Ubiquitination of tissue transglutaminase is modulated by interferon α in human lung cancer cells

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The addition of 2500 i.u./ml interferon α (IFN α) for 48 h induced apoptosis, and caused an approx. 4-fold increase in the activity and expression of tissue transglutaminase (tTG), in human lung cancer H1355 cells. However, the increase in mRNA levels for tTG was just 1.6-fold. On the basis of these data, we investigated whether tTG levels may be regulated through regulation of its degradation via ubiquitination. It was found that 2500 i.u./ml IFN α induced a time-dependent decrease in tTG ubiquitination. On the other hand, addition of the proteasome inhibitor lactacystin led to accumulation of the ubiquitinated form of the enzyme and to a consequent increase in its expression. Treatment of the cells with the two agents combined antagonized the accumulation of the ubiquitinated isoforms of tTG induced by

lactacystin and caused a potentiation of tTG expression. Moreover, the tTG inducer retinoic acid was also able to cause increased expression and ubiquitination of tTG in H1355 cells. The addition of monodansylcadaverine (a tTG inhibitor) to IFN α -treated H1355 cells completely antagonized growth inhibition and apoptosis induced by the cytokine. In conclusion, we demonstrate for the first time that tTG is ubiquitinated and degraded by a proteasome-dependent pathway. Moreover, IFN α can, at least in part, induce apoptosis through the modulation of this pathway.

Key words: apoptosis, cancer cells, interferon α , proteasome, transglutaminase, ubiquitin.

INTRODUCTION

Interferon α (IFN α) is a common cytokine that is used widely in the therapy of human cancer [1–5]. However, its mechanism of action is still unclear, although a means by which it can inhibit cell proliferation is the induction of apoptosis [6–12]. It has been demonstrated that IFN α induces apoptosis and increases the function and expression of the receptor for epidermal growth factor (EGF) in human head and neck tumour KB cells [13,14]. This effect is paralleled by activation of a stress pathway that has c-Jun N-terminal kinase 1 (JNK1) and p38 kinase as terminal enzymes [10].

An enzyme that has been reported to play a role in apoptosis is tissue transglutaminase (tTG; EC 2.3.2.13), a ubiquitous member of the transglutaminase enzyme family. This protein is normally expressed in mammalian cells, and is localized mainly in the cytoplasm [15]. tTG gene expression can be regulated by several factors that also modulate apoptosis [16–21]. Moreover, growth factors (i.e. EGF and transforming growth factor β) [22,23] and cytokines (i.e. interleukin-6) [24] can regulate tTG gene expression in cancer cells. It has been reported that IFN β , which binds to the same cellular receptor as IFN α , induces apoptosis in human non-small-cell lung cancer cells via stimulation of tTG, and that inhibition of tTG by monodansylcadaverine (MDC) reduces the formation of apoptotic bodies, but not DNA fragmentation, suggesting that the cells can enter multiple cell death pathways triggered by IFNs (tTG-dependent and -independent) [25]. It was also shown that IFN α stimulates apoptosis and tTG activation in human squamous carcinoma cell lines, and that it acts synergistically in triggering apoptosis with another tTG activator, retinoic acid (RA) [26]. However, the expression of tTG did not correlate with the subsequent

induction of apoptosis which, in turn, occurred with the induction of IFN regulatory factor 1 [27]. Moreover, it was reported previously that type I IFN (IFN β) can also induce differentiation and increase tTG expression in human non-small-cell lung cancer cells [28]. However, the mechanism of tTG modulation via type I IFN has not been completely elucidated. IFN can induce the transcription of the gene encoding tTG: several authors have reported an increase in tTG mRNA following treatment with type I IFN [27,28].

Another possibility that has not yet been investigated is that IFN may alter the intracellular stability of tTG via modulation of its ubiquitination. In fact, it has been reported that type I IFNs induce a 15 kDa protein exhibiting identity with ubiquitin [29], but with a distinct pathway of enzyme conjugation [30]. More recently it was described that IFN α can also induce two ubiquitin cross-reactive enzymes, UbcH5 and UbcH8 [31]. The IFN α -induced degradation of key proteins involved in control of the cell cycle has been correlated with the anti-proliferative activity of the cytokine, opening a new scenario on the mechanism of action of this cytokine [32].

