Gamma-glutamyltransferase expression during all-*trans* retinoic acid-induced differentiation of hematopoietic cell lines

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Received 16 May 1995; revised version received 20 June 1995

Abstract Gamma-glutamyltransferase activity, genes transcripts and differentiation by all-*trans* retinoic acid have been investigated in cultured HL-60, U937 and K562 cells. Acquisition of morphological and functional characteristics confirmed the terminal differentiation of HL-60 and U937 cells. All-*trans* retinoic acid increased gamma-glutamyltransferase activity in a cell type- and time-dependent manner. Treatments with all-*trans* retinoic acid isomers and structurally analogs showed that only retinoids with carboxylic acid group were able to induce enzyme activity in terminal differentiated cells. Additionally, the analysis of gamma-glutamyltransferase genes transcription products demonstrated clearly that, both in untreated and in RA treated cells, only mRNA type I transcribed from the gene 6, was expressed.

Key words: Differentiation; Gamma-glutamyltransferase; Hematopoietic cell line; Retinoic acid; Retinoid

1. Introduction

Gamma-glutamyltransferase (GGT, EC 2.3.2.2), a glycosylated plasma membrane enzyme, is one of the key enzymes involved in glutathione (GSH) metabolism, GSH-detoxification pathway and other metabolic processes including the conversion of leukotriene C_4 to leukotriene D_4 [1,2]. GGT expression is regulated by cytokines, growth factors, glucocorticoids and several xenobiotics [3–5]. The relative expression of GGT genes is not well established yet, however, Courtay et al. [6] reported that GGT genes are expressed in several tissues in a specific manner. Several blood cells and their hematopoietic precursors express GGT, but only little is known regarding the significance and the regulation of this enzyme, especially during normal or pathological hematopoiesis. The particular plasma membrane localization of GGT makes possible its implication in events stimulated by hematopoietic factors leading to proliferation and differentiation. Indeed, a novel role for GGT in the signal transduction pathway induced by hematopoietic cytokines, especially colony stimulating factors, has been described [3].

Retinoids, a group of naturally and synthetic molecules structurally related to vitamin A exert profound effects on the growth and differentiation of a wide variety of cells and tissues [7]. Direct antiproliferative effects of retinoic acid (RA) were observed in various cells such as F9 teratocarcinoma cells and HL-60 or U937 myeloid cells [8–10] concomitantly to differentiation and reversion of malignant phenotypes. Cell maturation often heralds the acquisition of differentiation markers. However, the majority of these markers are not specific for one particular mature phenotype. Novogrodsky et al. [11] suggested that the surface localization of GGT and the relatively easier determination of its activity should make this enzyme useful as a surface marker for normal and neoplastic lymphoid cells in various stages of differentiation. However, the role and the expression of GGT in normal and pathological hematopoietic cells have not been clearly elucidated. In addition, no information is available on the effect of RA on GGT expression in established hematopoietic cell lines. Tsao and Batist, demonstrated that RA induces GGT expression in a dose- and time-dependent manner in rat epithelial cells [12]. Additionally GGT is induced by RA in cultured rat osteoblasts (UMR 106) [13] and in cerebral endothelial cells (CR3) [14]. GGT activity is also induced in KG-1 cells stimulated by hematopoietic growth factors, supporting an eventual role for GGT in the cellular events occuring in myeloid cells response to hematopoietic growth factors [3].

The purpose of this work was to study the ability of all-*trans* retinoic acid to affect GGT genes expression and enzyme activity in HL-60, K562 and U937 cell lines.

2. Materials and methods

2.1. Cell lines and culture conditions

The human leukemic HL-60 (promyelocytic cells), K562 (early myeloid cells) and U937 (promonocytic cells) were from the European Collection of Animal Cell Cultures. The cells were grown in RPMI-1640 medium (Gibco-BRL, France) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Boehringer-Mannheim, France), 1% (v/v) antibiotic-antimycotic (Gibco-BRL, France) and 2 mM L-glutamine (Gibco-BRL, France). Cultures were maintained at 37°C in a humidified atmosphere of air/CO_2 (95%:5%).

