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γ -Glutamyltransferase activity in human atherosclerotic plaques—Biochemical similarities with the circulating enzyme

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Abstract

Background and purpose: Serum γ -glutamyltransferase (GGT) activity has been identified as a predictor of complications of atherosclerosis, with a prognostic value for cardiovascular diseases and stroke. Human atherosclerotic lesions contain active GGT, which can give rise to pro-oxidant molecular species; thus a direct contribution of GGT to atherosclerosis progression is conceivable. The relationship between plaque and serum GGT is however unclear.

Methods and results: Human carotid plaques obtained from 18 consecutive patients undergoing carotid endoarterectomy were analyzed, of which 6 were used for anion exchange and gel filtration chromatography/western blot studies, 7 for β -lipoprotein precipitation, and 5 for RNA extraction and determination of low molecular weight thiols. Mean GGT activity in crude plaque homogenates was 60.9 ± 21.5 (S.D.) mU/g tissue. The characteristics of GGT activity were compared in plaque homogenates and in serum obtained from controls (healthy blood donors). The methods employed (anion exchange and gel chromatography, western blot) showed the presence in plaque homogenates of two distinct complexes containing GGT activity, one of which comparable with plasma LDL/GGT complexes. Accordingly, precipitation of β -lipoproteins from plaque homogenates resulted in removal of GGT activity. RT-PCR indicated in plaques the presence of GGT mRNA transcribed from GGT-I gene. Analysis of plaque extracts also revealed the presence of enzyme product cysteinyl-glycine both as free and protein-bound form, confirming that GGT-dependent pro-oxidant reactions may occur within the plaque environment.

Conclusions: The results obtained suggest the presence in plaques of a serum-like GGT protein, indicating that a direct contribution of serum GGT to enzyme activity found within atherosclerotic lesions is possible. Data also indicate the occurrence of GGT-mediated redox reactions within plaque environment, which might influence plaque progression.

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1. Introduction

Although best known as a reliable index of hepatobiliary dysfunction and alcohol abuse [1], serum levels of γ -glutamyltransferase (EC 2.3.2.2; GGT) have been repeatedly proposed to play an independent role in clinical evolution of cardiovascular diseases related with atherosclerosis, including stroke [2,3]. A series of studies indicated that

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GGT is associated with overall mortality and cardiovascular events, either in unselected populations [4–7] or in patients with ascertained coronary artery disease, independently from potential confounders including liver disease and alcohol abuse [8,9]. Interestingly, although conditions associated with increased atherosclerosis such as obesity, elevated serum cholesterol, high blood pressure and myocardial infarction are positive determinants of serum GGT activity, all studies mentioned above found that GGT predictive value for cardiovascular disease is independent from that of these determinants [4–9]. These findings were finally confirmed by a recent prospective study in a population of over 160,000 subjects [10]: serum GGT, with a dose–response relationship, was shown to predict the occurrence of incident fatal events during coronary heart disease, congestive heart failure and stroke [10].

In the presence of its substrate glutathione, GGT enzyme activity is able to trigger the production of reactive oxygen species [11], thus promoting LDL oxidation [12] and other pro-oxidant reactions potentially involved during progression of atherosclerosis [13]. Cardiovascular morbidity and mortality are in strict correlation with underlying atherosclerotic disease. Thus, the possibility exists that circulating GGT may participate in the pathogenesis of cardiovascular atherosclerotic disease and its complications (plaque progression and rupture). Due to the antioxidant properties of plasma, it is highly unlikely that pro-oxidant reactions may be catalyzed by GGT in plasma. On the other hand, the fact that GGT has been found within cerebral, carotid, and coronary plaques, co-localizing with oxidized lipids and CD68⁺ foam cells [3,12,14] suggests that the pathogenetic action of GGT may involve its localization inside the arterial wall. The present paper was aimed to compare the biochemical/molecular characteristics of serum and plaque GGT, in order to verify the hypothesis that serum GGT may contribute to the accumulation of active GGT within atheromas.

2. Methods

2.1. Chemicals

Unless otherwise indicated, all reagents were from Sigma–Aldrich and BioRad.

2.2. Patients

We prospectively evaluated a total of 18 consecutive adult patients, with documented atherosclerotic carotid artery lesions undergoing carotid endoarterectomy, who gave informed consent in all instances. Clinical and laboratory data of patients are reported in Table 1. Serum control samples were obtained from healthy young adult male blood donors, periodically undergoing general check-ups (age range: 29–42; *N*=3). The study was approved by Institutional Ethics Committees, and con-

Table 1

Clinical and laboratory data (means \pm S.D.) of the patients studied

Age	64 \pm 10 (range 57–75)
Sex (M/F)	14/4
Serum GGT activity (U/L)	23.6 \pm 26.0 (range 6–181)
Smokers	10/18 (53%)
Drinkers ^a	11/18 (56%)
Diabetes	7/18 (39%)
Hypertension	18/18 (100%)
Medication	
Calcium-channel blockers	12/18 (67%)
Nitrates	2/18 (11%)
Beta-blockers	4/18 (22%)
ACE-inhibitors	12/18 (67%)
Statins	10/18 (56%)

^a Average daily alcohol intake: 32 \pm 17 g.

formed to the principles outlined in the Declaration of Helsinki.