The present paper describes an investigation into the effects of IFN α on apoptosis and on tTG expression and activity in human epidermoid lung cancer cells. Moreover, we have evaluated whether tTG can associate with ubiquitin, and if IFN α can affect the proteasome-dependent degradation of the enzyme.

EXPERIMENTAL

Cell culture and cell proliferation assays

The human lung epidermoid carcinoma H1355 cell line (obtained from American Type Tissue Culture Collection, Rockville, MD,

Abbreviations used: ECL, enhanced chemiluminescence; EGF, epidermal growth factor; IFN α , interferon α ; JNK, c-Jun N-terminal kinase; MAb, monoclonal antibody; MDC, monodansylcadaverine; RA, retinoic acid; RT-PCR, reverse transcription-PCR; tTG, tissue transglutaminase.

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U.S.A.) was grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 20 mM Hepes, 100 units/ml penicillin, 100 µg/ml streptomycin, 1% (w/v) L-glutamine and 1% (w/v) sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO₂ at 37 °C. For cell proliferation experiments, 1.5 × 10⁵ control, IFNα- or RA-treated H1355 cells were seeded in 6-well plates and incubated at 37 °C. At the selected times, cell number was determined by haemocytometric counting after adding Trypan Blue dye.

Internucleosomal DNA fragmentation (ladder)

DNA fragmentation was measured after extraction of low-molecular-mass DNA. Briefly, 10 × 10⁶ cells were resuspended in 900 µl of 10 mM Tris/0.1 mM EDTA buffer and lysed with 25 µl of 20% (v/v) SDS. DNA was precipitated in ethanol for 6 h in the presence of 5 M NaCl. The high-molecular-mass fraction was sedimented by high-speed centrifugation (20000 g, 10 min), and the fragmented DNA was extracted from the aqueous phase with phenol/chloroform (25:24, v/v) and then precipitated with ethanol. After resuspension in water, DNA was electrophoresed on a 1.5% (w/v) agarose gel and visualized by UV light following ethidium bromide staining.

Evaluation of apoptosis by DNA flow cytometry

Cells were centrifuged (800 g, 8 min) and stained directly in a propidium iodide solution (50 µg of propidium iodide in 100 µl of PBS containing 0.1% sodium citrate and 0.1% Nonidet P40, 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, U.S.A.) interfaced with a Hewlett Packard computer (model 310) for data analysis. To evaluate cell apoptosis, propidium iodide fluorescence was collected as FL2 (log scale) by the CellFIT software (Becton Dickinson). For the determination of annexin V expression, the cells were incubated for 1 h at 37 °C in the dark with an anti-annexin FITC-conjugated monoclonal antibody (MAb) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and analysed with FACScan. For the determination of annexin V expression, at least 10000 events for each point were analysed in at least three different experiments, giving an S.D. of < 5%.

Immunodetection of tTG in H1355 cells

For immunodetection of tTG, cells growing in complete α-Dulbecco's modified Eagle's medium (80% confluent) were washed three times with PBS, scraped and centrifuged for 5 min at 800 g. For cell extract preparation, the cells were centrifuged at 4 °C for 30 min at 20000 g in 250 µl of lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% IGEPAL-CA630, 5 mM MgCl₂, 1 mM EDTA, 2% glycerol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 25 µM NaF, 10 µM PMSF). A 5 µg aliquot of the total homogenate protein obtained from supernatants was subjected to SDS/PAGE using a 10% (w/v) polyacrylamide gel. Proteins were then electroblotted to type HA 0.45-mm pore nitrocellulose paper (Bio-Rad, Richmond, CA, U.S.A.). The primary antibody, i.e. anti-tTG MAb clone CUB 7402 (Dako, Milan, Italy) or anti-α-tubulin MAb clone DM1A (Calbiochem), was diluted 1:2000 with a blocking solution containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 10% (v/v) fetal bovine serum. The specific bands for tTG were detected with goat anti-rabbit or anti-mouse IgG (Santa Cruz) conjugated to peroxidase and subsequent enhanced chemiluminescence (ECL[®]) detection (Amersham, Milan, Italy). The intensities of the bands associated

with tTG were determined by laser scanning using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, U.S.A.).