2.2. Drug treatment induced differentiation

The retinoids tretinoin (all-*trans* retinoic acid), isotretinoin (13-*cis*-retinoic acid), acitretin (all-*trans*-9-(methoxy-2,3,6-trimethyl phenyl)3,7-dimethyl-2,4,6,8-non-atetraecanoic acid), RO 13-7410 (*p*-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl) propenyl] benzoic acid), RO 13-6307 ([all-E]-3-methyl-7-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-2,4,6-octatrienoic acid) were kindly provided by Dr. Schneider F. (Hoffman-La Roche, Basel, Switzerland). They were initially dissolved in ethanol (Merck, France) to obtain 10 mM stock solutions, stored in the dark at −20°C, and then diluted in

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Abbreviations: GGT, gamma-glutamyltransferase; PBS, phosphate-buffered saline; RA, all-*trans* retinoic acid; DMSO, dimethylsulfoxide; NBT, nitro-blue-tetrazolium; TPA, 12–0-tetradecanoylphorbol-13-acetate; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); APL, acute promyelocytic leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodysplasic syndrom.

SSDI 0014-5793(95)00753-9

medium to obtain appropriate final concentrations. Maximum concentration of ethanol in the culture (<0,1%) did not influence cell growth, differentiation or GGT activity. Following treatment, the cells (protected from light) were harvested by centrifugation (5 min, 400 × g) and cell pellets were washed twice with PBS (Eurolab, France) before storing at −80°C until use.

2.3. Enzyme assay

GGT activity was determined in the cell pellets treated with 0,1% (v/v) Triton X-100 (Sigma, France) and incubated on ice for 30 minutes. The cell homogenates were used for GGT activity determination with 6 mM L-γ-glutamyl-3-carboxy-4-nitroanilide (Boehringer-Mannheim, France) as a donor substrate and 150 mM glycyl-glycine (Merck, France) as the acceptor substrate in 0,1 M Tris-HCl pH 6.8 [15]. GGT activity was expressed as mU/mg proteins corresponding to nmoles/min/mg proteins. Proteins were determined as described by Lowry et al. [16] using bovine serum albumine (Sigma, France) as a standard.

2.4. Assessment of differentiation and cell proliferation

Reduction of Nitro-blue-tetrazolium (NBT) (Sigma, France), a typical marker of myelomonocytic differentiation was assayed colorimetrically as reported by Kasukabe et al. [17]. 200 μl of cell suspension (10^6 cells) were incubated at 37°C, for 2 h with an equal volume of a solution containing 1 mg/ml NBT and 100 ng/ml TPA (Sigma, France). The reaction was stopped by addition of 50 μl HCl (1 M final concentration). The blue/black formazan precipitate obtained, was solubilized in DMSO and the absorbance was determined at 540 nm.

Cell proliferation was assayed by MTT test according to Page et al. [18]. Briefly, 20 μl of MTT solution (5 mg/ml in PBS) (Sigma, France) were added to 200 μl of cell suspension and the mixtures were incubated at 37°C in 96 well plate dish for 4 hours. After centrifugation, the formazan precipitates were dissolved in DMSO and absorbances were determined at 540 nm. Results are expressed as percentage of growth inhibition according to untreated cells.

2.5. Cell adhesion

Cell adhesion was assayed as described by Baud et al. [19]. Briefly, after treatment, nonadherent cells were harvested by centrifugation, washed twice with PBS and solubilized in 3% (w/v) SDS. Resting adherent cells were washed with PBS and scraped directly in 3% SDS. Absorbance at 280 nm of solubilized cell pellets from both nonadherent and adherent cells were then measured. Results are expressed as a percentage of adherent cells calculated from the ratio between A_{280nm} of adherent cells and the sum of A_{280nm} of adherent and non-adherent cells.

