Regulation of Tissue Factor–Mediated Initiation of the Coagulation Cascade by Cell Surface Grp78

Gourab Bhattacharjee, Jasimuddin Ahamed, Brian Pedersen, Amr El-Sheikh, Nigel Mackman, Wolfram Ruf, Cheng Liu, Thomas S. Edgington

Objective—To test the hypothesis that Grp78 negatively regulates cell surface tissue factor (TF) procoagulant activity and whether this is mediated by physical interaction.

Methods and Results—Biopanning with phage-displayed peptidyl libraries has identified peptide probes that bind selectively in vivo to the surface of atherosclerotic plaque endothelium. The highest affinity peptide, EKO130, binds 78-kDa glucose regulated protein (Grp78). Grp78 participates in numerous pathological processes, including the regulation of the coagulation cascade, but the mechanism of Grp78 regulation of coagulation is unknown. To characterize this function, we analyzed the effect of Grp78 on TF-mediated procoagulant activity on murine brain endothelial cells (bEND.3) and macrophage-like (RAW) cells, which are relevant in mediation of atherothrombosis. We show that Grp78 is present on the surface of endothelium and monocyte/macrophage-like cells in atherosclerotic lesions. Inhibition of Grp78 resulted in increased procoagulant activity. We demonstrate that Grp78 negatively regulates procoagulant activity by interacting physically with the TF extracellular domain on the cell surface.

Conclusions—The evidence indicates that Grp78 negatively regulates TF functional activity via direct binding to and functional inhibition of TF. Identification of the mechanism by which Grp78 regulates TF function may advance insight into the pathobiology of atherosclerosis and associated arterial thrombosis. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: atherosclerosis • coagulation • endothelium • Grp78 • thrombosis • tissue factor

Cardiovascular disease resulting in chronic heart failure, myocardial infarction, and stroke is the major cause of morbidity and mortality in the Western hemisphere. Atherosclerosis is a major contributor to the pathogenesis of myocardial and cerebral artery thrombosis as well as unstable angina. It is a complex, progressive disease characterized by the accumulation of lipid-laden deposits (plaques) in the arterial wall. Plaque formation proceeds in 3 stages: (1) fatty streaks containing lipid-laden macrophages; (2) fibrous plaques containing foam cells covered by a fibrous cap; and (3) complex lesions that can invoke thrombus formation, fibrin, and platelet deposition and can result in occlusion of the arterial lumen producing myocardial infarction. The apolipoprotein E-knockout mouse (apoE−/−) is an accepted model for atherosclerosis because it mimics many of the typical features and progression of the human disease; and plaque formation in mice can be accelerated and aggravated by high-fat, high cholesterol diets.

We have previously used a phage expressed random combinatorial peptide library to perform in vivo interrogation of the atherosclerotic plaque endothelial surfaces of apoE−/− mice. This has resulted in identification of 93 peptide motif sets that selectively bind to the atherosclerotic plaque endothelial surface. The high-affinity peptide, EKO130, binds to an 78-kDa protein on endothelial cells in vitro. Identified as Grp78, a heat shock protein originally characterized as an endoplasmic reticulum chaperone, the presence has been demonstrated on the surface of cells under various pathologic conditions including rheumatoid fibroblasts, metastatic prostate cancer cells, and as a coreceptor for coxsackievirus A9. Grp78 is also associated with the low-density lipoprotein receptor-related protein, a scavenger receptor in the low-density lipoprotein superfamily, members of which are strongly implicated in atherogenesis. Recent studies have implicated Grp78 overexpression in having an inhibitory effect on cardiovascular disease progression by decreasing the procoagulant activity of tissue factor (TF), as well as inhibiting homocysteine induced gene expression.