Ubiquitination of whole-cell proteins and tTG

H1355 cells were seeded and treated with 2500 i.u./ml IFNα and/or 10 µM lactacystin and/or 10 µM RA for different times. At the time of the assay, cells were washed three times with PBS and cell proteins were extracted as described above. For ubiquitination of whole-cell proteins, the supernatants were run directly and electroblotted with anti-ubiquitin antibody, as described below. For the determination of tTG ubiquitination, the supernatants were subjected to immunoprecipitation with anti-tTG MAb clone CUB 7402 (Dako). tTG was precipitated from 300 µg of cell lysate using 5 µg of MAb for 12 h at 4 °C and 50 ml of Protein A-Sepharose (Sigma) [1:1 (w/v) suspension in PBS] for 12 h at 4 °C. Immunoprecipitated samples were washed four times with lysis buffer supplemented with 0.1% SDS, boiled in 20 µl of Laemmli buffer for 5 min and electrophoresed on SDS/10%-PAGE. Proteins were then electroblotted and probed with the anti-ubiquitin rabbit antiserum FL-76 (diluted 1:500) (Santa Cruz) or the anti-tTG MAb CUB 7402. The specific bands for ubiquitin or tTG were detected with goat anti-rabbit or anti-mouse IgG (Santa Cruz) conjugated to peroxidase and subsequent ECL detection.

tTG assay

Enzyme activity was assayed by measuring the incorporation of [³H]spermidine trihydrochloride (specific radioactivity 15 Ci/mmol; Amersham) into *N,N*-dimethylated casein. The assay mixtures (100 µl) containing 125 mM Tris/HCl buffer, pH 8.0, 10 mM dithiothreitol, 2.5 mM Ca²⁺, 50 nM [³H]spermidine and 0.2 mg of dimethylated casein were incubated at 37 °C for 1 h in the presence of various amounts of cellular homogenate. Blanks were run simultaneously with radioactive spermidine in the presence of 5 mM EGTA. The reactions were stopped by adding 1.0 ml of 10% trichloroacetic acid containing 2 mM unlabelled spermidine and the samples were then centrifuged (15000 g, 8 min). The resulting precipitates were washed twice by suspension in assay mixture (see above), dissolved in 1.0 ml of 0.1 M NaOH, and finally radioactivity was counted following the addition of 5 ml of Pico-Fluor 40 scintillation mixture (Packard).

Reverse transcription-PCR (RT-PCR)

mRNA isolated using an mRNA Capture Kit (Roche Diagnostics S.p.A., Milan, Italy) from H1355 cells incubated in the standard culture medium for 48 h in the absence or the presence of IFNα (2500 i.u./ml) was transcribed using reverse transcriptase (Superscript II; Gibco-BRL) at 37 °C for 1.5 h according to the manufacturer's protocol (final volume 20 µl). The cDNA contained in 2 µl of this reaction mixture was amplified in another reaction mixture containing, in a final volume of 25 µl, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 100 ng of both sense and antisense primers for tTG (sense, 5'-TATGGCCAGT-GCTGGGTCTTCGCC-3'; antisense, 5'-GGCTCCAGGGT-TAGGTTGAGCAGG3'), 100 µM deoxynucleoside triphosphate and 1 unit of *Taq* DNA polymerase (Roche Diagnostics S.p.A.). The reaction was carried out in a DNA thermal cycler (Promega). PCR was performed with 35 cycles in the exponential phase of amplification, following a 3 min denaturation step at 95 °C. Each cycle comprised 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min. A final step was carried out for 7 min at 72 °C. The PCR products were analysed by electrophoresis on a 1.2%

(w/v) agarose gel in Tris/borate/EDTA [32a]. The identities of the amplification products were confirmed by comparing their sizes with those expected from the known gene sequences. Co-amplification of different cDNA sequences was performed by adding into the amplification reaction mixture the β -actin gene primers (10 ng of both sense and antisense: sense, 5'-CGTGG-GCCGCCCTAGGCACCA-3'; antisense, 5'-TTGGCCTTAG-GGTTTCAGGGGGG-3'). No products were detectable in control amplifications performed in the absence of cDNA (results not shown).