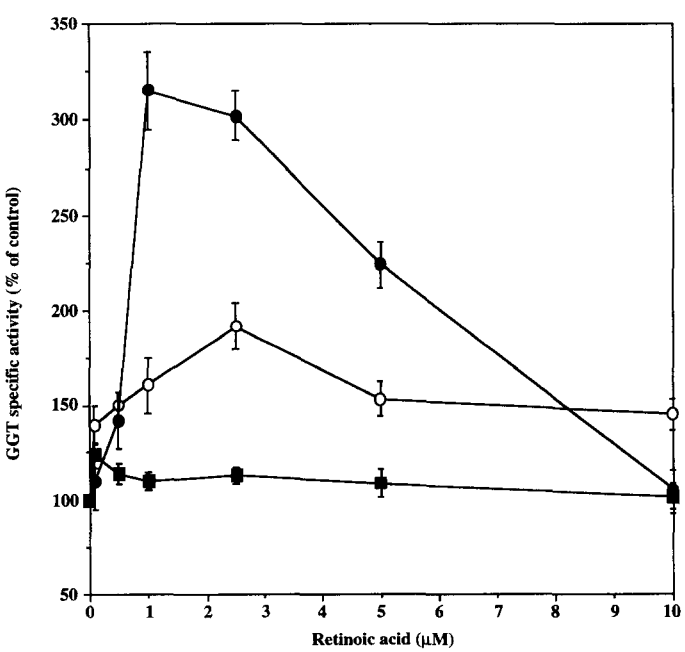

Fig. 1. Dose-dependent effect of retinoic acid treatment on GGT activity in HL-60, U937 and K562 cell lines. Cells were treated during 48 hours with various concentrations of RA under conditions described in section 2. GGT is expressed as a percentage of control values which were 40.55 ± 2.50, 71.90 ± 4.50 and 16.95 ± 3.50 mU/mg for HL-60 (●), U937 (○) and K562 (■) cells, respectively. The results represent the means ± S.D. of three separate measurements.

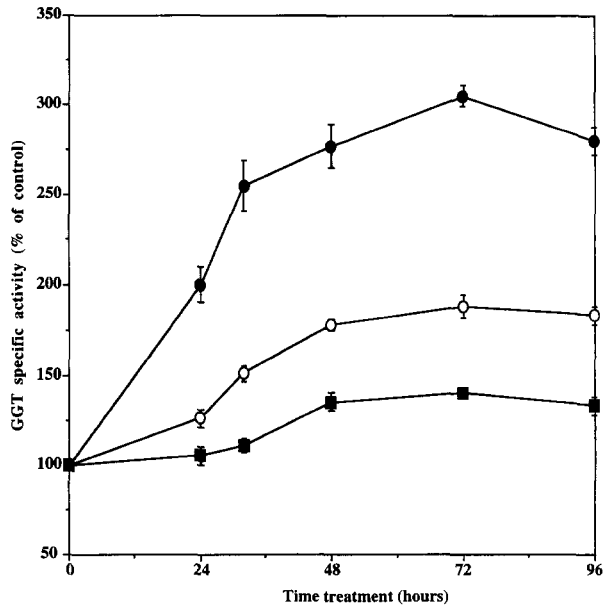

Fig. 2. Time course of GGT activity during retinoic acid treatment. U937 (○) and HL-60 (●) were treated waith 1 μM RA and K562 (■) cells with 2.5 μM RA. GGT is expressed as percentage of control values indicated in Fig. 1. The results represent the means ± S.D. of three separate measurements.

2.6. RT-PCR method and analysis of amplified products

Total RNA was isolated from untreated and treated HL-60, K562 and U937 cells using the guanidium thiocyanate method described by Chomczynski and Sacchi [20]. 5 μg of RNA were subjected to reverse transcription using MMLV-reverse transcriptase (Gibco-BRL, France) according to the experimental procedure described by the manufacturer. This was followed by standard PCR according to Courtay et al. [6] and using two probes: Amp 1 (5′-AATGGATGACTTCAGCTCT-3′) and Amp 2 (5′-TCTACAACCTCTGGTTCGGCT-3′) which represent the 5′ and 3′ primers, respectively (Eurogentec, Belgium). The presence of different GGT genes transcripts in amplified products was detected by Southern blot and hybridization with corresponding [γ-^{32}P]ATP-labeled oligonucleotides probes according to Courtay et al. [6]. The oligonucleotides probes used, specific for gene 1, 3 and 6, respectively, were:

Amp 5 (5′-CTGATATGTGTCACCCCTTTTCT-3′)
Mut 3 (5′-CTCTTGTCCATGTGCCTGAC-3′)
Mut 6 (5′-CCAACGAGTTTGGGGTACCC-3′)

Optimal washing temperatures were: 55°C, 67°C and 65°C for respectively Amp 5, Mut 3 and Mut 6 probes (Eurogentec, Belgium).

3. Results

3.1. Effect of retinoic acid on cell proliferation, differentiation and cell adhesion

It is well known that HL-60 and U937 cells treated with All-*trans* retinoic acid differentiate efficiently toward neutrophils and macrophages, respectively [9,10]. By contrast, RA did not induce differentiation of K562 cells [21]. We, first, examined the effect of RA on cell growth, cell adhesion and acquisition

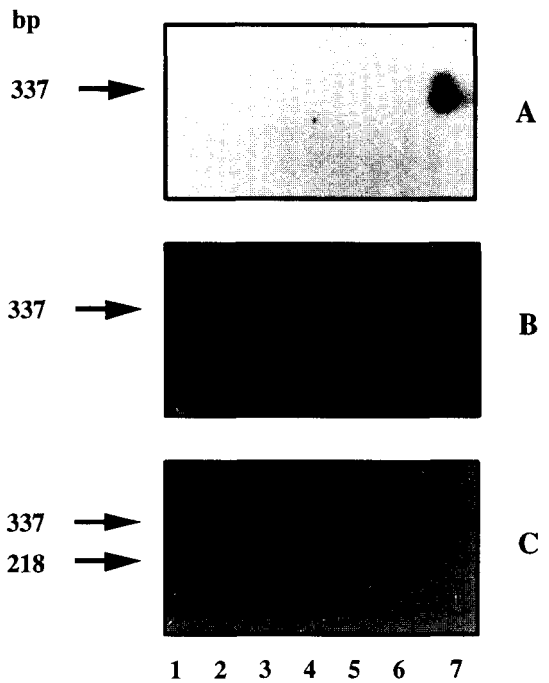

Fig. 3. Autoradiography of Southern blot hybridization of amplified RT-PCR transcripts of GGT genes. Cells were treated during 96 hours, under conditions described in section 2, before total RNA extraction. PCR products, from GGT genomic 1.6 Kb *Bgl* II fragment described by Courtay et al. [6] serve as positive controls. They are indicated by 337 pb fragments corresponding to gene 1 (lane 7A), gene 3 (lane 7B) and gene 6 (lane 7C). RNAs used in this study were isolated from U937 control cells (lane 1), 1 μM RA treated U937 cells (lane 2), HL-60 control cells (lane 3), 1 μM RA treated HL-60 cells (lane 4), K562 control cells (lane 5), 2.5 μM RA treated K562 cells (lane 6). Hybridation was performed with the following probes: A, Mut 3 ; B, Amp 5 and C, Mut 6.

of NBT reduction capacity by treated cells. In both HL-60 and K562 treated cells, a slight growth inhibition (6%) was observed when the cells were treated with 1 μM RA. The percentage of growth inhibition increased with the concentration of drug and reached 15% and 30% values for the HL-60 and K562 cells, respectively, when treated with 10 μM RA. More pronounced growth inhibition, reaching 45% was observed for U937 cells treated with increasing doses of RA ranging from 0,1 to 10 μM.

Functional differentiation process was followed by the evaluation of NBT reducing activity of untreated and RA treated cells. Cells were incubated with RA during 4 days at the final concentrations of 1 μM for HL-60 and U937 cells, and 2.5 μM for K562 cells. Table 1 shows NBT reduction in terms of variations in the absorbances at 540 nm. Data obtained showed that RA induced an increase of NBT reduction in both HL-60 and U937 cells, stimulated with TPA, as described by Breitman et al. [9] and Olsson et al. [10]. By contrast, NBT reduction activity was not modified in RA-K562 treated cells.

