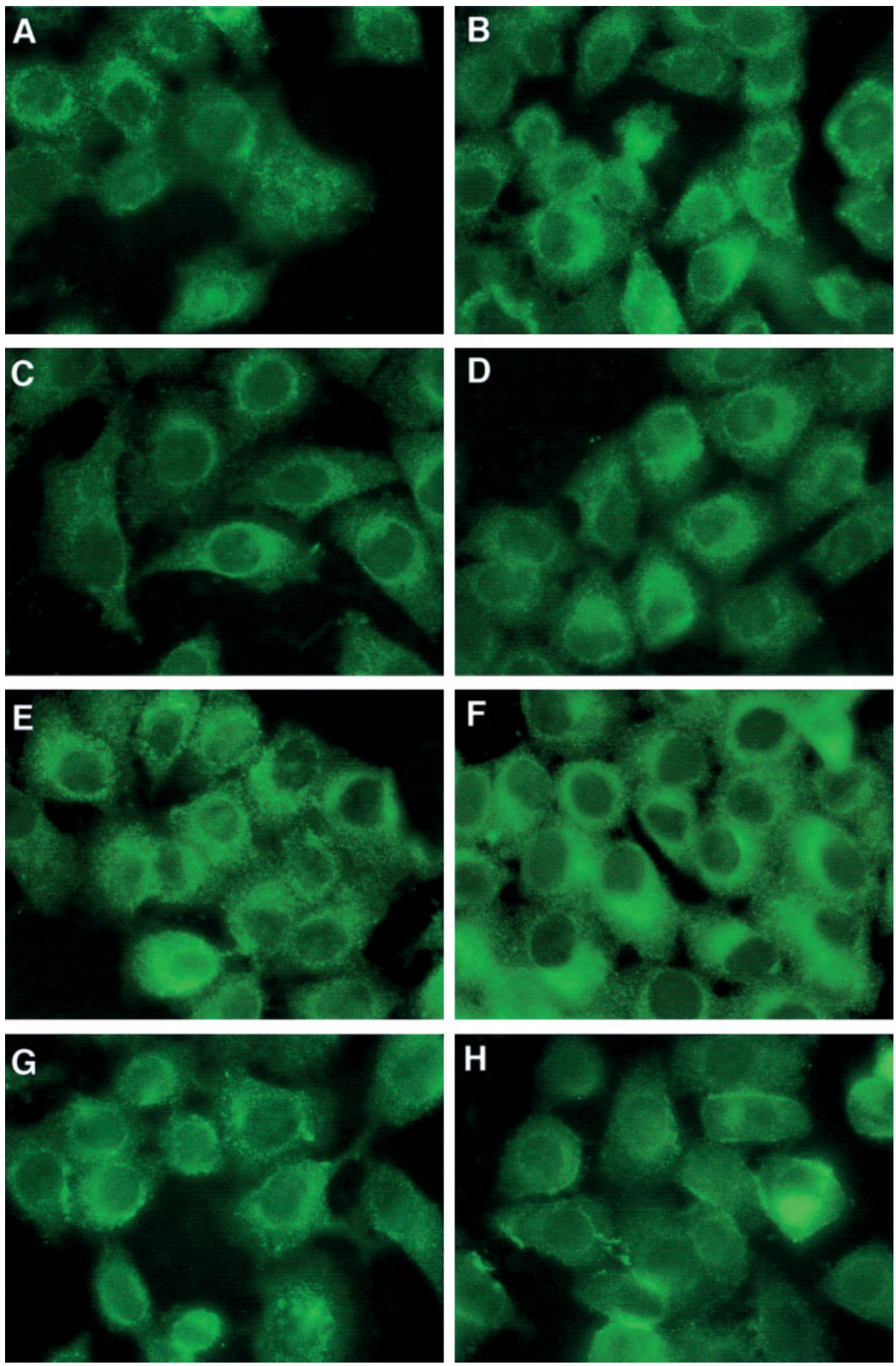

Fig. 2. Effects of IFNα and EGF on intracellular gelsolin distribution after transfection of WT gelsolin in epidermoid cancer KB cells. KB cells were transfected with WT gelsolin and/or treated with IFNα and EGF and gelsolin distribution was visualized with fluorescence microscope as described in "Materials and Methods." Gelsolin is shown as green fluorescence. **A**: Untreated KB parental KB cells; (**B**) 48 h 1,000 IU/ml IFNα-treated parental KB cells; (**C**) 12 h 10 nM EGF-treated parental KB cells; (**D**) 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated parental KB cells; (**E**) Untreated KB WT gelsolin transfected KB cells; (**F**) 48 h 1,000 IU/ml IFNα-treated WT gelsolin transfected KB cells; (**G**) 12 h 10 nM EGF-treated WT gelsolin transfected KB cells; (**H**) 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated WT gelsolin transfected KB cells. The experiment is representative of at least three different experiments that gave always similar results.