In situ tTG activity assay

For measurement of *in situ* tTG activity, H1355 cells were treated with 3.5 mM 5-(biotinamido)pentylamine (Pierce, Rockford, IL, U.S.A.) in standard culture medium or in combination with 2500 i.u./ml IFN α , and incubated at 37 °C in a humidified atmosphere containing 5% CO $_2$ and 95% air for 24 h. At the end of the incubation time, the cells were washed three times with PBS, scraped and centrifuged for 5 min at 800 *g*. For cell extract preparation, the cells were resuspended in a homogenizing buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 25 μ M NaF, 10 μ M PMSF) and sonicated on ice. A 40 μ g portion of total homogenate protein was subjected to SDS/PAGE using a 10% (w/v) polyacrylamide gel. Proteins were then electroblotted on to type HA 0.45 mm pore nitrocellulose paper (Bio-Rad). The blots were blocked in 5% (w/v) BSA in borate-buffered saline (100 mM boric acid, 20 mM sodium borate, 80 mM NaCl) overnight at 4 °C, and were then incubated with horseradish peroxidase-conjugated streptavidin (Pierce) diluted 1:1000 in borate-buffered saline containing 1% (w/v) BSA for 2 h at room temperature. The blots were rinsed for 3 $\times$ 30 min with borate-buffered saline containing 0.01% Nonidet P40, followed by four quick rinses with distilled water, and developed following the standard ECL protocol.

RESULTS

IFN α inhibits proliferation and induces apoptosis in human epidermoid lung H1355 cells

The effects of IFN α on the cell growth of epidermoid lung H1355 cancer cells were studied. KB cells were exposed to increasing concentrations of IFN α , and 2500 i.u./ml IFN α induced approx. 50% growth inhibition after 48 h of treatment without cytotoxicity, as evaluated by Trypan Blue assay (Figure 1A). Higher concentrations (5000 i.u./ml) of the cytokine were toxic, as determined by the count of floating cells and by trypan Blue assay, while 1000 i.u./ml IFN α was unable to induce significant growth inhibition (Figure 1A). Cell growth retardation was induced by 2500 i.u./ml IFN α , as demonstrated from the growth curve profile, but cell growth arrest was never reached (Figure 1A). Therefore a concentration of 2500 i.u./ml IFN α was chosen for all subsequent experiments.

We next evaluated whether it was possible to induce apoptosis under these experimental conditions. H1355 cells were analysed by FACS after DNA labelling with propidium iodide, and it was found that the exposure to 2500 i.u./ml IFN α for 48 h caused approx. 42% apoptosis (Figures 1B–1D). Analogous results were obtained in experiments assessing internucleosomal DNA fragmentation. A gel ladder was evident after 24 h of exposure to the cytokine, and fragmentation was increased further after 48 h (Figure 1E).

Figure 1 Effects of IFN α on the proliferation and apoptosis of H1355 cells

(A) H1355 cells were treated with various concentrations of IFN α , as described in the Experimental section: $\blacktriangle$, 1000 i.u./ml; $\bullet$, 2500 i.u./ml; $\blacksquare$, 5000 i.u./ml. At the indicated times the cells were counted in a haemocytometer after labelling with Trypan Blue. Values are expressed as growth inhibition, and are means $\pm$ S.D. of three different experiments. (B)–(D) H1355 cells were treated with 2500 i.u./ml IFN α for the indicated times and analysed by FACS after propidium iodide labelling, as described in the Experimental section. (B) Untreated cells incubated for 48 h; (C) treatment with 2500 i.u./ml IFN α for 24 h; (D) treatment with 2500 i.u./ml IFN α for 48 h. The percentage of apoptotic cells is shown. The experiment was performed three times, and S.E.M.s were always lower than 5%. (E) H1355 cells were treated with 2500 i.u./ml IFN α for the indicated times. Internucleosomal DNA fragmentation was assessed as described in the Experimental section. The experiment was performed at least three times, with similar results. MW indicates size markers.