Table 1
Influence of RA treatment on NBT reducing activity and cell adhesion

		NBT reducing activity (A_{540} nm/10^6 cells)	Cell adherence (%)
HL-60	Control	0.25 ± 0.01	2.05 ± 0.10
	RA (1 μM)	0.82 ± 0.02	26.45 ± 2.50
U937	Control	0.11 ± 0.10	2.40 ± 0.15
	RA (1 μM)	0.62 ± 0.15	28.00 ± 3.20
K562	Control	1.16 ± 0.20	2.40 ± 0.20
	RA (2.5 μM)	1.13 ± 0.18	2.70 ± 0.18

Cells were cultured under conditions described in section 2. RA was added to the culture medium, and both control and treated cells were cultured during 4 days before performing NBT reducing activity and cell adhesiveness measurement. NBT activity was performed for 10^6 cells and results are expressed as A_{540nm}. Cell adhesiveness was evaluated by cell scraping and measurement of A_{280nm} of SDS cell lysates.

Table 2
Effect of retinoids on GGT activity in HL-60 and U937 cells

	GGT activity (mU/mg protein)	
Retinoids	HL-60 cells	U937 cells
Control	39.60 ± 5.00	70.50 ± 5.00
Retinoic acid	142.20 ± 6.00	105.75 ± 5.00
RO 13-6307	143.50 ± 3.00	122.80 ± 3.00
Isotretinoine	134.68 ± 5.00	102.20 ± 9.00
RO 13-7410	130.68 ± 2.00	190.35 ± 6.00
Acitretine	99.00 ± 9.00	109.27 ± 8.00
RO 40-8757	50.88 ± 6.00	66.97 ± 15.00

Cells were exposed to 1 μM of several retinoids during 2 days and GGT activity was measured as described in section 2. Results represent mean ± S.D. of three separate measurements.

Concomitantly to cell growth inhibition, we observed an increase in cell adhesiveness of HL-60 and U937 RA treated cells (Table 1). By contrast, RA treatment did not affect K562 cell adherence, even at a higher concentration (10 μM). This is probably because of the lack in differentiation of these cells after RA treatment.

3.2. *Effect of RA on GGT specific activity*

In order to study the variation of GGT activity after cell differentiation, we treated our cell lines with progressively increasing doses of RA during 48 hours and measured GGT activity in both treated and control cells. Fig. 1 shows that the exposure of cells to various concentrations of RA during 48 hours leads to a cell type-specific modulation of GGT activity. A significant increase of GGT activity was observed in HL-60 and U937 RA treated cells, whereas GGT activity was slightly modified in K562 RA treated cells. The highest increase of GGT activity was obtained for RA concentrations ranging from 0.1 μM to 5 μM for both HL-60 and U937 cells. Treatment with higher concentrations of RA did not increase GGT activity in these two cell lines.

So far, time-course experience was choosen to describe the effect of RA on GGT activity in the three cell lines K562, HL-60 and U937, during various periods of treatment. GGT activity increases in a time dependent manner (Fig. 2). After 72 hours treatment, GGT induction was about 180% and 305% of control values for U937 and HL-60 cells, respectively. In K562 cells, GGT activity increases slightly and the maximal induction (130%) was observed after 48 hours of treatment.

The effect of five structural analogs of RA was then studied. Table 2 summarizes their effects on GGT activity in HL-60 and U937 cells. In comparison to RA effect, similar results were obtained when the HL-60 and U937 cells were treated with 1 μM RO 13-6307 and isotretinoin. Lower induction effect was observed when these cells were treated with acitretin and RO 40-8757. In contrast, RO 13-7410 treatment of U937 cells resulted in an induction of 270%, which was higher than that observed after RA treatment.