2.3. Homogenates of arterial tissues and atherosclerotic lesions

Surgically excised carotid plaques were collected on ice and immediately placed in liquid nitrogen until analysis. Plaques were used as follows: 6 for GGT activity determination/anion exchange and gel filtration chromatography/western blot; 7 for GGT activity determination/ β -lipoprotein precipitation; 5 for RNA extraction/low molecular weight thiols determination. In fact, the variable amount of tissue in each plaque did not allow the execution of all determinations on all plaques.

For GGT analysis, 13 plaques were rinsed in cold Dulbecco's phosphate buffer (PBS), cut into fragments and disrupted in 5 vol. of cold hypotonic lysis buffer (0.05 M sodium phosphate buffer pH 7.5, 0.15 M NaCl) using an Ultraturrax homogenizer (T25, IKA-Labortechnik). Crude homogenates were centrifuged at 500 \times *g* for 10 min, and the supernatant was used for characterization of plaque GGT. Aliquots of crude homogenates were centrifuged at 100,000 \times *g* (1 h, 4 °C) in order to separate a soluble fraction from insoluble (membranous) pellet. As controls, normal arterial wall samples were processed in the same way (mammary artery tissue, *N*. 5 specimens obtained from 5 distinct patients undergoing cardiac by-pass surgery; informed consent was obtained in these cases as well). The use as controls of plaque-free carotid segments from the patients undergoing endoarterectomy was not ethically justifiable. On the other hand, mammary arteries are generally atherosclerosis-resistant, probably due to their lower sensitivity to leukotrienes and/or increased secretion of prostacyclin, and they were thus considered as ideal controls for our study.

2.4. Enzyme assays

GGT activity was determined using gamma-glutamyl-*p*-nitroanilide as substrate and glycyl-glycine as transpep-

tidation acceptor [15]. The amounts of *p*-nitroaniline were detected at 405 nm (molar extinction coefficient 9200 L/mol cm). 1 Unit of GGT activity was defined as 1 μ mol of substrate transformed/mL/min. GGT activity of chromatographic fractions was measured in 96-wells microtiter plates mixing 100 μ L of sample with 200 μ L of substrate solution including glycyl-glycine. Plates were incubated at 37 °C and read in a multilabel plate reader (Wallac 1420-Victor³, PerkinElmer).

2.5. Anion exchange chromatography

In pellets and soluble fractions of plaque homogenates, GGT extraction and subsequent anion exchange chromatography were performed after sample solubilization, as described by Pettersen et al. [16]. Solubilization/papain digestion of samples was required in order to isolate GGT protein from macro-molecular complexes without altering its electric features. Samples were then centrifuged at 100,000 $\times g$ (1 h, 4 °C) and the buffer of supernatants was replaced with 50 mmol/L Tris–HCl pH 7.5 using desalting columns (PD10, Amersham-Biosciences).

Samples were analyzed by anion exchange chromatography using a MonoQ column (GE-Healthcare) equilibrated in 50 mmol/L Tris–HCl, pH 7.5, and run at a flow rate of 0.5 mL/min. The column was eluted with 7.5 mL, followed by a linear gradient up to 0.5 mol/L NaCl in 20 mL; fractions of 1 mL were collected.

The same procedures were employed for analysis of serum GGT.

2.6. Western blot analysis

Aliquots from plaque homogenates (approx. 1 mU GGT, 150 μ g protein) and serum (0.4 mU GGT, 150 μ g protein) were separated by 7.5% SDS-PAGE [17], and gels were blotted onto nitrocellulose membranes. Blots were incubated overnight with an anti-GGT antiserum (1:5000 in PBS, containing 0.01% Tween-20) direct against the C-terminal 20 aminoacids of human GGT heavy chain [18]. Visualization of protein bands was obtained using a peroxidase conjugated anti-rabbit IgG antibody and an enhanced chemiluminescence detection kit (Roche).

2.7. Molecular exclusion chromatography

Aliquots (1 mL) of soluble fractions of plaque homogenates were used. Gel chromatography was performed using a 16/60 Sephacryl S300 column (GE-Healthcare), and 0.05 mol/L Na-phosphate buffer, pH 7.5, containing 0.15 mol/L NaCl as eluant (flow rate: 12 mL/h, 1 mL fractions). The same procedure was employed for analysis of serum GGT and of GGT solubilized from U937 cell membranes (proteolysis) as described above. The column

was calibrated for molecular mass determination using a mixture of standard proteins.

2.8. β -Lipoprotein precipitation

β -Lipoproteins were precipitated from soluble fraction of plaque homogenates (100,000 $\times g$ supernatant) by sodium phosphotungstate and MgCl₂, according to the procedure described [19]. GGT activity and cholesterol content were determined before and after precipitation of β -lipoprotein fractions of plaque homogenates.

2.9. Reverse transcription and polymerase chain reactions

Total RNA was extracted from plaques using the GenElute Mammalian Total RNA Kit. 2 μ g of total RNA were treated with DNase I (1 unit, 15 min room temperature followed by 10 min at 70 °C) then submitted to reverse transcription. Reaction mixture contained 200 U reverse transcriptase M-MLV (Promega) and 0.5 μ g anchored oligo(dT)₂₃ in 31 μ L containing 50 mmol/L Tris–HCl, pH 8.3, 75 mmol/L KCl, 3 mol/L MgCl₂, 10 mmol/L DTT and 1 mmol/L of each dNTP. Reverse transcription reaction went on 120 min at 37 °C, and was stopped by 2 min incubation at 90 °C.