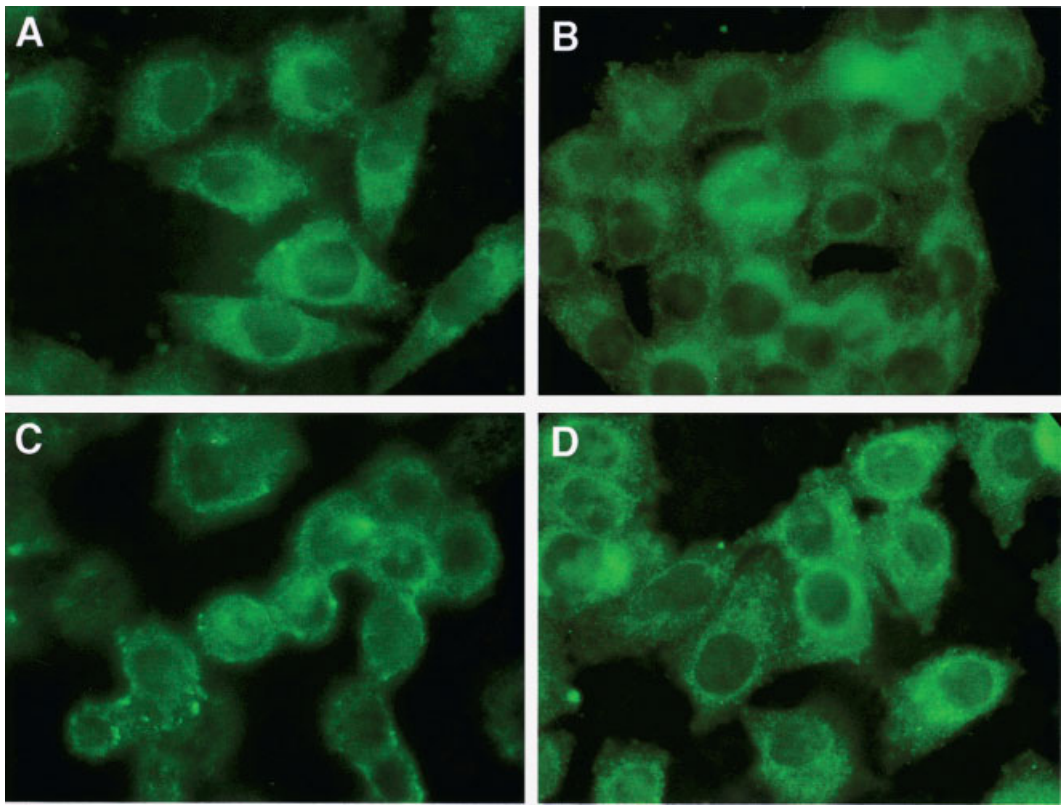

Fig. 3. Effects of IFNα and EGF on intracellular gelsolin distribution after the transfection with CTT gelsolin in epidermoid cancer KB cells. KB cells were treated with IFNα and EGF and gelsolin distribution was visualized with fluorescence microscope as described in "Materials and Methods." Gelsolin is shown as green fluorescence. **A**: Untreated KB CTT gelsolin transfected KB cells; (**B**) 48 h 1,000 IU/ml IFNα-treated CTT gelsolin transfected KB cells; (**C**) 12 h 10 nM EGF-treated CTT gelsolin transfected KB cells; (**D**) 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated CTT gelsolin transfected KB cells. The experiment is representative of at least three different experiments that gave always similar results.

fragment is significantly different in the EGF, and IFNα + EGF-treated cells. On the other hand, the treatment of KB cells with IFNα and/or EGF and the transfection with both WT and CTT gelsolin forms had no effects on the expression of other two cytoskeleton proteins, focal adhesion kinase, and vinculin (Fig. 4E and F, respectively). These data suggest a role of gelsolin and, consequently, of its fragmentation in the regulation of apoptosis induced by IFNα in KB cells again not depending upon the C terminal domain of the protein.

Effects of IFNα and EGF on caspase-3 activation and cytochrome c release in epidermoid cancer KB cells

Since IFNα induced a stress response paralleled by apoptosis in KB cells we have evaluated if such effect occurred together with caspase 3 activation mediated by mitochondrial changes. We have found that IFNα induced an increased activity of caspase-3 that reached a peak after 48 h of exposure of KB cells to the cytokine as evaluated with a fluorimetric assay (Fig. 5A). At 72 h of treatment with IFNα caspase-3 activity resembled that one of untreated control. The timing of the decrease of the caspase 3 activity is in agreement with our previously published data (see Caraglia et al., 2003). Moreover, the activity of caspase 3 was maximal after 48 h of IFNα treatment and, at this time, the maximal gelsolin degradation occurred. This effect, likely induced the peak of the 14 Kda gelsolin fragment expression recorded after 72 h from the beginning of IFNα treatment when the maximal apoptotic effect was recorded. EGF alone again did not induce any modification of the activity of the enzyme while it antagonized the effect of IFNα (Fig. 5A). These effects were paralleled by the fragmentation of caspase-3 induced by IFNα and by another activator of apoptosis (etoposide) as evaluated with a Western blot assay (Fig. 5B). The caspase-3 fragmentation induced by IFNα was again antagonized by EGF that was added in the last 12 of 48 h IFNα treatment (Fig. 5B). We have also found that IFNα induced a cytoplasmic release of cytochrome c that began 12 h after the exposure to the cytokine and reached the maximal level at 24 h of treatment of KB cells with IFNα (Fig. 5C). More prolonged exposure times did not induce a further change in cytochrome c release (Fig. 5C). These data support the hypothesis that the IFNα-induced apoptosis occurred through the mitochondrial-mediated activation of caspase-3.