3.3. GGT gene expression in RA treated cells

RT-PCR followed by hybridization with specific oligonucleotides probes was performed in order to characterize GGT genes expressed in studied cells. In all studied samples, a PCR product of 218 pb, corresponding to all possible GGT mRNAs was observed on an ethidium-bromide stained gel (data not shown). Hybridization experiments (Fig. 3) using specific probes for the GGT genes 1, 3 and 6 demonstrated that U937, HL-60 and K562 cells express only gene 6. Treatment of the cells with 1 μM RA did not result in the activation of transcription of any other gene than that expressed in untreated cells.

4. Discussion

GGT is one of the numerous enzymes supposed to be regulated during the differentiation process [11]. Actually, not enough data concerning GGT expression during hematopoietic cell differentiation are available. However, some previous studies showed that hematopoietic growth factors including some cytokines, induce GGT activity and mRNA level in KG-1 cell line [3]. Additionally, RA enhances GGT activity in several cells [12–14]. Retinoids capable of inducing cellular maturation in vitro have been proposed as therapeutical agents in diseases such as acute promyelocytic leukemia and myelodysplasic syndrom [22] which are characterized by a block in hematopoietic differentiation. Our results demonstrated that HL-60 and U937 treated with RA differentiated into mature cells. Concomitantly to cell differentiation, GGT activity was induced after RA treatment suggesting a typical regulation of this enzyme during differentiation process. In contrast, K562 cells which are known to lack in differentiation after RA treatment [21], did not exhibit a marked modification in GGT activity. In addition to RA, a series of retinoids, structurally apparented, have been studied for their ability to induce GGT activity in HL-60 and U937 cells. All retinoids tested, except RO 40-8757, induced GGT activity in these cells. However, there was a smaller increase in GGT activity in U937 cells which are known to be less sensitive than HL-60 cells [23]. Our results showed that only retinoids containing a terminal carboxylic acid group, such as tretinoin, RO 13-6307, isotretinoin, RO 13-7410 and acitretin, were able to induce GGT activity. Surprisingly, these retinoids are known to induce HL-60 and U937 cell differentiation [23]. Thus, our data clearly demonstrated that GGT induction in retinoids-treated HL-60 and U937 cells, seems to be a general phenomenon associated with the functional maturation and differentiation of both the promyelocytic and promonocytic studied cell lines.

GGT is encoded by a multigenic family of at least seven genes. Courtay et al. [6] reported that five GGT genes are expressed in man, in a tissue-restricted manner, suggesting that their products can exhibit distinct functions in various tissues. Gene 6 seems to be ubiquitously expressed in all tested tissues including lymphocytes and bone marrow [6]. By contrast, gene 1 was expressed only in placenta, sigmoid, thyroid and lung, while gene 3 expression was detected only in thyroid, sigmoid and placenta. Our purpose was to characterize gene 1, 3 and 6 transcripts in control and RA-treated cells. From our results, it became clear that only GGT gene 6 was expressed in HL-60, U937 and K562 cells, and that RA-induced GGT activity in both U937 and HL-60 cells, is related solely to the expression of gene 6.

In conclusion, we clearly demonstrated that GGT activity is induced during U937 and HL-60 cell differentiation process, by all-*trans* retinoic acid and some structurally related retinoids. However, it is at present unclear if the increased GGT activity level is due to increased transcription or increased post transcriptional stabilization of the GGT mRNAs. We do not know the significance and the mechanism by which RA differentially induced differentiation of HL-60 and U937 cells, but the HPLC analysis of methylated cytosine showed an hypomethylation of genomic DNA in these two cell lines during RA treatment (data not shown). Future studies to determine the GGT-specific genes methylation profils in these cells should clarify mechanisms and significance of its induction during cell proliferation and differentiation.

Acknowledgements: The authors thank Dr A. Visvikis for a critical reading of the manuscript, H. Bodaud for technical help and Dr F. Schneider (Hoffman-La Roche LTD, Basel, Switzerland) for providing us with the retinoids used in this work. H. Hachad was supported by grant from Ministère de la Recherche et de l'Enseignement supérieur-France, and H. Leh was supported by the Association pour la Recherche sur le Cancer (ARC-France).

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