GGT specific mRNA was detected using the Amp1/Amp2 primer couple, which amplifies a 217 bp cDNA fragment [20]. PCR was performed using 2 μ L of cDNA solution mixed with Taq polymerase (1.25 U, Takara), dNTPs (0.2 mmol/L) and primers (1 μ M) in a total volume of 50 μ L. After initial denaturation (2 min, 94 °C), samples were submitted to 30 cycles as follows: 94 °C (30 s), 56 °C (30 s), 72 °C (45 s).

RNA extracted from GGT-expressing U937 histiocytic lymphoma cells was used as positive sample.

2.10. HPLC analysis of thiol compounds in plaque extracts

Plaque specimens were disrupted in 3 vol. of cold trichloroacetic acid (TCA; 10%, w/v). After 30 min on ice, homogenates were centrifuged (10,000 $\times g$, 10 min, 4 °C) and supernatants diluted twice in 20 mmol/L EDTA, 0.2 mol/L Tris–HCl buffer, pH 8.2. Pellets were washed twice with 10% TCA and resuspended in 1 mL of 20 mol/L EDTA, 0.2 mol/L Tris–HCl buffer, pH 8.2 containing 1% SDS. Samples were then reduced and derivatized by the fluorogenic reagent SBD-F, as described by Pfeiffer et al. [21].

SBD-derivatized thiols were separated by reverse phase HPLC analysis using a System Gold apparatus (Beckman) equipped with a spectrofluorimetric detector (RF 551, Shimadzu; ex. 385 nm, em. 515 nm) and a Resolve C18 column (Waters). 5% methanol in 0.2 mol/L KH₂PO₄, pH 2.7 (1 mL/min) was used as mobile phase. Results were

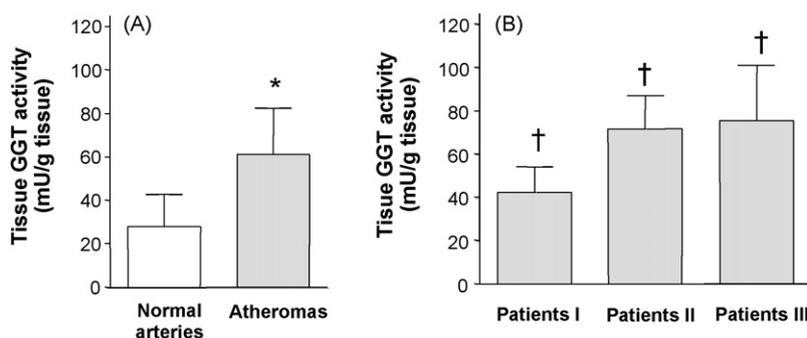


Fig. 1. GGT activity in carotid plaque tissue and its correlation with serum GGT levels in atherosclerotic patients. (A) GGT levels (means \pm S.D.) in atherosclerotic plaques ($N=13$), and in internal mammary artery tissue taken for control ($N=5$); (* \dagger) $P < 0.01$ (Student's t -test). (B) Mean GGT activities detected in plaques obtained from subjects with increasing serum GGT levels: ≤ 20 U/L (patients I, $N=5$), $>20 \leq 40$ U/L (patients II, $N=3$) and >40 U/L (patients III, $N=3$). (\dagger) $P < 0.03$ (one-way ANOVA with post-test for linear trend).

compared with cysteine, cysteinyl-glycine and glutathione standards.

2.11. Other materials and determinations

Total cholesterol content was determined by the enzymatic method [22] (reagent from Giesse Diagnostic, Italy). Protein content was determined by the method of Bradford [23] using the Bio-Rad protein assay reagent and bovine serum albumin as standard.

Statistical analysis of data was performed by parametric or paired Student's t -test, or one-way ANOVA with post-test for linear trend, as indicated in the corresponding figure legends.

3. Results

3.1. GGT activity in atherosclerotic plaques and its correlation with serum GGT levels

GGT activity of crude plaque homogenates was found to range between 30.0 and 103.5 mU/g tissue (mean \pm S.D.: 60.9 ± 21.5 mU/g tissue). A substantial portion of plaque GGT was recovered in $100,000 \times g$ ultracentrifugation pellet [31.8 ± 24.5 mU/g tissue (86.3% of total) as compared to 5.5 ± 7.2 (13.7%) in the supernatant]. Enzyme activity determined for comparison in normal arterial tissue (internal mammary arteries, sampled during coronary artery by-pass grafting) was significantly lower (Fig. 1A). When patients were divided in classes according to their serum GGT levels, mean plaque GGT concentrations were significantly correlated with increasing serum GGT values (Fig. 1B).

3.2. Anion exchange chromatography

In order to obtain additional insight with respect to the possible origin of plaque GGT, the electric properties of the enzyme extracted from plaques and serum were compared. When analyzed by anion exchange chromatography

(Fig. 2), GGT of serum (panel I) eluted as a single peak "b". GGT present in the supernatant of plaque homogenates (panel II) showed two peaks, one with the same elution as in serum (peak "b"), while the other (peak "a") eluted ear-