Effects of the overexpression of WT and CTT gelsolin on the apoptosis induced by IFNα and antagonized by EGF

We have transfected KB cells with the WT form of gelsolin and we have evaluated the apoptosis occurrence in these cells with or without the addition of IFNα and/or EGF. We have chosen to treat the cells with IFNα for

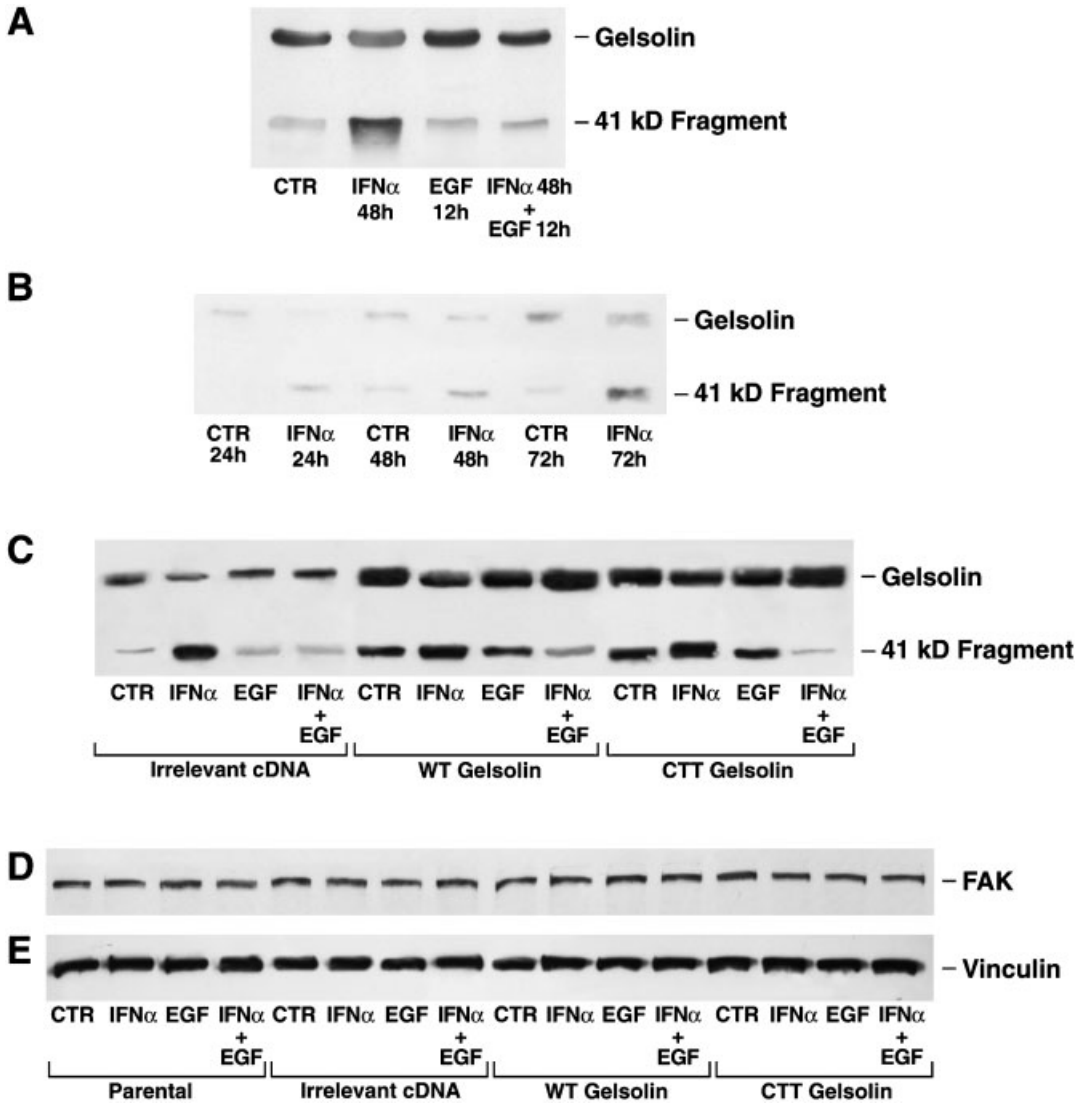

Fig. 4. Effects of IFNα and EGF on gelsolin fragmentation in epidermoid cancer KB cells. **A**: KB cells were seeded and treated with IFNα and/or EGF. After treatment cells were processed for the immunodetection of gelsolin with Western blotting as described in "Materials and Methods." CTR, Untreated cells; IFNα 48 h, 48 h 1,000 IU/ml IFNα-treated cells; EGF 12 h, 12 h 10 nM EGF-treated cells; IFNα 48 h + EGF 12 h, 48 h 1,000 IU/ml IFNα- and 10 nM EGF-treated cells. The experiment is representative of at least three different experiments that gave always similar results. **B**: KB cells were seeded and treated for the indicated times with IFNα. After treatment cells were processed for the immunodetection of gelsolin with Western blotting as described in "Materials and Methods." CTR 24 h, 24 h untreated cells; IFNα 24 h, 24 h 1,000 IU/ml IFNα-treated cells; CTR 48 h, 48 h untreated cells; IFNα 48 h, 48 h 1,000 IU/ml IFNα-treated cells; CTR 72 h, 72 h untreated cells; IFNα 72 h, 72 h 1,000 IU/ml IFNα-treated cells. The experiment is representative of at least three different experiments that gave always similar results. **C**: KB cells were transfected with an irrelevant cDNA or WT gelsolin or CTT gelsolin, seeded and treated with IFNα and/or EGF. After treatment cells were processed for the immunodetection of gelsolin with Western blotting as described in "Materials and Methods." CTR, Untreated cells; IFNα, 48 h 1,000 IU/ml IFNα-treated cells; EGF, 12 h 10 nM EGF-treated cells; IFNα + EGF, 48 h 1,000 IU/ml IFNα- and 10 nM EGF-treated cells. The experiment is representative of at least three different experiments that gave always similar results. Gelsolin was always visible as the intact form of 82 KDa and as the N-terminal fragment of 41 KDa. **D** and **E**: KB cells were transfected or not with an irrelevant cDNA or WT gelsolin or CTT gelsolin, seeded and treated with IFNα and/or EGF. After treatment cells were processed for the immunodetection of FAK (D) or vinculin (E) with Western blotting as described in "Materials and Methods." CTR, Untreated cells; IFNα, 48 h 1,000 IU/ml IFNα-treated cells; EGF, 12 h 10 nM EGF-treated cells; IFNα + EGF, 48 h 1,000 IU/ml IFNα- and 10 nM EGF-treated cells. The experiment is representative of at least three different experiments that gave always similar results.