TF is a 47-kDa integral membrane protein that binds to the serine protease, factor VII/VIIa, thereby initiating the coagulation protease cascade. TF is implicated in numerous pathologic conditions including atherosclerosis, postoperative thrombosis, and sepsis-linked disseminated intravascular coagulation. TF is constitutively expressed on the surface of several extravascular cell types, and it is transiently expressed on the plasmalemma of macrophages,
endothelial cells, and monocytes in response to a number of inflammatory agents, including bacterial lipopolysaccharide (LPS).18

We demonstrate here that Grp78 is present on endothelium overlying the atherosclerotic plaque and on foam cells within the plaque. In this study, we determined the role of Grp78 in regulating coagulative processes. Our data show that Grp78 negatively regulates TF procoagulant activity on both endothelial and macrophage cell types. We demonstrate that the mechanism by which this occurs is via direct binding of Grp78 and TF on the cell surface. Our studies indicate a possible protective role for Grp78 in the progression of atherothrombosis.

Materials and Methods

Cell Culture

Murine brain endothelial cells (bEND.3) and RAW murine macrophage cells (ATCC, Rockville, Md) were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine in 8%CO2.

Immunohistochemistry and Western Blotting

Immunohistochemical analysis was performed on frozen 5-μm sections of atherosclerotic aortic valves from high-fat diet-fed apoE−/− mice after perfusion with 4% paraformaldehyde. Plaque sections were permeabilized with acetone and stained with monoclonal anti-Grp78 (Anti-Grp78M, Pharmingen, San Diego, Calif) rat anti-mouse CD31, or rat anti-mouse CD14 (Pharmingen). Fluorescein isothiocyanate (FITC) or Texas Red conjugated secondary antibodies (Vector Labs; Burlingame, Calif) were used to visualize plaques via confocal microscopy using a Bio-Rad (Hercules, Calif) MRC1024 laser scanning confocal microscope.

For Western blot analysis, cells were lysed with 2× SDS reducing buffer and PAGE using 8% to 16% Tris-glycine gels (Invitrogen). The proteins were transferred onto polyvinylidene fluoride membranes and blocked with 20 mmol/L Tris, 140 mmol/L NaCl, 0.1% Tween 20, 10% powdered dry milk protein (TBS-T/milk). The probes were blotted with either goat polyclonal (anti-Grp78M; Santa Cruz Biotechnology, Santa Cruz, Calif) or anti-Grp78M antibody against Grp78 for 1 hour at room temperature. The blots were washed 3 times with TBS-T and incubated with horseradish peroxidase-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology) for 1 hour at room temperature. The blot was washed 3 times with TBS-T and developed with ECL reagent (Sigma, St. Louis, Mo).

Single-Stage Clotting Assay

Cells grown to confluence in 6-well polystyrene plates (~5×10⁶ cells/well) were incubated in the presence of anti-Grp78, or anti-Hsc70 (another member of the homologous heat shock family of proteins) for 30 minutes at 37°C and subsequently stimulated with or without LPS (1 μg/mL) for 4 hours at 37°C. Cells were then washed twice with phosphate-buffered saline, solubilized in 100 μL of 15 mmol/L n-Octyl-β-d-glucopyranoside in 25 mmol/L HEPES/saline solution, vortexed for 1 minute, and incubated at 37°C for 15 minutes. The lysate was mixed with 200 μL of 25 mmol/L HEPES/saline solution; 50 μL of this final solution was incubated with 50 μL of murine plasma (Sigma) using a START4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ) machine. After adding 25 μL of 25 mmol/L calcium chloride, the time to clot was recorded. Clot times were converted to TF units by reference to an established standard curve.

Cell-Based Factor Xa Generation Assay

Cells grown to confluence in 12-well polystyrene plates (~5×10⁶ cells/well) were incubated in the presence of anti-Grp78, or anti-Hsc70 for 30 minutes at 37°C and subsequently stimulated with or without LPS (1 μg/mL) for 4 hours at 37°C. Cells were then washed twice with HEPES (10 mmol/L)-buffered saline plus 5 mmol/L CaCl₂. Factor VIIa (10 nmol) and Factor X (50 nmol) were added. Reactions were stopped at selected time points by adding 100 mmol/L EDTA to the sample and the activity of the generated Xa in the medium was quantified with the chromogenic substrate Spectrozyme FXa (American Diagnostics, Greenwich, Conn). Absorbance (405 nm) was converted to nmol Xa generated per minute using an established standard curve.