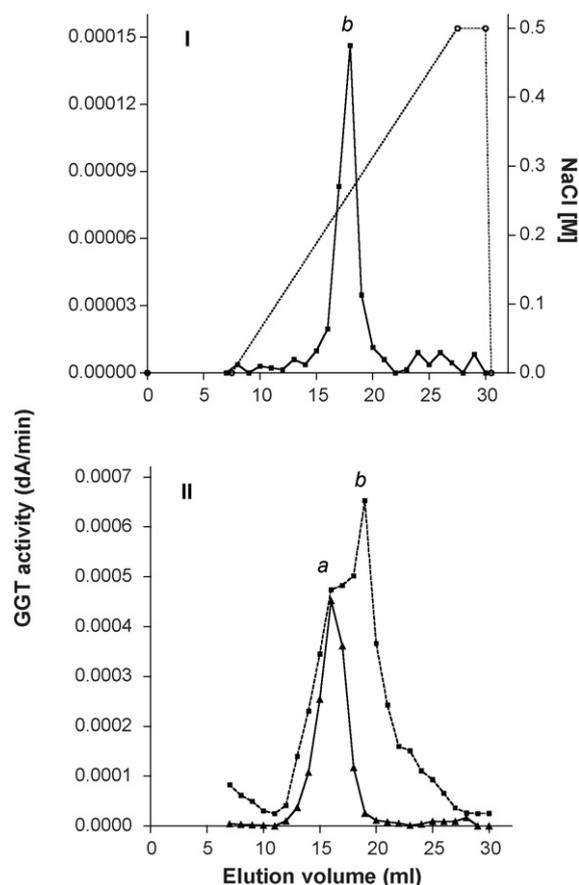


Fig. 2. Anion exchange chromatography of GGT activity recovered from different sources. Soluble GGT, obtained by papain digestion of samples, was subjected to anion exchange chromatography using a MonoQ column. The column was equilibrated in 50 mM Tris-HCl (pH 7.5) and was eluted with a linear gradient up to 0.5 M NaCl. GGT activity was measured in 1 mL fractions. Elution profiles of enzyme activity in serum (I) and plaque homogenate fractions (II; (■) supernatant; (▲) pellet).

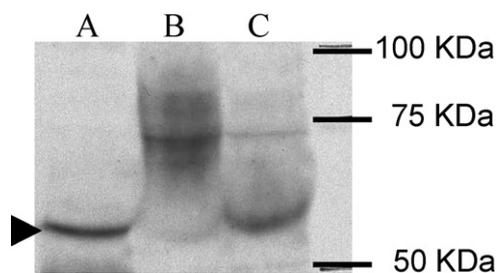


Fig. 3. Western blot analysis of GGT heavy chain. Proteins were separated by 7.5% SDS-PAGE and gels were blotted onto nitrocellulose membranes. Anti-GGT antiserum was directed against the C-terminal 20 aminoacids of human GGT heavy chain. Lane A: serum; lanes B and C: total homogenates obtained from two distinct plaques.

lier (corresponding to a less electronegative protein species). GGT extracted from pellet fractions of plaque homogenates showed only the earlier eluting peak “a”. Thus, plaque tissue appeared to contain a form of GGT protein having the electric characteristics of serum GGT, suggesting that at least part of plaque GGT may originate from blood-stream.

3.3. Western blot analysis

Molecular weights and antigenic properties of GGT protein (unproteolysed) present in plaques and serum were analyzed and compared by means of western blot. Using the antibody against GGT heavy chain, two immunoreactive bands were found in total plaque homogenates, presenting with an apparent MW of about 75 and 60 kDa, respectively (Fig. 3). These two bands were present in variable proportions in plaques deriving from different patients (lanes B and C); in particular, the lighter band corresponded to the 60 kDa GGT found in serum (lane A).

3.4. Molecular exclusion chromatography

The comparison of serum and plaque GGT was extended then to include analysis of molecular sizes. In agreement with previous studies [24], molecular exclusion chromatography of human serum (Fig. 4, panel I) showed three peaks of GGT activity, corresponding to GGT associated with plasma lipoproteins (peak “a”: VLDL/LDL; peak “b”: HDL), as well as to free GGT (peak “c”), with molecular weights ranging 700,000 to 55,000. Interestingly, plaque homogenate supernatants (Fig. 4, panels III–VI) again showed two peaks of GGT activity, suggesting the presence of two distinct forms of the enzyme protein: the one eluting first corresponded to VLDL/LDL-associated GGT (peak “a”) found in serum, while the second one corresponded to control cellular GGT obtained from U937 membranes (by papain proteolytic solubilization; panel II, peak “c’”). HDL-associated GGT of serum (peak “b”) did not have any correspondence in the plaque GGT elution profile.

3.5. β -Lipoprotein precipitation

In order to verify the association of plaque GGT with β -lipoproteins accumulated in the vessel wall, the soluble fractions of plaque homogenates were precipitated by sodium phosphotungstate and $MgCl_2$. Total content of cholesterol was measured before and after β -lipoprotein depletion, in order to evaluate the extent of precipitation. As shown in Fig. 5 (panel A) total cholesterol was approx. fivefold decreased after precipitation of β -lipoprotein. At the same time, a significant decrease of GGT activity was also observed (approx. fourfold; Fig. 5, panel B).

3.6. Reverse transcription and polymerase chain reactions

To investigate the possibility that plaque GGT may derive from local enzyme synthesis, rather than from accumulation of blood-borne enzyme, RT-PCR was conducted on RNA extracted from plaques. The results showed the presence, in all lesions examined, of GGT mRNA transcribed from GGT-I gene (Fig. 6); transcripts of the latter are known to be translated into a functional enzyme [20].

