γ-Glutamyl transpeptidase expression in Ewing's sarcoma cells: up-regulation by interferons

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The genetic hallmark of Ewing's sarcoma family of tumours (ET) is the presence of the translocation t(11;22)(q24;q12), which creates the ET fusion gene, leading to cellular transformation. Five human γ-glutamyl transpeptidase (γ-GT) genes are located near the chromosomal translocation in ET. γ-GT is a major enzyme involved in glutathione homoeostasis. Five human cell lines representative of primary or metastatic tumours were investigated to study whether γ-GT alterations could occur at the chromosomal breaks and rearrangements in ET. As shown by enzymic assays and FACS analyses, all ET cell lines consistently expressed a functional γ-GT which however did not discriminate steps of ET progression. As shown previously [Sancéau, Hiscott, Delattre and Wietzerbin (2000) Oncogene **19**, 3372–3383], ET cells respond to the antiproliferative effects of interferons (IFNs) type I (α and β) and to a much less degree to IFN type II (γ). IFN-α and -β arrested cells in the S-phase of the cell cycle. We found an enhancement of γ-GT mRNA species with IFN-α and -β by reverse transcriptase–PCR analyses. This is reflected by up-regulation of γ-GT protein, which coincides with the increase in γ-GT-specific enzymic activity. Similarly, IFNs up-regulate the levels of γ-GT in another IFN-responsive B cell line. Whether this up-regulation of γ-GT by IFNs is of physiological relevance to cell behaviour remains to be studied.

Key words: cancer, ectopeptidase, proliferation.

INTRODUCTION

Cell surface peptidases (ectopeptidases) are transmembrane enzymes present in a wide variety of tissues and cell types [1]. Dysregulated expression of some of them in human diseases has led to research on their value as disease markers [1]. Moreover, some of these ectoenzymes may influence major cell functions, such as metabolic regulation, growth, apoptosis and motility [1,2]. γ-Glutamyl transpeptidase (γ-GT) (CD224; EC 2.3.2.2) is a widespread ectopeptidase that catalyses the transfer of γ-glutamyl from γ-glutamyl peptides to other amino acid and peptide acceptors as well as the hydrolytic cleavage of the γ-glutamyl group of donor peptides [3]. The most characterized *in vivo* function of γ-GT is in the metabolism and transport of glutathione and its derivatives [4]. Enhanced γ-GT levels are detected in acute myeloid leukaemia, chronic myeloid leukaemia and high-grade non-Hodgkin's lymphoma [1,2]. By contrast, the loss of cell surface γ-GT has been associated with T-acute lymphocytic leukaemias and T/B-chronic lymphocytic leukaemias [1,5]. Overexpression of γ-GT in human liver carcinoma is accompanied by enhanced levels of serum γ-GT [1].

Human γ-GT is a multigenic family composed of at least five genes identified on the chromosome 22q11 [6–10] proximal to the chronic myeloid leukaemia point in the *break-point cluster-region* gene, which is involved in chromosomal breaks and rearrangements associated with various diseases [7]). Among γ-GT genes, the gene encoding the ubiquitously expressed γ-GT transcribes three mRNAs found in foetal liver (type A), HepG2 hepatoma (type B) and placenta (type C) [9–12]. Whether these mRNAs arise from the alternate splicing of one or multiple transcripts initiated on one or several promoters on the gene remains unknown [11,12].

Ewing's sarcoma family of tumours (ET) are childhood bone tumours, of neuroectodermal origin, characterized by a highly specific recurrent balanced chromosomal translocation t(11;22)(q24;q12) [13,14]. This translocation results in the fusion, in the der (22) chromosome, of the 5′ half end of a resident EWS (Ewing's sarcoma) gene of unknown function, with the 3′ portion of the chromosome 11-derived Fli-1 (Friend Leukaemia Integrator-1) gene carrying the Ets domain with a specific DNA-binding motif [15,16]. The resultant EWS/Fli-1 fused protein initiates cellular transformation and has been suggested to function as an aberrant transcription factor that may modulate the expression of a different set of target genes [17–21].

As γ-GT genes are located near the chromosomal translocation in ET, we tried to determine whether γ-GT alterations could occur at the chromosomal breaks and rearrangements in ET. In the present investigation, we determined that human cell lines derived either from primary or metastatic secondary ET tumours express γ-GT. Since previous studies [22] from our laboratory have demonstrated the antiproliferative action of interferons (IFNs) on ET cells, we also explored if γ-GT expression was affected by IFNs.

EXPERIMENTAL

Materials

Recombinant human (rHu)-IFN-α 2a (specific activity, 2×10^8 units/mg) was provided by Hoffman La Roche (Basel, Switzerland); rHu-IFN-β 1a (specific activity, 4×10^8 units/mg) was from Area-Serono International (Geneva, Switzerland); rHu-IFN-γ (specific activity 2×10^7 units/mg) was from Roussel Uclaf (Romainville, France). The biological activity of IFN was

Abbreviations used: ET, Ewing's sarcoma family of tumours; EWS, Ewing's sarcoma gene; IFN, interferon; γ-Glu-pNA, L-γ-glutamyl-*p*-nitroanilide; γ-GT, γ-glutamyl transpeptidase; RT, reverse transcriptase; STAT, signal transduction and activators of transcription; SF, specific fluorescence density.

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assessed by antiviral protection against vesicular stomatitis virus on human Wish cells (CCL-25; A.T.C.C., Manassas, VA, U.S.A.). L-γ-Glutamyl-*p*-nitroanilide (γ-glu-pNA), GlycylGlycine and acivicin [(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid] were provided by Sigma.