Biochemical Factor Xa Generation Assays

Proteolytic activation of factor X by functionally assembled TF, Vι/Vlla complex was analyzed using coupled chromogenic assays. TF at 25 nmol was incubated with varying amounts of recombinant Grp78 (Stressgen, Vancouver, BC) in the presence, or absence of anti-Grp78, for 4 hours at 22°C in HBSA buffer (20 mmol/L Hepes, 150 mmol NaCl, 0.5% bovine serum albumin; pH 7.4) with 5 mmol/L CaCl₂. Vιa (25 nmol) and X (25 nmol) were then added to start the reaction. After 10 minutes at 37°C, the reaction was arrested by the addition of 100 mmol/L EDTA. The generated Xa was quantified with chromogenic Spectrozyme FXa substrate (American Diagnostics) and the change in absorbance (405 nm) quantitated in a kinetic microtiter plate reader.

Protein Binding Assay

Recombinant TF extracellular domain (TF, Vι/Vlla) was expressed in mammalian cells and immobilized to Avigel (Pierce, Rockford, Ill) beads.19 TF beads were incubated with recombinant Grp78 alone and in the presence of excess TF, Vι/Vlla, for 4 hours at 22°C. Beads were washed extensively with Tris-buffered saline and resuspended in 2× SDS reducing sample buffer for Western blotting. Beads were probed using anti-Grp78 and anti-TF (loading control).

Immunoprecipitation and Confocal Microscopy

Ad5 serotype vectors expressing full-length human TF were described in detail.20,21 bEND.3 and RAW cells grown to 50% to 60% confluence were transduced with TF (50 virus particles per cell) for 3 hours, and grown for 48 hours. Cells were solubilized with 50 mmol/L n-Octyl-β-d-glucopyranoside and incubated with anti-Grp78M, Hsc70 (Santa Cruz) or 10H10 anti-TF antibody22 for 12 to 16 hours at 4°C followed by immunoprecipitation using protein A/G beads (Amersham Biosciences, Uppsala, Sweden). Precipitates were then analyzed by Western blotting using Anti-Grp78M. Immunoprecipitation was also performed on biotinylated cells. Cells were washed extensively with Hepes buffered saline (10 mmol/L Hepes, 0.9% saline), incubated with 5 mmol/L NHS-Sulfo-LC-Biotin (Pierce) for 20 minutes, 4°C, and washed again before immunoprecipitation as described. Precipitates were analyzed by Western blotting using streptavidin coupled to horseradish peroxidase (Vector Labs) in addition to Anti-Grp78.

For confocal imaging, nonpermeabilized cells were transduced with human TF-expressing adenovirus23 and grown to 90% confluence on cover slips and incubated with anti-Grp78 for 1 to 2 hours at 4°C, then followed by FITC-conjugated anti-mouse immunoglobulin (Vector Labs) for 1 hour at 4°C. Cells were then extensively washed and incubated with Texas Red-conjugated 10H10 anti-TF antibody for 1 hour at 4°C, followed by mounting in Vectashield with DAPI (Vector Labs). Cells were visualized via confocal microscopy using a Bio-Rad MRC1024 laser scanning confocal microscope. Control experiments were performed using anti-Hsc70.

Results

Grp78 Is Expressed on Endothelial Cells and Foam Cells Associated With Atherosclerotic Plaques

We have previously demonstrated that the EKO130 peptide binds selectively to atherosclerotic plaque endothelium in vivo and ex vivo and to Grp78 in vitro. Here we assessed the pathophysiological relevance of those findings by demon-
strating the presence of Grp78 on the endothelium overlying the atherosclerotic plaques and on monocyte/macrophage cells within the plaque. Our studies show that Grp78 is present on endothelium overlying the atherosclerotic plaque and colocalizes with CD31 (Figure 1A to 1C) as well as with CD14 on monocytes within the plaque (Figure 1D to 1F), both of which are cell surface markers. No colocalization and minimal staining was observed with secondary antibody alone. These data support the hypothesis that Grp78 may play a role in the progression of atherothrombosis.

