Molecular Immunology 45 (2008) 3639–3648

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

# Stable complexes formed by Grp94 with human IgG promoting angiogenic differentiation of HUVECs by a cytokine-like mechanism

Elisa Tramentozzi[a], Monica Montopoli[a], Genny Orso[a], Andrea Pagetta[a], Laura Caparrotta[a], Martina Frasson[b], Anna Maria Brunati[b], Paola Finotti[a,*]

[a] Department of Pharmacology and Anesthesiology, University of Padova, L.go E. Meneghetti 2, 35131 Padova, Italy
[b] Department of Biochemistry, University of Padova, Italy

ARTICLE INFO

*Article history:*
Received 16 April 2008
Accepted 24 April 2008
Available online 12 June 2008

*Keywords:*
Angiogenic proteins
Cell physiology
Endothelial cells
Heat-shock proteins
Immunoglobulins

ABSTRACT

To explore the molecular mechanisms by which complexes of Grp94 with IgG, purified from the plasma of diabetic subjects, could drive an inflammatory risk in vascular cells, native Grp94 was co-incubated with human, non-immune IgG to obtain the formation of complexes that were then tested on human umbilical vein endothelial cells (HUVECs). Co-incubation of Grp94 with IgG led to the formation of stable, SDS-resistant complexes that displayed effects partly similar and partly significantly different from those of Grp94 alone. Both Grp94 alone and with IgG stimulated the cell growth and promoted angiogenesis by a mechanism of autocrine/paracrine activation of the expression of heat shock protein (HSP)90 and HSP70. However, the most striking alterations in the cell cytoskeleton, characterized by dramatic rearrangement of actin and increased formation of podosomes, were induced by Grp94 with IgG, and were mediated by the enhanced expression of HSP90. At variance with Grp94 alone, Grp94 with IgG promoted the angiogenic differentiation by activating a signaling pathway apparently independent of the intense stimulation of the ERK1/2 pathway that was instead more directly involved in mediating the proliferative effects on HUVECs.

Results show unprecedented cytokine-like effects of Grp94 and a so far undisclosed capacity to bind irreversibly IgG, forming complexes that, with respect to Grp94 alone, display a more intense angiogenic transforming capacity that may predict an increased inflammatory risk in vascular cells *in vivo*.



## 1. Introduction

Grp94 is the most highly represented endoplasmic reticulum-resident heat shock protein (HSP)*. Besides its main property of chaperoning nascent and heat-stressed proteins (Lee, 2001), Grp94 has the highly specialized property of processing and delivering antigenic peptides to the MHC I processing pathway, activating both humoral and cellular immune responses (Li et al., 2002). Functional to this property is the unique proteolytic activity possessed by Grp94, due to the presence in the C-terminus of a linear amino acid sequence containing a serine protease motif (Menoret et al., 2001). Since the C-terminus of HSPs also contains the sequence necessary for binding peptides and proteins (Wearsch and Nicchitta, 1996), the possibility arises that chaperoning and proteolytic activities are functionally coupled in the Grp94 molecule, as in some bacterial and human tissue HSPs (Faccio et al., 2000).

The role of Grp94 in modulating the immune system is clear from numerous observations, showing that cytokine-like effects follow the increase in cell membrane expression and/or extra-cellular liberation of HSPs under various inflammatory and immunogenic stimuli (Asea et al., 2002; Li et al., 2002; Vabulas et al., 2002; Wallin et al., 2002). Thus, activation of heat shock factor 1 (HSF1) by immunogenic stimuli is known to lead to increased expression of both HSPs and cytokines (Inouye et al., 2004). Reciprocally, the cytokine-mediated stimulation of HSF1 is reported to induce the expression of HSPs, together with the production of IgG (Ferris et al., 1988; Suematsu et al., 1989), and elevated concentrations of both IgG and HSPs are common in several autoimmune diseases (Inouye et al., 2004). The cell-surface expression of Grp94 can induce the spontaneous appearance of systemic autoimmune diseases in transgenic mice (Liu et al., 2003), and Grp94 binding to macrophages is known to cause activation of the ERK1/2 pathway (Reed et al., 2003). The immunogenicity of extra-cellular HSPs has also been confirmed indirectly by findings showing elevated plasma concentrations of antibodies (Abs) against HSP90, HSP70 and Grp94 in diabetic patients (Pagetta et al., 2003; Qin et al., 2003) and in subjects with atherosclerotic lesions (Xu et al., 1993). The autoimmune diseases in which plasma concentrations of both HSPs

*Abbreviations:* Ab, antibody; ERK1/2, extra-cellular signal-regulated kinase1/2; HSP, heat shock protein; HUVEC, human umbilical vein endothelial cell; MEK, mitogen-activated extracellular kinase; MMP, matrix metallo protease.

* Corresponding author. Tel.: +39 0498275088; fax: +39 0498275093.
*E-mail address:* paola.finotti@unipd.it (P. Finotti).

0161-5890/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2008.04.020

and anti-HSP Abs are found increased are also characterized by a higher incidence of vascular complications (Pagetta et al., 2003; Qin et al., 2003; Xu, 2002; Xu et al., 1993).