Cell lines and culture

Five cell lines derived from ET were used in this study: (1) wild-type p53 EW-7 cells, primary tumour localized on scapula; (2) p53 mutated EW-1 cells, primary tumour localized on rib; (3) wild-type p53 COH cells, metastatic tumour localized on femur; (4) p53 mutated ORS cells from unknown localization and (5) wild-type p53 EW-24 cells, metastatic tumour from pleural effusion [23,24]. ET cell lines were maintained in RPMI 1640 (Gibco BRL, Life Technology) containing 2 mM L-glutamine and 10 μg/ml gentamicin (Flow Laboratories, Rockwell, MD, U.S.A.) in a 5% CO_2 humidified atmosphere at 37 °C. For IFN stimulation, cells [(1–10) × 10^5 cells/ml] were resuspended in a fresh medium containing fetal calf serum and 10 mM Hepes in tissue culture flasks, and were grown in the absence or presence of different concentrations of IFN. After various periods of incubation, cells were collected, washed twice, counted with a Coulter Counter ZM equipped with a Coultronic 256 channelizer, and their viability was determined by Trypan Blue exclusion.

Flow cytometry analysis

Intact cells were immunostained as described previously [25]. Anti-γ-GT (3A8, mIgG2a) was characterized previously [26]. The matched-isotype (mIgG2a) and FITC-conjugated goat F(ab′)2 anti-mouse IgG were from Coulter Beckman (Margency, France). FACS flow cytometer analyser (Becton-Dickinson, Mountain View, CA, U.S.A.) was used and 10000 events were recorded and analysed using Lysys II software (Becton Dickinson). Fluorescence data were expressed as relative fluorescence intensity (%) and antigen relative density per cell [specific fluorescence density (SF), which was obtained by subtracting the peak channel number of the negative control from the peak channel number of the corresponding experimental sample]. Cell cycle analysis, using the fluorescent DNA intercalating propidium iodide, was performed as described previously [27]. The percentage of cells in G_o/G_1, S and G_2/M populations was quantified from the DNA histogram using the Cell Quest program.

γ-GT assay

γ-GT activity at the surface of intact cells and whole cell lysates was assayed as described previously [28] using γ-Glu-pNA as a substrate of γ-GT hydrolytic activity and Glycylglycine as glutamate acceptor for the transpeptidation reaction. Total homogenates were obtained by solubilizing the cells at 4 °C in 100 mM Hepes buffer, pH 7.6 [0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$ (protease buffer), 1% (w/v) n-octyl-β-D-glucoside]. After lysis, the total extracts were centrifuged for 20 min at 10000 rev./min at 4 °C. Results were expressed as nmol of *p*-nitroaniline formed per 10^5 cells in 30 min at 37 °C or *p*-nitroaniline formed per μg in 30 min at 37 °C.

Reverse transcriptase (RT)–PCR

RNA extraction (by SV Total RNA Isolation System; Promega) from Ewing cells and subsequent cDNA syntheses were conducted as described previously [22]. Three different primer sets were used for detecting the three types of γ-GT cDNA [29]. Type A (foetal liver) cDNA (308 bp) was amplified using the sense primer 5′-CACAGGGGACATACAGTGAG-3′ and the antisense primer 5′-GAAATAGCTGAAGCACGCGC-3′, type B (HepG2) cDNA (300 bp) was amplified using the sense primer 5′-GGATTCTCCCAGAGATTGCC-3′ and the antisense primer 5′-GAAGGTCAA GGGAGGTTACC-3′ and type C (placenta) cDNA (386 bp) was amplified using the sense primer 5′-GCCCCAGAAGTGAGAGCAGTT-3′ and the antisense primer 5′-TCCAGAAAGCAGCTAGAGGG-3′. To normalize the PCR products, β_2-microglobulin cDNA (165 bp) was amplified using the sense primer 5′-CAT CCA GCG TAC TCC AAA GA-3′ and antisense primer 5′-GAC AAG TCT GAA TGC TCC AC-3′. The PCR products were visualized by electrophoresis in 1.5% agarose gel containing 0.2 μg/ml ethidium bromide. Bands of the linear amplification area were scanned with the NIH Image 1.44 b11 software.

RESULTS

Characterization of γ-GT in cells derived from ET

As shown in Figure 1(A), the five ET cell lines derived either from primary tumours (EW-7 and EW-1) or metastatic secondary tumours (COH, ORS and EW-24) expressed various levels of surface γ-GT. The primary EW-7 and EW-1 cell lines expressed disparate levels of cell surface γ-GT, whereas the three metastatic cell lines expressed γ-GT at levels almost comparable with those of EW-1 (Figure 1A). As expected, γ-GT-positive ET cells displayed, at their surface, a proteolytic activity towards γ-glu-