### Inhibition of Grp78 Increases TF-Dependent Single-Stage Clotting

To determine the potential participation of Grp78 in regulating the thrombogenic coagulation protease cascade, we first analyzed its role in a single-stage clotting assay using both viable endothelial and macrophage cells. To study the role of Grp78 on clotting time in basal and LPS-induced bEND.3 and RAW cells, endogenous Grp78 was blocked by incubating cells with anti-Grp78. In the bEND.3 cells, antibody-mediated Grp78 inhibition resulted in significantly accelerated clotting time from 200.9 ± 100.6 seconds to 150.6 ± 3.1 seconds in untreated cells and from 145.0 ± 9.2 seconds to 91.4 ± 3.5 seconds in LPS-induced cells (Table). This corresponds to a 2.5-fold increase in TF functional activity (59 mU) over basal (24 mU) and a 4.2-fold increase over LPS-induced TF-driven clotting (280 mU versus 66 mU) (Figure 2A). Inhibition of Grp78 in RAW cells resulted in increased basal clotting time from 56.5 ± 2.4 seconds to 26.8 ± 0.4 seconds (Table). This corresponds to a 9.5-fold increase in TF functional activity (11.87 units) over basal (1.25 units) (Figure 2B). When RAW cells were stimulated with LPS, Grp78 inhibition decreased clotting time from 27.3 ± 0.9 seconds to 24.6 ± 0.3 seconds (Table), which corresponds to a 1.3-fold increase in TF initiation of coagulation protease activity (12.6 units versus 16.6 units) (Figure 2B). As a control experiment, we used an antibody against another member of the heat shock protein family (Hsc70), which had no effect on control and LPS-induced single stage clotting. These data demonstrate that Grp78 negatively regulates cell surface TF-dependent procoagulant activity. Similar results were observed in single stage clotting assays performed on whole cells (data not shown).

### Inhibition of Grp78 Results in Increased TF-Dependent Factor Xa Generation

To further analyze the effect of Grp78 inhibition on TF-dependent initiation of coagulation, we examined Xa generation by both viable bEND.3 and RAW cells expressing TF. Our results were akin to those observed for single-stage clotting. In the bEND.3 cells, antibody (anti-Grp78)-mediated inhibition of Grp78 resulted in a 3.6-fold increase in basal Xa generation (0.8 pmol/L/L/min versus 2.9 pmol/L/L/min) and a 2-fold increase over LPS-induced Xa generation (280 pmol/L/L/min versus 66 pmol/L/L/min) (Figure 3A). Inhibition of Grp78 in RAW cells resulted in increased basal clotting time from 56.5 ± 2.4 seconds to 26.8 ± 0.4 seconds (Table). This corresponds to a 9.5-fold increase in TF functional activity (11.87 units) over basal (1.25 units) (Figure 3B). When RAW cells were stimulated with LPS, Grp78 inhibition decreased clotting time from 27.3 ± 0.9 seconds to 24.6 ± 0.3 seconds (Table), which corresponds to a 1.3-fold increase in TF initiation of coagulation protease activity (12.6 units versus 16.6 units) (Figure 3B). As a control experiment, we used an antibody against another member of the heat shock protein family (Hsc70), which had no effect on control and LPS-induced single stage clotting. These data demonstrate that Grp78 negatively regulates cell surface TF-dependent procoagulant activity. Similar results were observed in single stage clotting assays performed on whole cells (data not shown).

### Effect of Grp78 on TF-Mediated Coagulation and Xa Generation

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<th>Treatment</th>
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<th>RAW Clotting Time, sec</th>
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<th>RAW TF activity, mU</th>
<th>bEND.3 Xa generation, pmol/L/min</th>
<th>RAW Xa generation, pmol/L/min</th>
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<td>280.2</td>
<td>16561.4</td>
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**Figure 1.** Grp78 expression by atherosclerotic endothelial and monocytic cells. Left panel shows a representative hematoxylin and eosin-stained section of aortic valve from an atherosclerotic apolipoprotein E (apoE) knockout mouse. In the right panel, endothelial cells overlying the aortic plaque section (A to C) and monocytic cells within the subendothelial portion of the lesion (D to F) from apoE−/− mice were stained using mouse monoclonal Grp78 antibody (visualized with FITC-conjugated anti-mouse IgG). Anti-CD31 was used as positive endothelial control and anti-CD14 as a positive monocytic control. A, Anti-CD31 (red). B, Anti-Grp78 (green). C, Colocalization of CD31 and Grp78 (white). D, Anti-CD14 (red). E, Anti-Grp78 (green). F, Colocalization of CD14 and Grp78 (white). Sections were visualized by confocal microscopy and quantitated using ImageJ colocalization software from National Institutes of Health.
pmol/L/min versus 5500 pmol/L/min) (Figure 3B). In the noncell-based biochemical assay, recombinant Grp78 inhibited factor Xa generation in a dose-dependent manner and this activity is inhibited by anti-Grp78G (Figure 3C). These data are consistent with the single-stage clotting assay indicating that endogenous Grp78 acts as a negative regulator of TF cofactor functions. The results from the clotting and Xa generation assays are summarized in the Table.