48 h because the maximal expression of gelsolin in transfected cells occurred at this time point (data not shown). We have found that the transfection of an irrelevant cDNA did not cause any significant change in apoptosis and that when these cells were treated with 1,000 IU/ml IFNα an approximately 50% apoptosis was recorded (Fig. 6A–C). The treatment of these cells with EGF alone caused an approximately 15% apoptosis and it was able to completely antagonize the programmed cell death when added to IFNα-treated KB cells (Fig. 6D and E, respectively). We have found that the transfection alone with WT gelsolin is able to induce an approximately 30% of apoptosis as evaluated with FACS analysis after labeling with PI (Fig. 6F). The exposure of

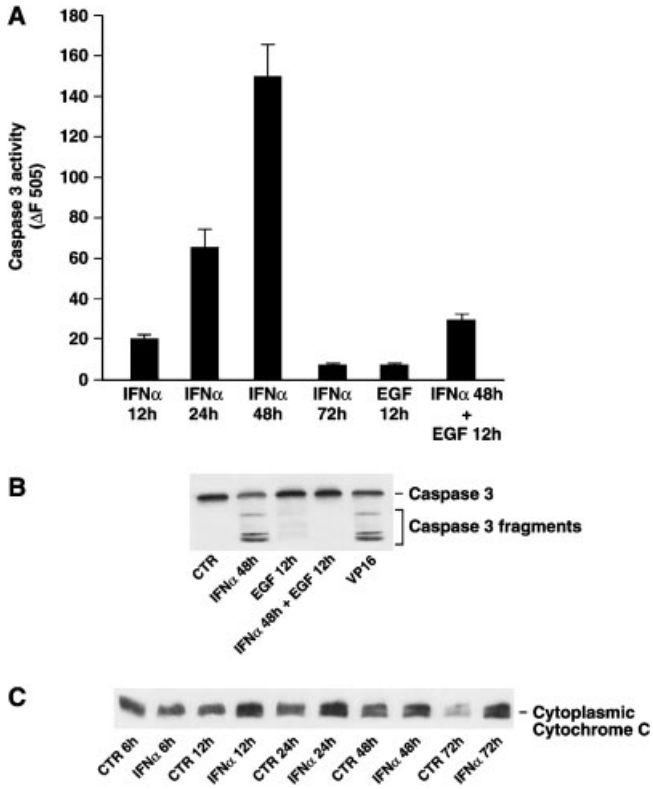

Fig. 5. Effects of IFNα and EGF on caspase-3 activation and cytochrome c release in epidermoid cancer KB cells. **A**: KB cells were seeded and treated with 1,000 IU/ml IFNα and/or 10 nM EGF for the indicated times. The specific caspase activity was determined with a fluorimetric assay as described in "Materials and Methods." The data are expressed as ΔF at 505 nM of emission wave length obtained subtracting the fluorescence emitted by an untreated control. IFNα 12 h, 12 h 1,000 IU/ml IFNα-treated cells; IFNα 24 h, 24 h 1,000 IU/ml IFNα-treated cells; IFNα 48 h, 48 h 1,000 IU/ml IFNα-treated cells; IFNα 72 h, 72 h 1,000 IU/ml IFNα-treated cells; EGF 12 h, 12 h 10 nM EGF-treated cells; IFNα 48 h + EGF 12 h, 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated cells. The experiment was performed three times and SDs were always less than 10%. Bars, SD. **B**: KB cells were seeded and treated with IFNα and/or EGF. After treatment cells were processed for the immunodetection of caspase-3 with Western blotting as described in "Materials and Methods." CTR, Untreated cells; IFNα 48 h, 48 h 1,000 IU/ml IFNα-treated cells; EGF 12 h, 12 h 10 nM EGF-treated cells; IFNα 48 h + EGF 12 h, 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated cells; VP16, 12 h 80 μM etoposide-treated cells. The presence of fragmented caspase-3 was a sign of enzyme activation. The experiment is representative of at least three different experiments that gave always similar results. **C**: KB cells were seeded and treated with 1,000 IU/ml IFNα for the indicated times. After treatment cells were processed, the cytoplasmatic fractions were extracted and immunodetection for cytochrome c was performed with Western blotting as described in "Materials and Methods." CTR 6 h, 6 h untreated cells; IFNα 6 h, 6 h 1,000 IU/ml IFNα-treated cells; CTR 12 h, 12 h untreated cells; IFNα 12 h, 12 h 1,000 IU/ml IFNα-treated cells; CTR 24 h, 24 h untreated cells; IFNα 24 h, 24 h 1,000 IU/ml IFNα-treated cells; CTR 48 h, 48 h untreated cells; IFNα 48 h, 48 h 1,000 IU/ml IFNα-treated cells; CTR 72 h, 72 h untreated cells; IFNα 72 h, 72 h 1,000 IU/ml IFNα-treated cells. The experiment is representative of at least three different experiments that gave always similar results.