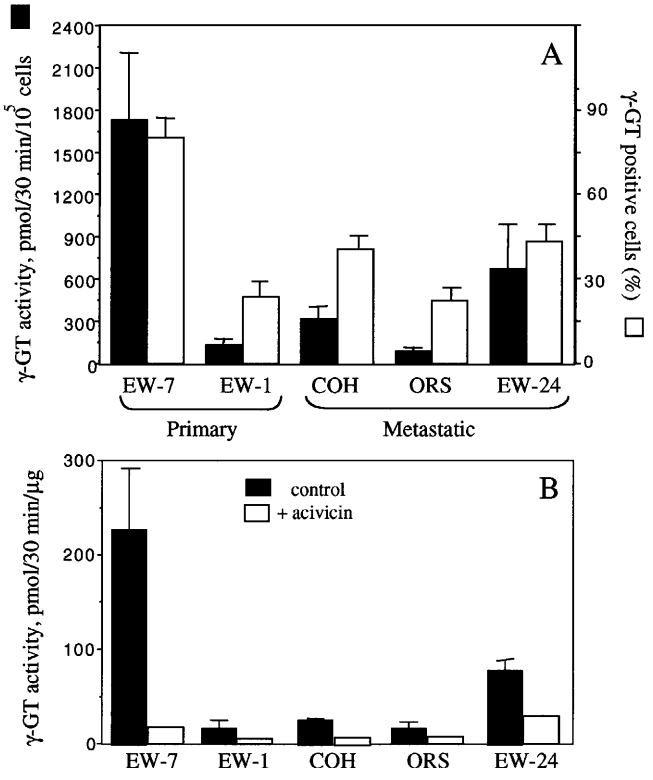

Figure 1 γ-GT expression and associated enzymic activity in ET cells

(**A**) EW-7, EW-1, COH, ORS and EW-24 cells were assayed for γ-GT activity (pmol · 30 min^{-1} · μg^{-1}) (black bars, means for five separate experiments) and for γ-GT cell surface expression (%) (open bars, means for two experiments). (**B**) Whole ET cell homogenates were assayed for γ-GT activity (pmol · 30 $min^{-1} \cdot \mu g^{-1}$) in the absence or presence of 10^{-3} M acivicin (means for four separate experiments).

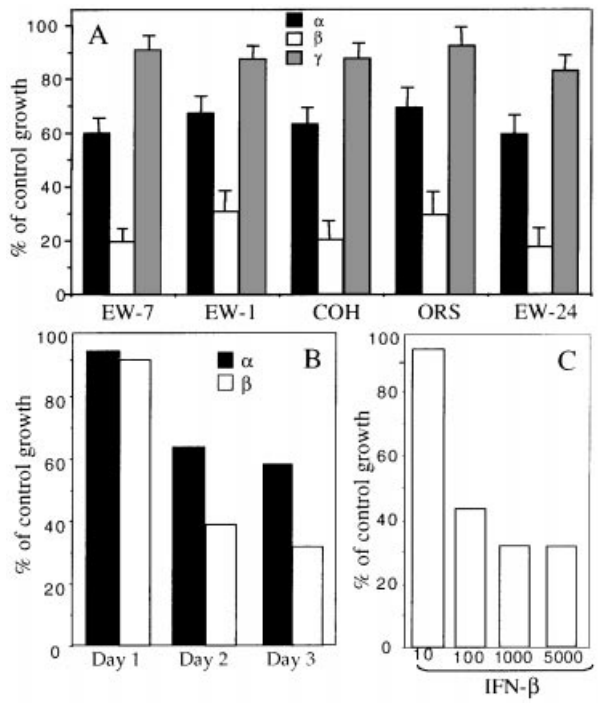

Figure 2 Growth inhibition of ET cell line after IFN treatment

(**A**) After incubation for 3 days without or with 1000 units/ml IFN, ET cells were harvested and counted. The percentage of inhibition of cell growth is calculated taking cell growth in untreated cultures as 100% proliferation. Values are means for separate experiments. (**B**) EW-7 cells were cultured in the absence or in the presence of 1000 units/ml IFN-α or IFN-β for up to 3 days. One representative experiment of two independent experiments is shown. (**C**) EW-7 cells were cultured in the absence or presence of increasing concentrations of IFN-β from 10 to 5000 units/ml for 3 days. An experiment representative of three separate experiments is shown.

pNA that reflected the levels of cell surface γ-GT (Figure 1A). Acivicin, which specifically blocks γ-GT activity [30], almost totally inactivated γ-glu-pNA hydrolysis in whole cell homogenates (Figure 1B) as well as that expressed at cell surface (results not shown), confirming that ET cells exhibited a proteolytically active γ-GT.

Inhibitory effects of IFNs on proliferation of ET cells

In a previous study [22], we observed a suppressive effect on the proliferation of ET cells after incubation with IFNs type I (α, β) and type II (γ). In all cell lines, after 3-day treatment, IFN-γ and IFN-α (1000 units/ml) exhibited 10–40% inhibition of cell proliferation, whereas treatment with IFN-β inhibited growth by more than 70% (Figure 2A). Kinetic studies shown here with the EW-7 cell line indicated that optimal inhibition was observed almost within 72 h of treatment (Figure 2B) and that maximal effects of IFNs were obtained for doses $\geqslant$ 1000 units/ml (Figure 2C for IFN-β; results not shown for IFN-α). The nature of the growth inhibition was further analysed using propidium iodide-based flow cytometry. As shown in Table 1, EW-7 cells are cells with 2N (70% of cells) and 4N (30% of cells) DNA content. IFNs did not modify the percentage of diploid and tetraploid cell populations. The G_o/G_1, S and G_2/M phase-specific distribution of EW-7 cells treated for 3 days with IFN-γ, was almost comparable with that of untreated EW-7 cells (Table 1). By contrast, there was a significant accumulation of IFN-α- and IFN-β-treated cells in the S-phase of the cell cycle (Table 1).

Table 1 Enhanced IFN-induced S arrest in EW-7 cells

EW-7 cells were cultured for 3 days in the absence or presence of 1000 units/ml IFNs. Cell cycle analyses were performed as described in the Experimental section. Data are means for two experiments.