3.7. Free and protein-bound low molecular weight thiols in plaque extracts

A crucial question is whether plaque GGT retains its enzyme activity, once it is accumulated within lesions. The metabolism of glutathione by GGT activity would yield the dipeptide cysteinyl-glycine, which in turn is able to oxidize protein –SH groups forming disulfide bonds [25] (*protein S-cysteyl-glycylation*). Indeed, HPLC analysis of plaque acid extracts showed the presence of both free and protein-bound cysteinyl-glycine, as well as of cysteine and GSH, indicating the occurrence of GGT-mediated redox reactions within the plaque environment (Table 2).

4. Discussion

Our previous studies have documented that active GGT enzyme is present in atherosclerotic lesions as well. Histochemical evidence of GGT activity was detected in the intimal layers of human atherosclerotic plaques, where it is apparently expressed in CD68⁺ macrophage-derived foam cells [3,13,14]. GGT-positive foam cells were found to co-localize with immunoreactive oxidized LDL [3], and catalytically active GGT could also be detected in microthrombi adhering to the surface of atheromas [13]. Against this background, the question arises whether GGT is involved in the pathogenic process during progression of atherosclerosis and related diseases.

The present study shows that significantly higher levels of GGT activity are present in atheromas as compared to normal arterial tissue (mammary arteries) (Fig. 1A). GGT

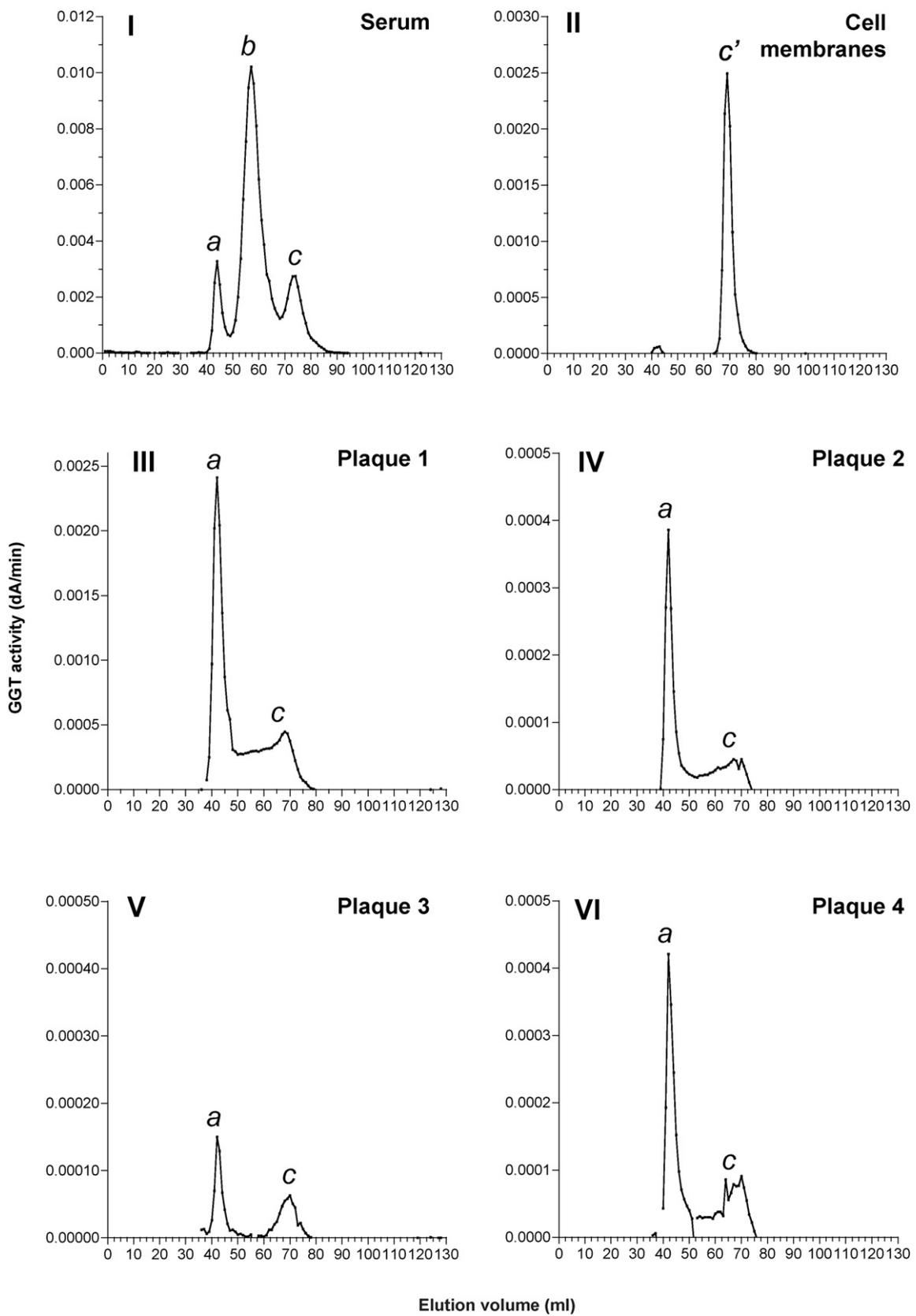


Fig. 4. Molecular exclusion chromatography of GGT activity recovered from different sources. High resolution gel chromatography was performed on a Sephacryl S-300 column eluted with 0.05 M Na-phosphate buffer (pH 7.5) containing 0.15 M NaCl. GGT activity was measured in 1 mL fractions. Elution profiles of GGT activity in one representative serum (I), of GGT solubilized by proteolysis from cell membranes (GGT-positive U937 cells, II), and in four distinct plaques (homogenate supernatants, III–VI) are shown.