transfected cells to 1,000 IU/ml IFNα for 48 h caused apoptosis in approximately 80% of cell population (Fig. 6G). The addition of 10 nM EGF for 12 h to untreated transfected cells almost completely abolished apoptosis while it significantly reduced apoptosis (30%) in IFNα-pretreated transfected cells (Fig. 6H and I, respectively). Interestingly, the transfection of KB cells with a mutant form of gelsolin, containing a deletion of the C-terminal fragment, gave similar results. In fact, the transfection alone with the CTT gelsolin caused an approximately 32% of apoptosis and the treatment of these cells with IFNα determined a 85% of apoptosis (Fig. 6J). Also, in this case the addition of 10 nM EGF had recovering effects in both untreated and IFNα-treated transfected KB cells (Fig. 6J). Therefore, the transfection of KB cells with the WT or CTT gelsolin determined per se a slight increase of apoptotic events and enhanced the apoptosis induced by IFNα. Moreover, the anti-apoptotic effects of EGF could be, at least in part, based on the antifragmentation effects on gelsolin.

Effects of caspase-3 inhibition on apoptosis and gelsolin cleavage in human epidermoid KB cells

We have studied if the specific inhibition of caspase-3 activity could prevent apoptosis and gelsolin cleavage. We have indeed found that the exposure of KB cells to the caspase-3 inhibitor DEVD 2 h before the beginning of the treatment with IFNα completely antagonized the apoptosis induced by the cytokine as suggested by the inhibition of DNA fragmentation induced by IFNα (Fig. 7A). In fact, IFNα alone induced approximately 45% apoptosis while the caspase-3 inhibitor DEVD had no effect on programmed cell death as demonstrated with FACS analysis after PI labeling (Fig. 7C and D, respectively). However, when KB cells were pretreated with DEVD 2 h before the exposure to IFNα the apoptosis was completely prevented (Fig. 7E). At the same time the specific inhibition of caspase-3 completely antagonized also the cleavage of gelsolin induced by IFNα (Fig. 7F). The exposure of KB cells to DEVD really antagonized the activation of caspase-3 by IFNα as demonstrated with a fluorimentric assay (Fig. 7G). These data suggest that the apoptosis and gelsolin cleavage were two events mediated by the activation of caspase-3 enzyme activity.

DISCUSSION

We have previously demonstrated that IFNα induces an increased expression and activity of EGF-R in human epidermoid cancer cells and these effects are paralleled by the induction of apoptosis that can be antagonized by the exposure of KB cells to EGF (Caraglia et al., 1995; Caraglia et al., 1999; Caraglia et al., 2003). The exposure of KB cells to IFNα causes also the activation of a stress pathway that has as downstream effectors JNK-1 and p38 kinase (Caraglia et al., 1999); these effects are again antagonized by EGF (Caraglia et al., 1999). On the basis of these findings we have speculated that the increased activity and expression of EGF-R could be part of a protective response of cancer cells to antiproliferative stimuli induced by IFNα. In this article, we have evaluated the effects of IFNα and of EGF on cytoskeletal components that are involved in the execution of the apoptotic stimuli in order to understand the mechanism of the counteracting effects of the two agents. We have found that IFNα induced a reorganization of the cytoskeletal architecture and intracellular actin fiber distribution that is compatible with the triggering of