	Apoptotic cells (%)	Diploid cells (%)		Tetraploid cells (%)			
		G_0/G_1	S	G_0/G_1	S	G_2/M	Total S
EW-7 control	11±7	67±0	33±0	68±1	23±1	9±1	29±1
EW-7 + IFN-α	15±2	62±1	37±1	62±1	31±2	6±1	36±1
EW-7 + IFN-β	12±1	54±1	46±1	51±1	49±1	0	47±1
EW-7 + IFN-γ	4±1	72±1	28±0	73±0	21±0	6±0	25±1

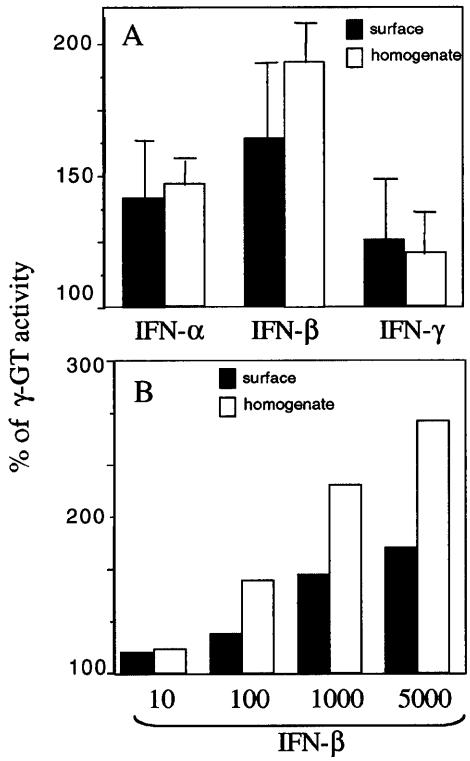

Figure 3 Modulation of γ-GT activity of EW-7 cells by IFNs

(**A**) After 72 h culture with or without 1000 units/ml IFNs, intact EW-7 cells (surface) or EW-7 homogenates were assayed for γ-GT (expressed as the percentage of the control activity). S.D. values are for three (homogenates) and four (intact cells) separate experiments. (**B**) After culturing for 72 h with or without increasing concentrations of IFN-β (from 10 to 5000 units/ml), intact EW-7 cells or EW-7 cell homogenates were assayed for γ-GT (expressed as the percentage of the control activity).

Stimulatory effects of IFNs on γ-GT of ET cells

At day 3, the growth inhibition of EW-7 cell line by IFN-α and IFN-β was accompanied by an up-regulation of their surface γ-GT activity, with the best effect observed with IFN-β (Figure 3A). By contrast, IFN-γ, which failed to alter EW-7 cell proliferation, did not markedly affect γ-GT activity (Figure 3A). The stimulatory effects of IFN-α and IFN-β on γ-GT activity were observed within 48–72 h of treatment (results not shown).

Table 2 Effects of IFN-β on the levels of γ-GT protein and activity of ET cell lines

ET cells were cultured for 3 days in the absence or presence of 1000 units/ml IFN-β. Specific fluorescence intensities of γ-GT (%) of intact cells and cell density (SF) were determined by means of fluorimetry (means $\pm$ S.D. for two separate experiments). Intact cells or cell homogenates were assayed for γ-GT activity (expressed as the percentage of the control activity $\pm$ S.D. for four determinations).

	% γ-GT positive cells (SF)	% cell surface γ-GT activity
EW-7 control	80 ± 10 (27 ± 7)	100
IFN-β-EW-7	90 ± 10 (51 ± 1)	161 ± 25
EW-1 control	17 ± 3 (8 ± 3)	100
IFN-β-EW-1	26 ± 5 (16 ± 8)	165 ± 15
COH control	40 ± 11 (34 ± 29)	100
IFN-β-COH	50 ± 13 (44 ± 30)	184 ± 8
ORS control	20 ± 4 (33 ± 16)	100
IFN-β-treated ORS	30 ± 5 (38 ± 25)	164 ± 14
EW-24 control	32 ± 7 (80 ± 31)	100
IFN-β-treated EW-24	52 ± 5 (80 ± 17)	147 ± 9

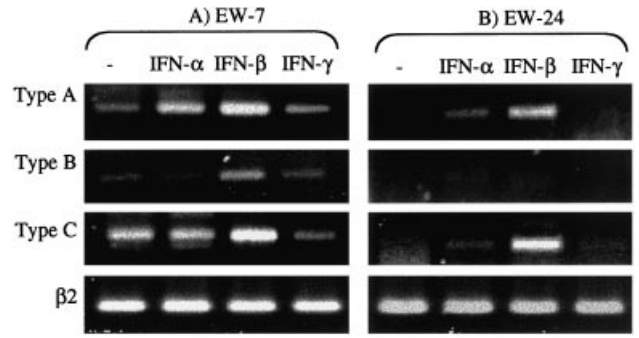

Figure 4 RT–PCR analysis of γ-GT transcripts in EW-7 and EW-24 cells stimulated with IFNs

cDNA from untreated or treated ET cells by IFN-α, -β and -γ (1000 units/ml) cultured for 48 h were used as templates for PCR reactions using specific primers for γ-GT (type A, foetal liver; type B, HepG2; type C, placenta) and β_2-microglobulin (control). PCR products were run on 1.5% agarose gels. (**A**) EW-7 cells, (**B**) EW-24 cells.