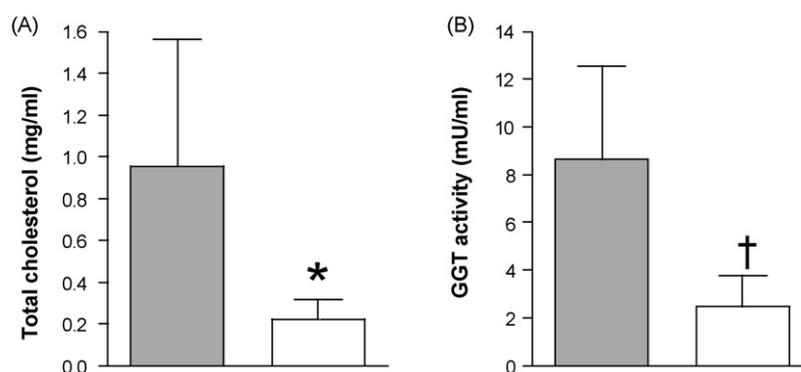


Fig. 5. β -Lipoprotein precipitation from plaque homogenates. Precipitation was performed on soluble homogenate fractions using sodium phosphotungstate 0.4% (w/v) and $MgCl_2$ (0.04 M). Total content of cholesterol (A) and GGT activity (B) was measured before precipitation (grey bars) and after, in the β -lipoprotein-depleted sample (white bars). Data are means \pm S.D. of 7 separate determinations. (*) $P=0.02$; (†) $P=0.002$ (paired Student's *t*-test).

activity was found in plaque tissue as gross intimal deposits, while intima was negative in mammary arteries (data not shown). In the latter, sparse positivity was only detectable in adventitia, likely due to the presence of blood cells in those areas. On the basis of the epidemiological data mentioned above the question thus arises, whether relationships exist between circulating GGT and the enzyme found in atherosclerotic lesions. Indeed, the data reported suggest the existence of a relationship between the two. A significant correlation was in fact observed between plaque GGT deposits and corresponding serum GGT levels (Fig. 1B). Moreover, the serum/plaque relationship was confirmed by molecular characterization studies. When the charge characteristics of plaque GGT protein were analyzed, one isoform detected in supernatant of plaque homogenates (peak “b” in Fig. 2) was comparable with serum GGT. On the other hand, GGT from plaque pellet fraction (eluting as peak “a”) represents a less electronegative form of protein, possibly originating from protein desialylation due to action of inflammatory cells. Peak “a” is actually present in the supernatant fraction as well, possibly suggesting that desialylation may be accompanied by an increased trend towards protein aggregation. This interpretation is further supported by the observation that GGT detectable in plaque supernatants accounts for a minor portion of total, while approx. 80% of activity was recovered in plaque pellets (data not shown).

Immunoblot studies indicated the substantial antigenic identity of plaque GGT and the serum protein (Fig. 3). The two distinct bands observed in plaque homogenates (lanes

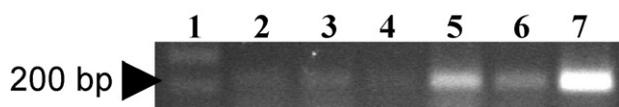


Fig. 6. Detection of GGT mRNA in plaques. After reverse transcription of RNA extracted from five plaques, GGT mRNA of type I was amplified in PCR with the specific Amp1/Amp2 primer couple, which amplifies a 217 bp cDNA fragment. Lane 1: DNA molecular weight marker; lanes 2–6: plaques; lane 7: U937 cells (positive control).

B and C) can be interpreted as the concurrence of protein species with different glycosylation status. Indeed, while GGT enzyme protein is transcribed from a single gene, several distinct glycosylation forms have been described, resulting in different molecular weights [1]. The different patterns observed in different patients suggest that additional, subject-specific aspects exist; future studies are needed in order to elucidate this point.

Importantly, the envisaged serum/plaque GGT relationship is further supported by size exclusion chromatography data, suggesting the presence in plaques of a GGT/lipoprotein complex with a molecular weight comparable to the GGT/LDL complex detectable in serum [24] (compare peaks “a” in the various panels of Fig. 4). In addition, the finding that plaques contain no forms comparable with serum GGT/HDL complexes (peak “b” in Fig. 4, panel I) points to the possible involvement of specific serum GGT-complexes.

Collectively, the three approaches employed to date suggest the possibility that GGT may accumulate in the arterial wall as the result of serum GGT entry, and that this may occur in the form of complexes of GGT with LDL lipoprotein. This interpretation is further supported by the finding that soluble GGT in plaque homogenates is indeed largely associated with β -lipoprotein; the precipitation of the latter in fact resulted in the removal of both cholesterol and GGT activity from samples (Fig. 5). In plasma, most of circulating GGT is indeed associated with lipoproteins, and in particular with the β -fraction (density gradient ultracentrifugation as well as agarose gel electrophoresis) [26]. The same was observed by us in atherosclerotic patients presenting with varying levels of plasma GGT: increases in total serum GGT were accompanied by increases in plasma lipoprotein-bound enzyme levels (density gradient ultracentrifugation) [27]. An alternative interpretation of data would point to a possible diffusion (secretion?) of accumulated β -lipoprotein-GGT complexes or of free enzyme protein from atherosclerotic plaques into the bloodstream, where it would become associated with serum β -lipoprotein. This seems however unlikely, since it was observed that incubation of detergent-solubilized

GGT with isolated lipoprotein *in vitro* failed to produce GGT-lipoprotein complexes [28].