IFN α induces tTG activity and expression in H1355 cells

Since activation of tTG has been correlated with apoptotic events, the effects of IFN α on tTG activity and expression in H1355 cells were studied. IFN α at 2500 i.u./ml increased tTG activity, reaching a peak (increase of approx. 4-fold) after 24–48 h of treatment with the cytokine (Figure 2A). After 72 h of exposure to IFN α , tTG activity had decreased to an approx. 2.5-fold enhancement compared with baseline. To determine whether the increased tTG activity *in vitro* co-incided with increased cross-linking activity *in situ*, an assay was performed using 5-(biotinamido)pentylamine, a labelled amine substrate which penetrates cells and is subsequently incorporated into substrate proteins [33]. Treatment with 2500 i.u./ml IFN α for 24 h resulted in a significant increase in the tTG-catalysed incorporation of 5-

Figure 2 Effects of IFN α on tTG activity and expression

(A) H1355 cells were cultured for various times in the absence (○) or presence (■, 12 h; ▲, 24 h; ◆, 48 h; ●, 72 h) of 2500 i.u./ml IFN α . Then cells were processed for the determination of tTG activity, as described in the Experimental section. The experiment was performed three times, and values are means $\pm$ S.D. Inset: for measurement of tTG activity *in situ*, H1355 cells were treated with 5-(biotinamido)pentylamine (5-BP) in standard culture medium (lane 2) or in combination with 2500 i.u./ml IFN α (lane 3) and incubated at 37 °C. The control (lane 1) shows untreated cells not exposed to 5-(biotinamido)pentylamine. At the end of the incubation time the cells were washed three times with PBS, scraped and centrifuged for 5 min at 1000 rev./min. Then cells were processed and the lysates obtained were run and electroblotted as described in the Experimental section. The blots were incubated with horseradish peroxidase-conjugated streptavidin and developed using the standard ECL protocol. The experiment was performed three times with similar results. (B) H1355 cells were cultured in the absence (CTR) or presence of 2500 i.u./ml IFN α for the indicated times. Then cells were processed for the immunodetection of tTG or α -tubulin by Western blotting as described in the Experimental section. The experiment is representative of at least three different experiments that produced similar results. (C) Laser scan of the specific band associated with tTG. The intensities of the bands are expressed in arbitrary (relative) units (%). CTR, untreated cells. (D) H1355 cells were treated with 2500 i.u./ml IFN α and/or 50 μ M MDC for 48 h. Internucleosomal DNA fragmentation was assessed as described in the Experimental section. The experiment was performed at least three times with similar results. CTR, untreated cells; MW, size markers. (E–H) H1355 cells were treated with 2500 i.u./ml IFN α and/or 50 μ M MDC for 48 h and analysed by FACS after labelling with an anti-(annexin V) FITC-conjugated MAb, as described in the Experimental section. Treatment: (E) untreated cells; (F) 2500 i.u./ml IFN α ; (G) 48 h 50 μ M MDC; (H) 2500 i.u./ml IFN α plus 50 μ M MDC. The percentage of apoptotic cells is shown by the horizontal bar. The experiment was performed three times, and S.E.M.s were always < 5%. (I) H1355 cells were treated with 2500 i.u./ml IFN α and/or 50 μ M MDC for 48 h, and the cell number was determined. CTR, untreated cells. Cell proliferation is shown as % growth inhibition. Values are means $\pm$ S.E.M. of three experiments.