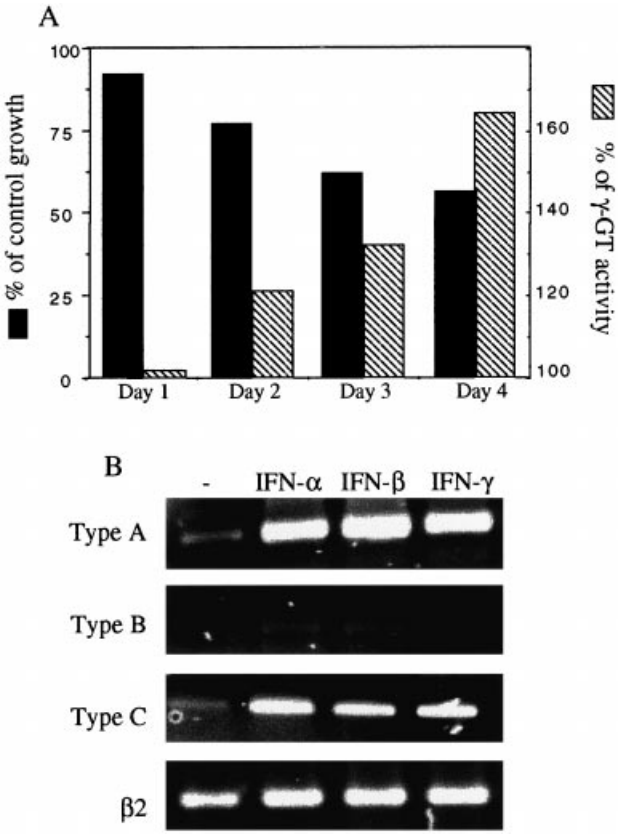

Figure 5 Effects of IFNs on cell growth and γ-GT of RPMI 8266 cells

(**A**) RPMI 8266 cells were cultured in the absence or presence of 1000 units/ml IFN-f. Cells were assayed for cell growth and cell surface γ-GT activity. Cell proliferation is expressed as the percentage of growth of untreated cells. γ-GT activity is expressed as the percentage of activity of unstimulated cells (100% = 19000 pmol $\cdot$ 30 $min^{-1} \cdot \mu g^{-1}$). (**B**) γ-GT transcripts of RPMI 8266 cells cultured in the absence or presence of IFNs (1000 units/ml) for 24 h were run on 1.5% agarose gels. PCR products for β2 microglobulin served as controls.

IFN-mediated up-regulation of γ-GT was similarly observed in whole cell homogenates (Figure 3A). The EW-7 cell line was cultured for up to 3 days in the presence of increasing concentrations (from 10 to 5000 units/ml) of IFN-β. IFN-β increased the γ-GT activity in a dose-dependent manner both at the cell surface and intracellularly (Figure 3B).

FACS analysis confirmed that the IFN-mediated up-regulation in cell surface γ-GT activity of EW-7 cells was associated with the increase in cell surface γ-GT protein levels (Table 2; increase in % and/or SF). IFN-β similarly up-regulated cell surface γ-GT activities in other ET cell lines, and such an enhancement was mirrored by augmented levels of surface γ-GT (Table 2). IFN-β similarly increased intracellular γ-GT activities of all ET cells (results not shown).

Modulation of γ-GT mRNA species levels in IFN-treated ET cells

To determine whether the up-regulation of γ-GT in ET cells by IFNs was related to alterations at the level of γ-GT mRNAs, further reverse transcriptase (RT)–PCR analyses were performed (Figure 4). RT–PCR was performed on the β_2-microglobulin (β2) gene, which gave bands of the linear amplification area, and with the specific primer sets for RT–PCR corresponding to the human γ-GT mRNA of foetal liver (type A), HepG2 cells (type B) and placenta (type C). As seen in Figure 4(A, control), γ-GT mRNA expression was polygenic in EW-7 cells, with the prevalence of types A and C higher than type B. Stimulation with IFN-α or IFN-β led to an enhancement of type A mRNA product (Figure 4A), whereas type B and type C were increased only upon IFN-β treatment (Figure 4A). IFN-γ did not significantly affect the expression of three γ-GT mRNA entities (Figure 4A). Up-regulation of γ-GT transcripts by IFNs was a long-lasting process, since the responses were observed within 24–48 h of IFN treatment (results not shown).

The types of γ-GT mRNAs in the metastatic EW-24 cell line were also analysed. Undetectable γ-GT messages were found in untreated EW-24 cells (Figure 4B). As observed above for EW-7 cells, IFN-α and IFN-β induced type A mRNA product and IFN-β induced type C mRNA product (Figure 4B). IFN-γ had no effect on any γ-GT mRNA species (Figure 4B).

Modulation of γ-GT in the IFN-responsive RPMI 8226 cell line

We investigated whether our observations on ET cells were validated on another model cell line, the RPMI 8226 B cells, in which both IFNs type I and II are known to inhibit cell growth equally [31]. Figure 5(A) shows that the treatment of RPMI 8266

cells with 1000 units/ml IFN-α inhibited cell growth and that there was a significant increase in γ-GT activity. Results of PCR showed a significant increase in type A and C γ-GT mRNA species after addition of IFN-α, -β and -γ (Figure 5B).

DISCUSSION

In the present study, we investigated γ-GT expression in human cell lines established from ET at different stages of the disease. Although these cell lines exhibited a similar phenotype, the expression and the status of various genes, e.g. the p53 gene, differed. Three of the five ET cell lines studied, EW-7, COH and EW-24 expressed wild-type p53, whereas EW-1 and ORS cells expressed a mutated p53 gene. All ET cell lines consistently displayed a functional γ-GT, whose expression however did not discriminate steps of ET progression. Moreover, no apparent link between γ-GT expression and p53 mutation was detected. Most importantly, this is the first study to show the stimulation of γ-GT by IFNs.