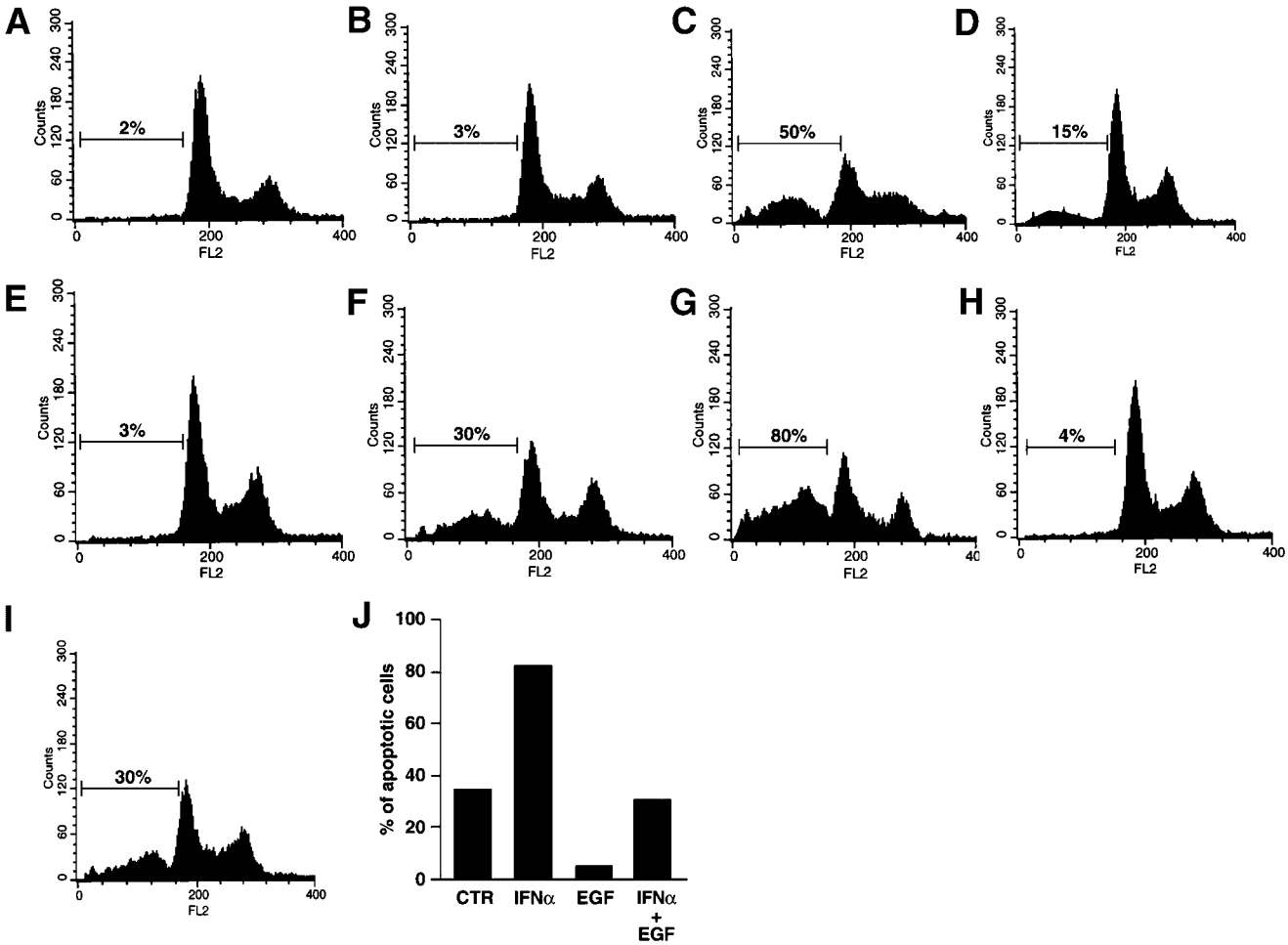

Fig. 6. Effects of the overexpression of the WT or CTT form of gelsolin on the apoptosis induced by IFNα and antagonized by EGF in human KB cells. The cells were electroporated or not with an irrelevant cDNA or with the WT form of gelsolin, seeded and treated with 1,000 IU/ml IFNα and/or 10 nM EGF as described above. At the time of the experiment KB cells were FACS analyzed after detachment and DNA labeling with propidium iodide (PI) as described in "Materials and Methods." The experiments were performed at least three different times and the results gave always less than 5% SD. In each experimental point 10,000 events were analyzed. **A**: Untreated KB cells; (**B**) Untreated KB irrelevant cDNA-transfected KB cells; (**C**) 48 h 1,000 IU/ml IFNα-treated irrelevant cDNA-transfected KB cells; (**D**) 12 h 10 nM EGF-treated irrelevant cDNA-transfected KB cells; (**E**) 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated irrelevant cDNA-transfected KB cells; (**F**) Untreated KB WT gelsolin transfected KB cells; (**G**) 48 h 1,000 IU/ml IFNα-treated WT gelsolin transfected KB cells; (**H**) 12 h 10 nM EGF-treated WT gelsolin transfected KB cells; (**I**) 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated WT gelsolin transfected KB cells; (**J**) Effects of CTT gelsolin transfection on the apoptosis induced by IFNα and antagonized by EGF. CTR, Untreated KB CTT gelsolin transfected KB cells; IFNα, 48 h 1,000 IU/ml IFNα-treated CTT gelsolin transfected KB cells; EGF, 12 h 10 nM EGF-treated CTT gelsolin transfected KB cells; IFNα + EGF, 48 h 1,000 IU/ml IFNα- and 12 h 10 nM EGF-treated CTT gelsolin transfected KB cells.