(biotinamido)pentylamine into proteins (Figure 2A, inset, lane 3) compared with the incorporation of the labelled substrate into proteins from untreated cells (Figure 2A, inset, lane 2). These results demonstrate that tTG is active in H1355 cells treated with IFN α .

Next we evaluated whether the increased tTG activity was due to the induction of tTG expression. IFN α at 2500 i.u./ml induced an approx. 4-fold increase in tTG expression after a 12 h exposure (Figures 2B and 2C). Interestingly, the expression of tTG

subsequently remained almost unchanged during exposure to the cytokine, while the increase in tTG activity was time-dependent.

In order to correlate the induction of tTG activity and expression with the antiproliferative and apoptotic effects induced by IFN α , H1355 cells were treated with the tTG activity inhibitor MDC alone or in combination with IFN α . Treatment with 50 μ M MDC for 48 h antagonized growth inhibition and apoptosis induced by IFN α (Figures 2D and 2H). IFN α and MDC alone induced 50 % and 20 % apoptosis respectively

Figure 3 Effects of IFN α on tTG mRNA levels

(A) H1355 cells were cultured for various times as indicated in the absence (CTR) or presence of 2500 i.u./ml IFN α . The mRNA was isolated, amplified by RT-PCR and run on a 1.2% (w/v) agarose gel in Tris/borate/EDTA buffer as described in the Experimental section. MW, size markers. The experiment is representative of at least three different experiments that produced similar results. (B) Laser scan of the specific band associated with tTG. The intensities of the bands are expressed as arbitrary (relative) units (%).

(Figures 2F and 2G respectively), while combined treatment with both agents caused apoptosis in only 10% of the cell population (Figure 2H). Likewise, MDC and IFN α alone caused 18% and 48% growth inhibition respectively, but combined treatment did not affect cell proliferation (Figure 2I). These data suggest that the apoptosis induced by IFN α was probably due to activation of tTG.

In order to investigate whether the increase in tTG expression was due to enhancement of transcript levels, the levels of mRNA for tTG were determined by RT-PCR. IFN α induced approx. 1.3- and 1.6-fold increases in tTG transcript levels at 6 h and 12 h respectively from the beginning of treatment (Figures 3A and 3B). At earlier and later time points the expression of tTG mRNA was almost unchanged in IFN α -treated cells (Figures 3A and 3B). On the basis of these data, it can be concluded that the increase in the activity and expression of tTG was only partially due to the enhancement of transcripts for the enzyme.

Ubiquitination and proteasome-dependent degradation of tTG is inhibited by IFN α

Since the increase in tTG protein expression was greater than the enhancement of tTG mRNA levels, we investigated whether IFN α affect tTG ubiquitination and, consequently, its proteasome-dependent degradation. We found that 2500 i.u./ml IFN α induced a time-dependent decrease in the ubiquitination of tTG. In fact, the exposure of H1355 cells to IFN α caused a 40% decrease at 24 h in tTG ubiquitination, and decreases of 60% and 80% at 48 h and 72 h respectively, as evaluated by Western blotting for ubiquitin after immunoprecipitation of tTG (Figures

4A and 4B). Since IFN α can induce ubiquitin cross-reactive enzymes that can lead to the degradation of key proteins involved in control of the cell cycle, the profile of intracellular protein ubiquitination induced by IFN α in our experimental model was investigated [31,32]. IFN α differentially modulated the ubiquitination of intracellular proteins, as demonstrated by Western blotting of whole-cell extracts (Figure 4C). The ubiquitination of some protein species was increased by IFN α , while that of others was decreased. We have not identified the proteins that were differentially ubiquitinated by IFN α treatment. However, we propose that the high-molecular-mass band whose ubiquitination was increased by the cytokine could be the receptor for EGF (which is overexpressed in these cells and appears at the same level in Western blotting experiments). Thus enhancement of the ubiquitination of tTG is likely to be due to specific, as yet unknown mechanisms elicited by IFN α (Figure 4C).