In addition to their antiviral activity, type I (α, β) and type II (γ) IFNs has potent antiproliferative and immunomodulatory effects [32,33]. Although ET cells expressed type I and type II IFN receptors [22,34], they are more responsive to the antiproliferative effects of IFN-α and IFN-β when compared with that of IFN-γ [22]. Moreover, cell cycle analyses indicated that IFN-α and IFN-β arrested the growth of EW-7 cells in the S-phase. The effects of IFNs on the cell cycle appear diverse [35–40]. For example, IFN-α inhibits the growth of Daudi cells at the G_o/G_1 boundary of the cell cycle [35]. IFN-β induces accumulation of transformed cells, but not of normal cells, in the S-phase [36,37]. Similarly, IFN-α and IFN-β inhibit glioma cell growth due to accumulation of cells in the S-phase [38]. Alternatively, IFNs arrest various cell types in a cell cycle phase-independent manner [39,40].

Concomitant to the IFN-mediated cell growth arrest, IFN, in a dose- and time-dependent manner, increased γ-GT (intracellular) and membrane expression in ET cells. This up-regulation was associated with an enhancement of γ-GT mRNA species. These observations on ET cells were validated on another human IFN-responsive B cell line in which IFN similarly up-regulated γ-GT at the mRNA and membrane levels.

In IFN signalling, activation of the associated tyrosine kinase JAK1 (Janus kinase)/TYK2 (for IFN-α and IFN-β) or JAK1/JAK2 (for IFN-γ) leads to phosphorylation of latent transcriptional factors, such as signal transduction and activators of transcription (STAT), followed by their dimerization, nuclear translocation and site-specific DNA binding leading to gene activation [41–43]. IFN-α, IFN-β1 and STAT2 heterodimerize and activate transcription by binding to an IFN-stimulated response element in conjunction with a 48 kDa protein of a different DNA binding family [41]. IFN-γ activated STAT1, which bound to an IFN-γ-activated site (GAS) to induce various gene promoters, including the interferon responsive factor-1 (IRF-1) gene [41,42]. It was also the case for the ectopeptidase dipeptidyl peptidase IV (DPPIV/CD26), which was up-regulated by IFNs through the signalling pathway involving STAT1 and the GAS response element of its promoter [44]. Newly synthesized IRF-1 bound interferon regulatory factor element sequences which overlapped with IFN-stimulated response element sequences [45,46]. Among IRF-1-activated genes, several gene coding for enzymes have been identified, which include 2′,5′-oligo A-synthetase, inducible nitric oxide synthase and interleukin-converting enzyme [45,46]. IFN-γ is capable of inducing the ectopeptidase aminopeptidase N/CD13 on human epithelial cells but direct activation of the CD13 gene remains to be demonstrated [47]. Due to the complexity of the human γ-GT gene details of sites for transcription factors have not been established. Potential sites for AP1, AP2, cAMP-response-element-binding protein, GRE and stimulating protein-1 transcription factors have been recently identified [12]. A previous study [22] from our laboratory showed STAT1 activation and IRF1 induction in IFN-stimulated EW-7 cells. However, given the slow kinetics of γ-GT mRNA accumulation and protein increase, the enhancing effects of IFNs on γ-GT could be mediated through indirect IFN-mediated signals.

Little is known about the regulation of γ-GT by physiological stimuli. Interleukin-15 increased the levels of γ-GT mRNA in human activated T cells [48]. Treatment of the myeloid KG-1 cell line with interleukin-1β or TNF-α resulted in a major increase in mRNA and enzyme activity levels [49]. Unstimulated or IFN-stimulated EW-7 and RPMI 8226 cells did not produce TNF-α and this excludes the eventual participation of this cytokine in the enhancing effect produced by IFN on γ-GT. Whether the induction of γ-GT by IFNs is mediated through endogenous cytokines produced in an autocrine loop remains to be elaborated.

A crucial question arising from our study is whether an increase in γ-GT is required to maintain the ET growth suppression by IFNs, i.e. whether up-regulation of γ-GT confers a selective advantage or disadvantage on IFN-treated T cells. Previous studies have implicated γ-GT in the regulation of cell growth and survival. Our recent observations however suggest a lack of participation of γ-GT in these cellular processes [50]. Interestingly, a molecular association between cell surface γ-GT and some members of the tetraspan 4 family, including CD53, CD81 and CD82 has been reported [24]. Whether such γ-GT-associated molecules mediate signal transduction involved in the modulation of cell responses has to be considered. Alternatively, we cannot exclude the possibility that the participation of γ-GT is marginal to ET cell behaviour. The full significance and function of γ-GT in IFN-treated ET cells require further study.

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REFERENCES

1 Antczak, C., De Meestner, I. and Bauvois, B. (2001) Ectopeptidases in pathophysiology. Bioessays **23**, 251–260
2 Antczak, C., De Meester, I. and Bauvois, B. (2001) Transmembrane proteases as disease markers and targets for therapy. J. Biol. Regul. Homeost. Agents **15**, 130–139
3 Tate, S. S. and Meister, A. (1981) γ-Glutamyltranspeptidase: catalytic, structural and functional aspects. Mol. Cell. Biochem. **39**, 357–368
4 Whitfield, J. B. (2001) γ Glutamyl transferase. Crit. Rev. Clin. Lab. Sci. **38**, 263–355
5 Woodlock, T. J., Brown, R., Mani, M., Pompeo, L., Hoffman, H., Segel, G. B. and Silber, R. (1990) Decreased L system amino acid transport and decreased γ-glutamyl transpeptidase are independent processes in human chronic lymphocytic leukemia B-lymphocytes. J. Cell. Physiol. **45**, 217–221
6 Bulle, F., Mattei, M. G., Siegrist, S., Pawlak, A., Passage, E., Chobert, M. N., Laperche, Y. and Guellaen, G. (1987) Assignment of the human γ-glutamyl transferase gene to the long arm of chromosome 22. Human Genet. **76**, 283–286
7 Heistekamp, N. and Groffen, J. (1988) The first BCR gene intron contains breakpoints in Philadelphia chromosome positive leukemia. Nucleic Acids Res. **16**, 8045–8056
8 Morris, C., Courtay, C. and Geurts van Kessel, A. (1993) Localization of a γ-glutamyl-transferase-related gene family on chromosome 22. Human Genet. **1**, 31–36
9 Courtay, C., Heisterkamp, N., Siest, G. and Groffen, J. (1994) Expression of multiple γ-glutamyltransferase genes in man. Biochem. J. **297**, 503–508