Fig. 7. Effects of caspase-3 inhibition on apoptosis and gelsolin fragmentation in human epidermoid KB cells. (**A**–**D**) FACS analysis after PI labeling of KB cells treated with IFNα and/or 20 μM DEVD as described in "Materials and Methods." (**A**) Untreated cells; (**B**) 48 h 1,000 IU/ml IFNα-treated cells; (**C**) 48 h 20 μM DEVD-treated cells; (**D**) 48 h 1,000 IU/ml IFNα- and 48 h 20 μM DEVD-treated cells. Arrows show the apoptotic peak with the relative % of apoptotic events. The experiment is representative of at least three different experiments that gave always similar results. In each experiment at least 10,000 events were analyzed and the interexperimental SD was always less than 5%. **E**: KB cells were seeded and treated with IFNα and/or 20 μM DEVD (caspase-3 inhibitor) and the internucleosomic DNA fragmentation was assessed as described in "Materials and Methods." CTR 48 h, 48 h untreated cells; IFNα 48 h, 48 h 1,000 IU/ml IFNα-treated cells; DEVD, 48 h 20 μM DEVD-treated cells; IFNα 48 h + DEVD, 48 h 1,000 IU/ml IFNα- and 48 h 20 μM DEVD-treated cells; MW, molecular weights. The experiment is representative of at least three different experiments that gave always similar results. **F**: KB cells were seeded and treated with IFNα and/or DEVD. After treatment cells were processed for the immunodetection of gelsolin with Western blotting as described in "Materials and Methods." CTR, 48 h untreated cells; IFNα, 48 h 1,000 IU/ml IFNα-treated cells; DEVD, 48 h 20 μM DEVD-treated cells; IFNα + DEVD, 48 h 1,000 IU/ml IFNα- and 48 h 20 μM DEVD-treated cells. The experiment was performed three times and the results were always similar. (**G**) KB cells were seeded and treated with IFNα and/or 20 μM DEVD. The specific caspase activity was determined with a fluorimetric assay as described in "Materials and Methods." The data are expressed as ΔF at 505 nM of emission wave length obtained subtracting the fluorescence emitted by an untreated control. CTR, 48 h untreated cells; IFNα, 48 h 1,000 IU/ml IFNα-treated cells; DEVD, 48 h 20 μM DEVD-treated cells; IFNα + DEVD, 48 h 1,000 IU/ml IFNα- and 48 h 20 μM DEVD-treated cells. The experiment was performed three times and SDs were always less than 10%. Bars, SD.

apoptosis and these effects were antagonized by EGF. Actin filament formation and polymerization are regulated by gelsolin that is an 82-kD protein that has been shown to be also involved in the regulation of apoptosis. In fact, gelsolin is cleaved by caspase-3 between residues Asp-352 and Gly-353, and the N-terminal gelsolin fragment contributes to the morphologic changes of apoptosis while intact protein has a protective role (Geng et al., 1998; Koya et al., 2000). We have also demonstrated that the transfection of both WT and CTT