**Grp78 Interacts With TF**

To determine whether inhibition of TF by Grp78 is attributable to direct protein–protein interaction with TF, the ability of Grp78 to bind TF was analyzed by incubation of recombinant Grp78 with TF bound to beads. Our studies demonstrate that Grp78 binds to TF (Figure 4A). This binding is inhibited in the presence of excess recombinant soluble TF containing only the extracellular domain (TF1to218) (Figure 4A and 4B). Grp78 exhibited no binding to beads alone or control protein coupled to beads (data not shown). These
There is no coprecipitation with the anti-Grp78G antibody that coprecipitates with TF in both cell types (Figure 5A, upper). Hsc70, followed by Western blotting. We found that Grp78 binds to the extracellular domain of TF, we overexpressed human TF in bEND.3 and RAW cells by adenovirus-mediated transduction. Immunoprecipitation was then performed on these cells using antibodies against Grp78 (anti-Grp78M, upper panel) or anti-TF (lower panel). Lanes denote Grp78 bound to TF beads alone and in the presence of equimolar, 2-fold molar excess, and 10-fold molar excess TF1 to 214 (lower panel). Lanes denote Grp78 bound to TF beads alone and in the presence of equimolar, 2-fold molar excess, and 10-fold molar excess TF1 to 214. B, Quantitation of TF1 to 214 competition of Grp78 binding to TF beads. Recombinant Grp78 exhibited no binding to beads alone or control protein coupled to beads (data not shown).

In vivo phage display is a powerful competitive selection technique that we used for the selection of a population of peptides that bound exclusively to the surface of tumor vascular endothelium. This resulted in a profile of numerous proteins that demonstrated differential expression on tumor and normal tissues as well as the discovery of tumor vascular selective targets. This methodology has been extended to analysis of the endothelium of atherosclerotic lesions. We reduced the combinatorial complexity of a library of peptides to those that bind predominantly to atherosclerotic plaque endothelium. The endothelial cell surface target of the highest affinity peptide from these search experiments is the 78-kDa glucose-regulated protein (Grp78). In this study we extend this initial discovery to demonstrate that Grp78 can play a significant functional role in the attenuation of the TF-driven thrombogenic cascade in vitro, a function that may, in turn, result in an atheroprotective role in vivo.

The present experimental data demonstrate that Grp78 attenuates the TF-mediated coagulation and thrombogenic protease cascades on both endothelial cells and macrophages, 2 of the major cell types comprising the atherosclerotic plaque. Moreover, Grp78 significantly suppresses TF activity on both basal and LPS-induced endothelial cells, whereas for the RAW macrophage cell type the LPS-induced suppression is less effective than for the unstimulated cell. This suggests that Grp78 is able to suppress low levels of TF as seen on stressed or unstressed endothelial cells and unstressed macrophages but is not effective beyond a certain higher level TF expression (LPS-induced macrophages can be as high as 43,000 molecules per cell surface). Similar results were observed in other endothelial cells and mononcytic cells (THP-1) (unpublished results). The possible protective role of Grp78 in atherothrombosis is indicated by the inhibitory in vitro effects on TF-dependent plasma clotting and Xa generation that we demonstrate in this study. In particular, the Xa generation assay (Figure 2) indicates that cell surface Grp78 negatively regulates TF procoagulant activity. It is of note that only the antibody against the C-terminal portion of Grp78 (anti-Grp78C) inhibited the suppressive effect of...
Grp78 and resulted in enhanced TF activity. This indicates that the putative structural locus in Grp78 that mediates suppression of TF activity is located in the C-terminal portion of the protein. Our immunoprecipitation studies (Figure 5A) support this result in that the monoclonal antibody against Grp78 (anti-Grp78α) coprecipitated TF, whereas anti-Grp78α did not. The N-terminal domain of Grp78 is known to mediate ATPase activity25 and our studies imply that this function may be independent of TF regulation because we observed no effect with antibody against the N-terminal portion of Grp78 (data not shown). Future studies involving Grp78 mutants that are deficient in ATPase activity26 may provide further direct evidence of the functional requirements of Grp78-mediated regulation of cell surface TF initiation of the coagulation protease cascades.