10 Collins, J. E., Mungall, A., Badcock, K. L., Fay, J. M. and Dunham, I. (1997) The organization of the γ-glutamyl transferase genes and other low copy repeats in human chromosome 22q11. Genome Res. **7**, 522–531
11 Chikhi, N., Holic, N., Guellaen, G. and Laperche, Y. (1999) γ-Glutamyl transpeptidase gene organization and expression: a comparative analysis in rat, mouse, pig and human species. Comp. Biochem. Physiol. Biochem. Mol. Biol. **122**, 367–380
12 Visvikis, A., Pawlak, A., Accaoui, M. J., Ichino, K., Leh, H., Guellaen, G. and Wellman, M. (2001) γ-Glutamyltranspeptidase-dependent glutathione catabolism results in activation of NF-kB. Eur. J. Biochem. **268**, 317–325
13 Aurias, A., Rimbaut, C., Buffe, C., Dubousset, J. and Mazabraud, A. (1984) Translocation involving chromosome 22 in Ewing's sarcoma. A cytogenetic study of four fresh tumors. Cancer Genet. Cytogenet. **12**, 21–25
14 Horvath, C. and Darnell, Jr, J. (1997) The state of the STATs: recent developments in the study of signal transduction to the nucleus. Curr. Opin. Cell Biol. **9**, 233–239
15 Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G. et al. (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature (London) **359**, 162–165
16 Bailly, R. A., Bosselut, R., Zucman, J., Cormier, F., Delattre, O., Roussel, M., Thomas, G. and Gysdael, J. (1993) DNA-binding and transcriptional activation properties of the EWS–FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. Mol. Cell. Biol. **14**, 3230–3241
17 May, W. A., Lessnick, S. L., Braun, B. S., Klemsz, M., Lewis, B. C., Lunsford, L. B., Hromas, R. and Denny, C. T. (1993) The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol. Cell Biol. **13**, 7393–7398
18 Rao, V. N., Ohno, T., Prasad, D. D., Bhattacharya, G. and Reddy, E. S. (1993) Analysis of the DNA-binding and transcriptional activation functions of human Fli-1 protein. Oncogene **11**, 1049–1054
19 Kovar, H., Aryee, D. N., Henockl, J. G., Schemper, M., Delattre, O., Thomas, G. and Gadner, H. (1996) EWS/FLI-1 antagonists induce growth inhibition of Ewing tumor cells *in vitro*. Cell Growth Differ. **7**, 429–437
20 Tanaka, K., Iwakuma, T., Harimaya, K., Sato, H. and Iwamoto, Y. (1997) EWS-Fli1 antisense oligodeoxynucleotide inhibits proliferation of human Ewing's sarcoma and primitive neuroectodermal tumor cells. J. Clin. Invest. **99**, 239–247
21 Jaishanker, S., Zhang, J., Roussel, M. F. and Baker, S. J. (1999) Transforming activity of EWS/FLI is not strictly dependent upon DNA-binding activity. Oncogene **18**, 5592–5597
22 Sancéau, J., Hiscott, J., Delattre, O. and Wietzerbin, J. (2000) IFN-β induces serine phosphorylation of Stat-1 in Ewing's sarcoma cells and mediates apoptosis via induction of IRF-1 and activation of caspase-7. Oncogene **19**, 3372–3383
23 Kovar, H., Auinger, A., Jug, G., Aryee, D., Zoubek, A., Salver-Kuntschik, M. and Gadner, H. (1993) Narrow spectrum of infrequent p53 mutations and absence of MDM2 amplification in Ewing tumours. Oncogene **8**, 2683–2690
24 Hamelin, R., Zucman, J., Melot, T., Delattre, O. and Thomas, G. (1994) p53 mutations in human tumors with chimeric EWS/FLI-1 genes. Int. J. Cancer **57**, 336–340
25 Bauvois, B., Van Weyenbergh, J., Rouillard, D. and Wietzerbin, J. (1996) TGF-β1-stimulated adhesion of human mononuclear phagocytes to fibronectin and laminin is abolished by IFN-γ: dependence on α5 β1 and β2 integrins. Exp. Cell. Res. **222**, 209–217
26 Nichols, T. C., Guthridge, J. M., Karp, D. R., Molina, H., Fletcher, D. R. and Holers, V. M. (1988) Monocytoid differentiation of freshly isolated human myeloid leukemia cells and HL-60 cells induced by the glutamine antagonist acivicin. Eur. J. Immunol. **28**, 4123–4129
27 Remvikos, Y., Tominaga, O., Hammel, P., Laurent-Puig, P., Salmon, R. J., Dutrillaux, B. and Thomas, G. (1992) Increased p53 protein content of colorectal tumours correlates with poor survival. Br. J. Cancer **66**, 758–764
28 Bauvois, B., Laouar, A., Rouillard, D. and Wietzerbin, J. (1995) Inhibition of γ-glutamyl transpeptidase activity at the surface of human myeloid cells is correlated with macrophage maturation and transforming growth factor β production. Cell Growth Differ. **6**, 1163–1170