Next, H1355 cells were exposed to IFN α and/or the specific proteasome inhibitor lactacystin in order to evaluate effects on tTG ubiquitination and expression. It was again found that 2500 i.u./ml IFN α reduced tTG ubiquitination and increased enzyme expression after 12 and 24 h of treatment (Figures 4D and 4E respectively). The addition of 10 μ M lactacystin for 12 and 24 h, inhibiting the proteasome-dependent degradation of ubiquitinated tTG, caused an accumulation of the ubiquitinated form of the enzyme, and a consequent increase in its expression (Figures 4D and 4E respectively). Treatment of the cells with the two agents combined antagonized the accumulation of ubiquitinated isoforms of tTG induced by lactacystin (Figure 4D). This effect was probably due to the decrease in tTG ubiquitination caused by IFN α . Under these experimental conditions a potentiation of tTG expression was recorded (Figure 4E). In order to demonstrate that the modulation of tTG ubiquitination was not a specific effect induced by IFN α , H1355 cells were treated with RA, another activator of tTG. RA also induced a decrease in the ubiquitination of tTG, as demonstrated by Western blotting for ubiquitin after immunoprecipitation of tTG (Figure 4F, upper gel). Concomitant treatment with RA and lactacystin again enhanced the accumulation of tTG induced by lactacystin alone (Figure 4F, lower gel).

These data suggest that tTG is ubiquitinated and degraded by a proteasome-dependent pathway in H1355 cells, and that IFN α decreased tTG polyubiquitination and, consequently, its degradation. Moreover, modulation of the ubiquitination of tTG was not induced specifically by IFN α , but may represent a general mechanism for the regulation of tTG expression.

DISCUSSION

IFN α is a cytokine that has shown well defined but still limited activity against human tumours [1–5]. The mechanism(s) by which tumour cell growth is suppressed by IFN α is not well understood. It has been reported that IFN α induces apoptosis of human squamous cancer [9,10], glioma [11] and virus-infected [12] cells. Therefore it is likely that this cytokine acts, at least in part, through triggering programmed cell death. We have also demonstrated that the growth inhibition induced by IFN α is due to apoptosis triggered by a stress response leading to the activation of JNK1 [10].

A possible mechanism of action of IFN α is the activation of tTG, a Ca²⁺-dependent enzyme involved directly in several cell functions correlated with the control of eukaryotic cell growth and apoptosis [15–24]. It has been shown that IFN α can induce apoptosis and tTG activation in several squamous cell lines [28]. In the present study we found that treatment with 2500 i.u./ml IFN α for 48 h resulted in inhibition of cell growth through the

Figure 4 Effects of IFN α , RA and the proteasome inhibitor lactacystin on ubiquitination and expression of tTG

(A) H1355 cells were cultured for the indicated times in the absence (CTR) or presence of 2500 i.u./ml IFN α . Then cellular proteins were extracted and immunoprecipitated with an anti-tTG or anti- α -tubulin MAb as described in the Experimental section. The immunoprecipitate was subsequently run on SDS/PAGE and immunoblotted for ubiquitin. The experiment shown is representative of at least three different experiments giving similar results. ub tTG, ubiquitinated tTG. (B) Laser scan of the specific band associated with ubiquitinated tTG (from A). The intensities of the bands are expressed in arbitrary units (%). (C) H1355 cells were cultured for the indicated times in the absence (CTR) or presence of 2500 i.u./ml IFN α . Then cellular proteins were extracted and Western blotting for ubiquitin was performed as described in the Experimental section. MW, molecular size markers. (D, E) H1355 cells were exposed or not (CTR) to 2500 i.u./ml IFN α and/or 10 μ M lactacystin (LC; a specific proteasome inhibitor) and at the indicated times were processed for the determination of tTG ubiquitination (D) or expression (E) as described in the Experimental section. Asp, Protein A–Sepharose without lysates; ub tTG, ubiquitinated tTG. The experiment shown is representative of at least three different experiments giving similar results. (F) H1355 cells were exposed or not (CTR) to 10 μ M RA and/or 10 μ M lactacystin (LC) and at the indicated times were processed for the determination of tTG ubiquitination (upper gel) or expression (lower gel) as described in the Experimental section. The experiment shown is representative of at least three different experiments giving similar results. ub tTG, ubiquitinated tTG.