To determine whether these in vitro results translate to an in vivo system, we demonstrated that Grp78 is present on the endothelium overlaying the atherosclerotic plaques of apo E knockout mice and in monocytes within the plaque (Figure 1). Our protein–protein interaction studies demonstrate that Grp78 physically binds to TF and that the ability of recombinant TF extracellular domain to competitively inhibit the TF/Grp78 interaction supports the idea that the negative regulation of TF-mediated coagulation occurs at the cell surface (Figure 4). This is further confirmed by immunoprecipitation studies in which TF coprecipitates with Grp78 and cell surface staining demonstrating colocalization of the 2 proteins (Figure 5).

Our data are consistent with recent studies which have implicated Grp78 overexpression as having an inhibitory effect on cardiovascular disease progression by decreasing the procoagulant activity of TF,31 as well as inhibiting homocysteine induced gene expression.12 We extend these studies by demonstrating a direct interaction between TF and Grp78.

Previous work has demonstrated that Grp78 overexpression has varying effects on the levels of the coagulation protease cascade. Secretion of factor VII and a mutant form of tissue plasminogen activator is increased in response to Grp78 overexpression whereas von Willebrand factor and factor VIII secretion is decreased.28-29 TF-dependent thrombogenic activity is also decreased.11 Noting that TF is the major physiological initiator of the coagulation cascade, it forms the functional bimolecular complexes with factor VII/VIIa leading to the activation of factors IX and X, resulting in thrombin generation, fibrin deposition, and activation of cellular signaling via cell surface protease-activated receptors.30,31 One major concern regarding the cell surface expression of Grp78 is that the protein lacks a transmembrane domain. However, a recent study has demonstrated that the mechanism by which Grp78 is translocated to the cell surface is via the murine tumor cell Dnap1-like protein 1 (MTJ-1).32 In addition, recent studies have demonstrated that Grp78 overexpression can allow membrane incorporation of some Grp78 and there are 4 potential membrane-spanning domains in the Grp78 protein.33 Once on the cell surface, our work and that of others indicate that Grp78 plays numerous roles that are independent of its function as an intracellular chaperone. In addition to the interaction, we demonstrate with TF, Grp78 also mediates the signaling function of the plasma protease alpha2-macroglobulin,34 binds MHC class I antigens,34,35 and mediates uptake of coxsackievirus A9.34 and dengue virus serotype 2.36 Regarding atherothrombosis, studies have shown that cholesterol accumulation causes ER stress in macrophages leading to apoptotic cell death.37 Recent studies have also demonstrated that the ER stress/unfolded protein response occurs at all stages of atherosclerotic development.38 This finding could be relevant to TF activation in that Grp78 is induced by ER stress; however, it is highly unlikely because the majority of work performed on TF indicates that it is overwhelmingly a cell surface molecule.17

Our studies are consistent with recent work demonstrating the validity of Grp78 as a therapeutic target in pathological models. Specifically, Grp78 binding peptides were shown to target tumor cells in vivo and ex vivo and tumor growth was suppressed by fusion of these peptides to an apoptosis-inducing sequence.39 This work reinforces the biological significance of cell surface Grp78 expression as a marker of pathophysiology associated with TF procoagulant functions.

To further assess the role of Grp78 in coagulation and atherosclerosis, in vivo animal modeling is required. Future in vivo studies involving Grp78 knockout mice or transgenic mice overexpressing Grp78 may provide further insight as to the possible protective role of Grp78 in atherosclerosis.

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References


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