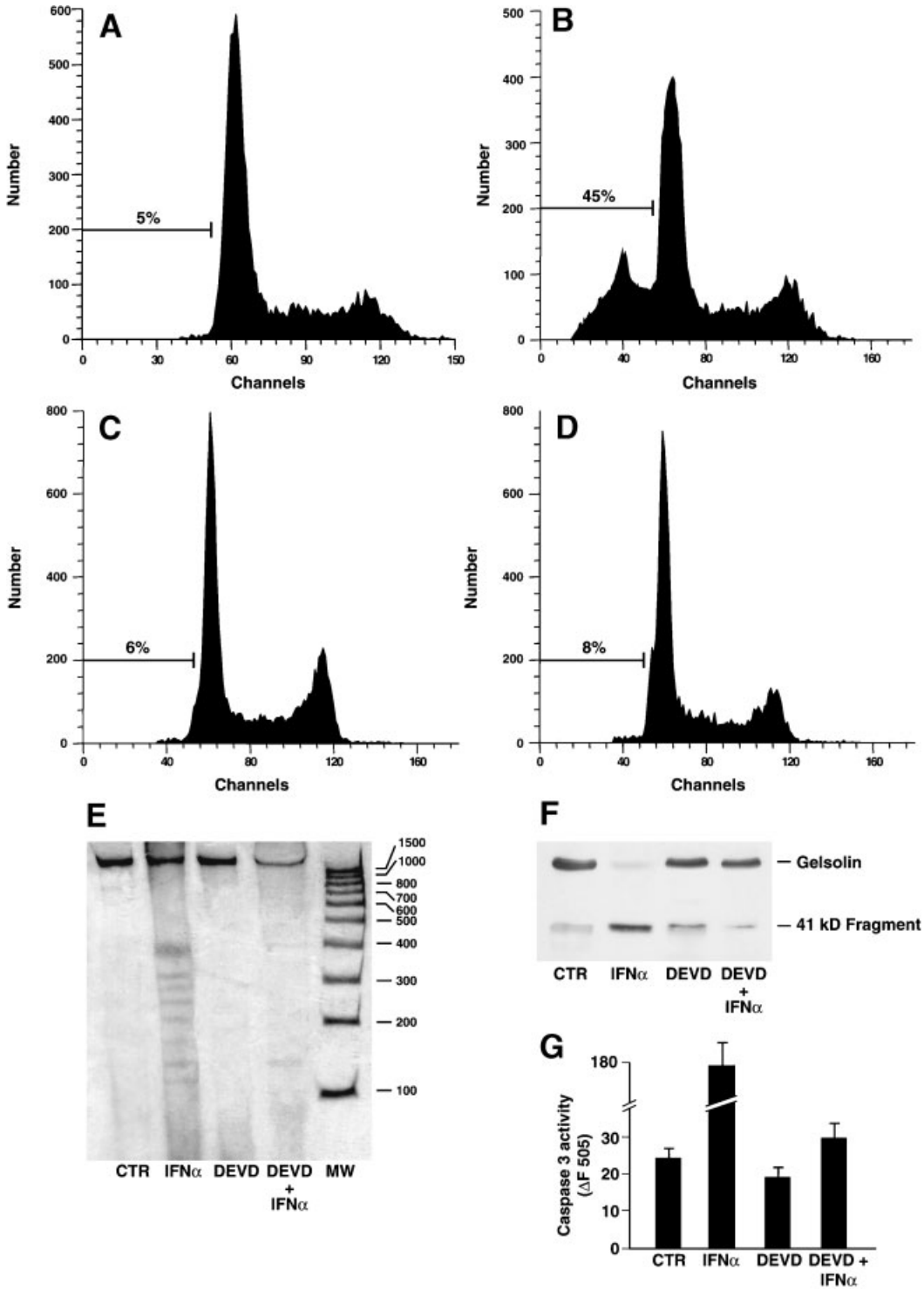

Fig. 7.

forms of gelsolin were able to induce similar cell shape changes compatible with apoptosis occurrence and that EGF was still able to antagonize these effects. We have demonstrated that the structural reorganization of cytoskeleton induced by exposure to IFNα or transfection with either WT or CTT gelsolin occurred together with intracellular polarization and membrane capping of gelsolin that appeared also fragmented in IFNα-treated cells. These effects were paralleled by caspase-3 activation and cytochrome c release from mithocondria and were completely antagonized by EGF. The transfection of KB cells with the WT or the CTT form of gelsolin induced per se apoptosis confirming the involvement of the protein in the regulation of apoptotic program in KB cells. Moreover, the transfection of both proteins sensitized the cancer cells to apoptosis induced by the cytokine. EGF was again able to antagonize these effects in these experimental conditions. The effects of gelsolin overexpression on cytoskeleton and apoptosis were independent from the presence of the C terminal domain of the protein. In this regard, it has been demonstrated that the N-terminal fragment is essential for the regulation of apoptosis by gelsolin and that the C-terminal domain is involved in the regulation of cell motility and metastatization (Ohtsu et al., 1997; Fujita et al., 2001). Therefore, our data suggest that gelsolin, and specifically its N-terminal fragment, could be indeed involved in the regulation of apoptotic program (Fujita et al., 2001) and the changes in gelsolin expression and intracellular localization were not simply secondary to the morphological modifications occurring during apoptotic program. Moreover, the changes of gelsolin expression and its fragmentation induced by IFNα and EGF were specific since other cytoskeleton proteins such as vinculin and FAK were not modulated neither by IFNα or EGF in the same experimental conditions. The increased cytochrome c release in the cytoplasm supports the hypothesis that the apoptotic effects induced by IFNα could be mediated by a stress response and not by the activation of a specific death pathway. Moreover, we have found that the specific inhibition of the caspase-3 activation can prevent both apoptosis and gelsolin cleavage mediated by IFNα. The latter effect suggests a link between IFNα, caspase-3 activation and gelsolin cleavage with the subsequent cytoskeletal reorganization. Our data suggest that the effects induced by IFNα on cell morphology are mediated by the caspase-3 dependent cleavage of gelsolin and that the counteracting effect of EGF could occur through the disruption of this pathway antagonizing caspase-3 activation. In fact, it has been reported that EGF-mediated signal transduction pathway can lead to the activation of the anti-apoptotic serine-threonine kinase Akt/PKB that can cause the inhibition of the activity of caspase-9 and contributes to the preservation of mitochondrial membrane permeability through the inhibition of Bad (Datta et al., 1999; Azuma et al., 2000; Gibson et al., 2002). Cytochrome c release with the consequent formation of the apoptosome between APAF-1 and caspase-9 and the subsequent proteolysis of the latter leads to caspase-3 activation and the consequent cleavage of the intracellular targets such as gelsolin. The inhibition of this pathway by EGF could prevent the morphological changes and apoptosis induced by IFNα. On the other hand, it has been reported that Rac migrates to the cell membrane following EGF stimulation in fibroblasts and that gelsolin is an essential effector of rac-mediated actin dynamics, acting downstream of rac recruitment to the membrane (Azuma et al., 1998). Therefore, the full-length gelsolin is the molecular target of the motility stimulating properties of EGF (Chen et al., 1996; Azuma et al., 1998). Moreover, it has been demonstrated that the full-length gelsolin can function as an effector of the Ras → PI3K → Rac invasion-signaling pathway that can be also activated by EGF (De Corte et al., 2002). However, at our knowledge, no data exist about the survival effects and on the restoration of intracellular levels of integral gelsolin induced by EGF on cancer cells.

In conclusion, our data are the first demonstration that IFNα can induce morphological cell changes that are peculiar of apoptosis onset through the caspase-3-mediated cleavage of gelsolin. Moreover, we have demonstrated for the first time that EGF is able to antagonize these effects through the inhibition of caspase-3 activation. Our data could be useful in understanding the mechanism of action of IFNα and in developing new anti-cancer therapeutic strategies based on the combined use of IFNα and biological agents that block the EGF-dependent signaling or gene transfer strategies based on the delivery of plasmids encoding for pro-apoptotic genes such as N-terminal gelsolin fragment.

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