29 Tsutsumi, M., Sakamuro, D., Takaida, A., Zang, S., Furukawa, T. and Taniguchi, N. (1996) Detection of a unique γ-glutamyl transpeptidase messenger RNA species closely related to the development of hepatocellular carcinoma in humans: a new candidate for early diagnosis of hepatocellular carcinoma. Hepatology **23**, 1093–1097
30 Allen, L., Meck, R. and Yunis, A. (1980) The inhibition of γ-glutamyl transpeptidase from human pancreatic carcinoma cells by (αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125; NSC 163501). Res. Commun. Chem. Pathol. Pharmacol. **27**, 175–182
31 Lasfar, A., Wietzerbin, J. and Billard, C. (1994) Differential regulation of interleukin-6 receptors by interleukin-6 and interferons in multiple myeloma cell lines. Eur. J. Immunol. **24**, 124–130
32 Billiau, A. and Vandenbroeck, K. (2000) IFN-γ. In Pharmacology of Cytokines (Mantovani, A., Dinarello, C. A. and Ghezzi, P., eds.), pp. 641–688, Oxford University Press, Oxford
33 Tossing, G. (2001) New developments in interferon therapy. Eur. J. Med. Res. **6**, 47–65
34 Rosolen, A., Todesco, A., Colamonici, O. R., Basso, G. and Frascella, E. (1997) Expression of type I interferon receptor in solid tumors of childhood. Mod. Pathol. **10**, 55–61
35 Teifenbrun, N., Melamed, D., Levy, N., Resnitzky, D., Hoffmann, I., Reed, S. and Kimchi, A. (1996) α-Interferon suppresses the cyclin D3 and cdc25A genes, leading to a reversible Go-like arrest. Mol. Cell. Biol. **16**, 3934–3944
36 Muro, M., Naomoto, Y. and Orita, K. (1991) Mechanism of the combined antitumor effect of natural human tumor necrosis factor-α and natural human interferon-α on cell cycle progression. Jpn J. Cancer Res. **82**, 118–126
37 Qin, X. Q., Runkel, L., Deck, C., DeDios, C. and Barsoum, J. (1997) Interferon-β induces S phase accumulation selectively in human transformed cells. J. Interferon Cytokine Res. **17**, 355–367
38 Naidu, K. A., Wiranowska, M., Phuphanich, S. and Porckop, L. D. (1996) Modulation of glioma cell growth and 5-lipoxygenase expression by interferon. Anticancer Res. **16**, 3475–3482
39 Preisler, H. D., Gopal, V., Banavali, S. D., Finke, D. and Bokari, S. A. (1992) Multiparameter assessment of the cell cycle effects of bioactive and cytotoxic agents. Cancer Res. **52**, 4090–4095
40 Joshi, C. V., Supriya, P. and Ajitkumar, P. (1999) Growth inhibition of human promonocytic leukaemic U937 cells by interferon γ is irreversible and not cell cycle phase-specific. Cytokine **11**, 673–678
41 Decker, T. and Kovarik, P. (2000) Serine phosphorylation of STATs. Oncogene **19**, 2628–2637
42 Seidel, H. M., Lamb, P. and Rosen, J. (2000) Pharmaceutical intervention in the JAK/STAT signaling pathway. Oncogene **19**, 2645–2656
43 Ramana, C., Chatterjee-Kishore, M., Nguyen, H. and Stark, G. R. (2000) Complex roles of Stat1 in regulating gene expression. Oncogene **19**, 2619–2627
44 Bauvois, B., Djavaheri-Mergny, M., Rouillard, D., Dumont, J. and Wietzerbin, J. (2000) Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. Oncogene **19**, 265–272
45 Vaughan, P. S., Van Wijnen, A. J., Stein, J. L. and Stein, G. S. (1997) Interferon regulatory factors: growth control and histone gene regulation – it's not just interferon anymore. J. Mol. Med. **75**, 348–359
46 Harada, H., Taniguchi, T. and Tanka, N. (1998) The role of interferon regulatory factors in the interferon system and cell growth control. Biochimie **80**, 641–650
47 Riemann, D., Kehlen, A. and Langner, J. (1995) Stimulation of the expression and the enzyme activity of aminopeptidase N/CD13 and dipeptidylpeptidase IV/CD26 on human renal cell carcinoma cells and renal tubular epithelial cells by T cell-derived cytokines, such as IL-4 and IL-13. Clin. Exp. Immunol. **100**, 277–283
48 Karp, D. R., Carlisle, L., Mobley, A. B., Nichols, T. C., Oppenheimer-Marks, N., Brezinschek, R. I. and Holers, V. M. (1999) γ-Glutamyl transpeptidase is up-regulated on memory T lymphocytes. Int. Immunol. **11**, 1791–1800
49 Miller, A. M., Sandler, E., Kobb, S. M., Eastgate, J. and Zucali, J. (1993) Hematopoietic growth factor induction of γ-glutamyl transferase in the KG-1 myeloid cell line. Exp. Hematol. **21**, 9–15
50 Antczak, C., Karp, D. R., London, R. E. and Bauvois, B. (2001) Reanalysis of the role of γ-glutamyl transpeptidase in the cell activation process. FEBS Lett. **508**, 226–230

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