The fact that 80–90% of total of plaque GGT was recovered in ultracentrifugation pellets of crude homogenates probably indicates its association with gross membranous structures or large macromolecular complexes. However, microscopic analysis showed that a substantial part of plaque GGT activity is not associated with cellular elements [14]. It has been recently reported that large aggregates are formed in the vessel wall following aggregation and fusion of modified LDL [29]. In particular, it has been proposed that during LDL oxidation the supramolecular structure of apoB may become destabilized due to alterations of the water-lipid interface, leading to protein misfolding, aggregation and accumulation [30]. Conceivably, the composition of such macromolecular aggregates might include GGT protein, thus explaining the precipitation of part of plaque GGT with ultracentrifugation pellets. More details could be obtained from a direct comparison of molecular features of GGT isolated from complexes circulating in serum with those of enzyme isolated from aggregates recovered from plaque material; current studies in our laboratory are addressing this important issue.

Additional insights were provided by RT-PCR analysis, which revealed in plaque extracts the presence of GGT mRNA transcribed from GGT-I gene (Fig. 6). This data indicates that plaque GGT may at least in part also derive from local synthesis of the protein. A likely endogenous source of GGT could be represented by cells of macrophagic lineage [31–33]. This could explain the presence of two distinct molecular forms of GGT in anion exchange as well as western blot analysis of plaques (Figs. 2II and 3). Inflammatory cells present in the vessel wall may also induce modifications in protein sialylation status [34]; interestingly, it was observed that modifications of this kind can facilitate the aggregation of apoB-containing lipoproteins [35,36].

In conclusion, the present study provides important clues in order to elucidate the origins of GGT activity detectable in human atherosclerotic plaques. In particular, the data presented suggest that plaque GGT may derive both from insudation of specific GGT-rich circulating β -lipoprotein fractions, as well as from endogenous synthesis by cellular elements of likely inflammatory nature. Once accumulated in the plaque environment, GGT retains its enzymatic activity, as shown by previous histochemical studies [3,14], and can promote pro-oxidant effects. As shown in Table 2, signifi-

cant levels of protein-bound cysteinyl-glycine were detected in plaque material; such a biochemical marker in fact documents that GGT-mediated redox reactions have taken place [25]. Redox events can play relevant roles in several processes favouring the evolution of atherosclerotic plaques towards instabilization and rupture [37,38], which could at least partly explain the reported association of increased serum GGT with unfavourable prognosis of cardiovascular diseases. Increasing evidence – in unselected populations or patients with atherosclerotic disease – is documenting an independent association of serum GGT (upper reference range, 25–40 U/L) with stroke, non-fatal infarction and cardiac death [39]. The identification of molecular GGT-containing complexes in serum specifically related with enzyme forms accumulating in plaques could provide a means for a better evaluation of risk in atherosclerotic patients, through a stratification based on the serum levels of specific GGT forms.

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References

- [1] Whitfield JB. Gamma-glutamyl transferase. *Crit Rev Clin Lab Sci* 2001;38:263–355.
- [2] Pompella A, Emdin M, Passino C, Paolicchi A. The significance of serum gamma-glutamyltransferase in cardiovascular diseases. *Clin Chem Lab Med* 2004;42:1085–91.
- [3] Emdin M, Passino C, Donato L, Paolicchi A, Pompella A. Serum gamma-glutamyltransferase as a risk factor of ischemic stroke might be independent of alcohol consumption. *Stroke* 2002;33:1163–4.
- [4] Conigrave KM, Saunders JB, Reznik RB, Whitfield JB. Prediction of alcohol-related harm by laboratory test results. *Clin Chem* 1993;39:2266–70.
- [5] Wannamethee G, Ebrahim S, Shaper AG. Gamma-glutamyltransferase: determinants and association with mortality from ischaemic heart disease and all causes. *Am J Epidemiol* 1995;142:699–708.
- [6] Brenner H, Rothenbacher D, Arndt V, et al. Distribution, determinants, and prognostic value of gamma-glutamyltranspeptidase for all-cause mortality in a cohort of construction workers from south Germany. *Prev Med* 1997;26:305–10.
- [7] Jousilahti P, Rastenyte D, Tuomilehto J. Serum gammaglutamyltransferase, self-reported alcohol drinking, and the risk of stroke. *Stroke* 2000;31:1851–5.
- [8] Karlson BW, Wiklund O, Hallgren P, et al. Ten-year mortality amongst patients with a very small or unconfirmed acute myocardial infarction in relation to clinical history, metabolic screening and signs of myocardial ischaemia. *J Intern Med* 2000;247:449–56.

Table 2

Free and protein-bound low molecular weight thiols in plaque extracts

	Free	Protein-bound
GSH	161.1 ± 74.1	13.6 ± 8.2
Cys-Gly	6.0 ± 1.3	3.3 ± 0.4
Cys	152.4 ± 85.8	61.4 ± 2.4

Low molecular weight thiols were determined through HPLC analysis of plaque acid extracts. Data are expressed as nmol/g tissue, and are means ± S.D. of separate determinations on 3 distinct representative plaques.