triggering of apoptosis in human epidermoid cancer H1355 cells. This effect occurred without apparent cytotoxicity, and was paralleled by significant increases in tTG expression and activity. Interestingly, we demonstrated, by using the 5-(biotinamido)-pentylamine as amino-donor substrate, that tTG is more active in intact H1355 cells treated with IFN α . It has been shown that the presence of functional tTG activity is indispensable for a correct programme of apoptosis [34]. The activation of tTG in dying cells results in the assembly of highly cross-linked intracellular proteins. In fact, several proteins have been shown to undergo tTG-dependent polymerization in apoptosis ([18] and references therein). We have found, moreover, that inhibition of tTG activity by MDC completely antagonized the growth inhibition and apoptosis induced by IFN α . This suggests an important role for tTG in the regulation of programmed cell death induced by IFN α in H1355 cells.

The mechanisms by which IFN α modulates tTG expression, thus leading to cell growth modulation, are in turn still unclear. It has been reported that type I IFN can induce transcription of the gene encoding tTG [27,28]. On the basis of these considerations, we evaluated whether IFN α could increase tTG transcript levels in our experimental system. An increase in tTG mRNA expression was found, but to a much lesser extent than the increases in activity and protein expression of the enzyme.

Another mechanism used by cells to increase the expression of a protein is its intracellular stabilization due to decreased degradation [35]. Protein degradation can be driven by the proteasome-dependent pathway following covalent addition to the proteins of several small molecules of 14 kDa, called ubiquitin [36]. This event leads to the subsequent delivery of the protein to a macromolecular complex called the proteasome, which determines the proteolysis and degradation of the protein [36]. It has been reported that type I IFNs induce a 15 kDa protein exhibiting identity with ubiquitin [29], but showing a distinct pathway of enzyme conjugation [30]. More recently, it was shown that IFN α can induce two ubiquitin cross-reactive enzymes [31], as well as the degradation of key proteins involved in control of the cell cycle [32]. On the other hand, it has never been reported that tTG can be ubiquitinated.

We found that tTG was ubiquitinated in H1355 cells, and that IFN α could decrease the ubiquitination of the protein. Moreover, the proteasome inhibitor lactacystin was able to increase the levels of the ubiquitinated form of tTG, demonstrating delivery of the latter in the proteasome complex. At the same time the expression of tTG was increased, demonstrating that the enzyme is degraded by the proteasome. The simultaneous addition of IFN α to lactacystin-treated cells antagonized the accumulation of ubiquitin-conjugated tTG and further increased the levels of

the enzyme. Another demonstrated pathway of tTG degradation is that dependent on caspase-3. However, it has been found that, under the same experimental conditions, IFN α induced caspase-3 activity, which therefore does not correlate with the increased tTG expression [37]. The ubiquitin-dependent mechanism of regulation of tTG expression does not appear to be induced specifically by IFN α , since RA, another tTG activator, can also increase tTG expression through a decrease in tTG ubiquitination and proteasome-dependent degradation. Moreover, in our experimental model IFN α can differentially regulate the ubiquitination of intracellular proteins, which does not exclude the occurrence of the previously described increase in the proteasome-dependent degradation of some intracellular proteins induced by this cytokine [31,32]. Therefore IFN α can induce differential modulation of the proteasome, either decreasing or increasing the degradation of proteins involved in cell growth and apoptosis. On this basis, one could propose the existence of another, yet to be discovered, ubiquitinating enzyme, which is negatively regulated by IFN α .

In conclusion, this study has demonstrated for the first time that tTG is ubiquitinated and degraded by a proteasome-dependent pathway. Moreover, it has been shown that IFN α modulates the expression and activity of tTG, at least in part through a decrease in its ubiquitination, and consequently leads to the intracellular stabilization of tTG. These biochemical effects are paralleled by retardation of cell growth and apoptosis, and therefore appear to be relevant in cell proliferation control.

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