- [9] Emdin M, Passino C, Michelassi C, et al. Prognostic value of serum gamma-glutamyl transferase activity after myocardial infarction. *Eur Heart J* 2001;22:1802–7.
- [10] Ruttman E, Brant LJ, Concin H, et al. γ -Glutamyltransferase as a risk factor for cardiovascular disease mortality. An investigation in a cohort of 163,944 Austrian adults. *Circulation* 2005;112:2130–7.
- [11] Dominici S, Paolicchi A, Corti A, Maellaro E, Pompella A. Prooxidant reactions promoted by soluble and cell-bound gamma-glutamyltransferase activity. *Methods Enzymol* 2005;401:484–501.
- [12] Paolicchi A, Minotti G, Tonarelli P, et al. Gamma-glutamyl transpeptidase-dependent iron reduction and LDL oxidation—a potential mechanism in atherosclerosis. *J Investig Med* 1999;47:151–60.
- [13] Dominici S, Paolicchi A, Lorenzini E, et al. Gamma-glutamyltransferase-dependent prooxidant reactions: a factor in multiple processes. *Biofactors* 2003;17:187–98.
- [14] Paolicchi A, Emdin M, Ghiozeni E, et al. Human atherosclerotic plaques contain gamma-glutamyl transpeptidase enzyme activity. *Circulation* 2004;109:1440.
- [15] Huseby NE, Stromme JH. Practical points regarding routine determination of gamma-glutamyl transferase (gamma-GT) in serum with a kinetic method at 37 degrees C. *Scand J Clin Lab Invest* 1974;34:357–63.
- [16] Pettersen I, Andersen JH, Bjornland K, et al. Heterogeneity in γ -glutamyltransferase mRNA expression and glycan structures. Search for tumor-specific variants in human liver metastases and colon carcinoma cells. *Biochim Biophys Acta* 2003;1648:210–8.
- [17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;22:680–5.
- [18] Hanigan MH, Frierson Jr HF. Immunohistochemical detection of gamma-glutamyl transpeptidase in normal human tissue. *J Histochem Cytochem* 1996;44:1101–8.
- [19] Burstein M, Scholnick HR, Morfin R. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J Lipid Res* 1970;11:583–95.
- [20] Courtay C, Heisterkamp N, Siest G, Groffen J. Expression of multiple γ -glutamyltransferase genes in man. *Biochem J* 1994;297:503–8.
- [21] Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. *Clin Chem* 1999;45:290–2.
- [22] Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem* 1974;20:470–5.
- [23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- [24] Wenham PR, Horn DB, Smith AF. Physical properties of γ -glutamyltransferase in human serum. *Clin Chim Acta* 1984;141:205–18.
- [25] Corti A, Paolicchi A, Franzini M, et al. The S-thiolating activity of membrane gamma-glutamyltransferase: formation of cysteinyl-glycine mixed disulfides with cellular proteins and in the cell microenvironment. *Antioxid Redox Signal* 2005;7:911–8.
- [26] Huseby NE. Multiple forms of serum gamma-glutamyltransferase. Association of the enzyme with lipoproteins. *Clin Chim Acta* 1982;124:103–12.
- [27] Paolicchi A, Emdin M, Passino C, et al. Beta-lipoprotein- and LDL-associated serum gamma-glutamyltransferase in patients with coronary atherosclerosis. *Atherosclerosis* 2006;186:80–5.
- [28] Artur Y, Wellman-Bednawska M, Jacquier A, Siest G. Associations between serum gamma-glutamyltransferase and apolipoproteins: relationships with hepatobiliary disease. *Clin Chem* 1984;30:1318–21.
- [29] Oorni K, Pentikainen MO, Ala-Korpela M, Kovanen PT. Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions. *J Lipid Res* 2000;41:1703–14.
- [30] Ursini F, Davies KJ, Maiorino M, Parasassi T, Sevanian A. Atherosclerosis: another protein misfolding disease? *Trends Mol Med* 2002;8:370–4.
- [31] Khalaf MR, Hayhoe FGJ. Cytochemistry of gamma-glutamyl transferase in haemic cells and malignancies. *Histochem J* 1987;19:385–95.
- [32] Grisk O, Kuster U, Ansoerge S. The activity of gamma-glutamyl transpeptidase (gamma-GT) in populations of mononuclear cells from human peripheral blood. *Biol Chem Hoppe Seyler* 1993;374:287–90.
- [33] Sener A, Yardimci T. Activity determination, kinetic analyses and isoenzyme identification of gamma-glutamyltransferase in human neutrophils. *J Biochem Mol Biol* 2005;38:343–9.
- [34] Pshezhetsky AV, Ashmarina M. Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Prog Nucleic Acid Res Mol Biol* 2001;69:81–114.
- [35] Tertov VV, Orekhov AN, Sobenin IA, et al. Three types of naturally occurring modified lipoproteins induce intracellular lipid accumulation due to lipoprotein aggregation. *Circ Res* 1992;71:218–28.
- [36] Mel'nichenko AA, Tertov VV, Ivanova OA, et al. Desialylation decreases the resistance of apo B-containing lipoproteins to aggregation and increases their atherogenic potential. *Bull Exp Biol Med* 2005;140:51–4.
- [37] Stocker R, Keaney Jr JF. Role of oxidative modifications in atherosclerosis. *Physiol Rev* 2004;84:1381–478.
- [38] Emdin M, Pompella A, Paolicchi A. Gamma-glutamyltransferase, atherosclerosis, and cardiovascular disease: triggering oxidative stress within the plaque. *Circulation* 2005;112:2078–80.
- [39] Emdin M, Passino C, Pompella A, Paolicchi A. Gamma-glutamyltransferase as a cardiovascular risk factor. *Eur Heart J* 2006;27:2145–6.