We recently reported that complexes of IgG with Grp94 purified from the plasma of type 1 diabetic subjects are characterized by an irreversible binding (Pagetta et al., 2007), a condition that besides underlining the difference with common immune complexes (in which antigen is reversibly linked to the Ab), points to the formation *in vivo* of fusion protein(s) with novel antigenic properties. It was also observed that complexes of IgG with Grp94, present in the pool of purified IgG, may be responsible for cell growth stimulation and angiogenic transformation of HUVECs (Tramentozzi et al., in press), a finding that reinforced the hypothesis that immune complexes with Grp94 are causally related to the development of vascular alterations in pathologies like type 1 diabetes. However, since the proof for this direct involvement is lacking – it is technically impossible to separate in the bulk of IgG those specifically linked to Grp94 (Tramentozzi et al., in press) – in this work we tried to overcome this difficulty by incubating native Grp94 with human, non-immune IgG in *in vitro* experiments, to obtain the formation of complexes that mimic as closely as possible those purified from diabetic plasma. This approach allowed us to analyze separately the effects due to Grp94 alone and with IgG on HUVECs, comparing also the molecular mechanisms and cellular pathways involved in either one condition.

Results indicate that native Grp94 binds tenaciously also non-immune IgG, forming complexes that display a stronger capacity, with respect to Grp94 alone, to promote angiogenesis by a mechanism of autocrine/paracrine activation of the expression of MMP-9, HSP90 and HSP70.

## 2. Materials and methods

### 2.1. Reagents and antibodies

β casein from bovine milk was from Fluka (Fluka Chemie, GmbH, Buchs, Switzerland); EBM medium, FBS, antibiotics, recombinant human endothelial growth factor (rHEGF), bovine brain extract and hydrocortisone were from Cambrex (Cambrex Bioscience Inc., Walkersville, MD, USA); human non-immune IgG, gelatin, BSA and Mowiol 40-88 were from Sigma (Sigma Chemicals, St. Louis, MO, USA); anti-human HSP90α/β rabbit polyclonal Abs were from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-human rat HSP90α monoclonal, rabbit HSP70 polyclonal and mouse HSP70 monoclonal Abs were from StressGen (StressGen Biotechnologies, Victoria, BC, Canada); mouse anti-β actin monoclonal Abs were from Cell Signaling & Neuroscience (St. Louis, MI, USA); anti-MMP-9 mouse monoclonal Abs were from Calbiochem (Merk KGaA, Darmstadt, Germany) and mitogen-activated extracellular kinase (MEK) inhibitor U0126 was from Promega (Promega Corporation, Madison, WI, USA). All other reagents were of analytical grade from Sigma.

### 2.2. Grp94 purification

Rat liver microsomal fractions were prepared as previously reported, by submitting the fractions to a DEAE-Sepharose column followed by a Heparin-Sepharose column (Lasa et al., 1997). The Grp94-containing peak, eluted from Heparin-Sepharose at 0.5 M NaCl, was chromatographed on FPLC-Superdex 200 (10 mm × 300 mm) previously equilibrated with buffer A (20 mM Tris–HCl, pH 7.5, 5 mM magnesium acetate, 1 mM EGTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 10% glycerol, 0.05 mM PMSF, 0.02% $NaN_3$) containing 500 mM NaCl. Fractions of 0.2 ml each eluted at a flow rate of 0.4 ml/min. The Grp94-containing fractions were collected and passed through a Con A-Sepharose column (5 ml) previously equilibrated with buffer B (20 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 M $CaCl_2$, 10 mM β-mercaptoethanol, 10% glycerol, 0.05 mM PMSF). Grp94 was subsequently eluted with buffer B containing 0.6 M α-D-methylmannoside, and its purity tested by immunoblotting with specific Abs.

The Grp94 preparation was also submitted to the QCL-1000 chromogenic LAL end-point assay (Cambrex BioScience Inc., Walkersville, MD, USA) to exclude any endotoxin contamination.

### 2.3. Preparation of Grp94 and IgG solutions and sedimentation velocity analysis

The purified Grp94 preparation was dialyzed on Spectrapor membrane tubing of 3500 MWCO (A.H. Thomas, Philadelphia, PA, USA) overnight at 4 °C against Tris buffer (20 mM Tris–HCl, pH 7.5) and then submitted to ultra-filtration on Amicon Centriplus YM-3 of 3000 MWCO (Millipore Corp., Bedford, MA, USA). The protein concentration was measured with the method of Bradford (Bradford, 1976). Samples of purified Grp94 were stored at −20 °C in 50-μl aliquots ready to use.

The purity of human non-immune IgG was assessed by Western blotting with sheep anti-human whole IgG polyclonal Abs (The Binding Site, Birmingham, U.K.) and goat anti-Fab polyclonal and mouse γ chain-specific monoclonal Abs (Sigma). In experiments aimed at evaluating complex formation with IgG, Grp94 (200 μg/ml) was co-incubated with human IgG (1:1 molar ratio) at 37 °C for 1 h. Control solutions of Grp94 and IgG, at the same concentration as those used in the co-incubation experiment, were also incubated individually. A 240-μl aliquot of each of the incubated sample solutions (100 μg proteins) were subjected to glycerol density gradient centrifugation with 10–40% glycerol in 25 mM Hepes buffer (pH 7.4), containing 1 mM EDTA and 1 mM dithiothreitol. After centrifugation at 100,000 × *g* for 18 h in a Beckman SW60Ti rotor at 4 °C, the gradient was separated into 18 fractions of 200 μl each, submitted to Western blot analysis. Glutamate dehydrogenase (62 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa, from Sigma) were used as standards for estimating the molecular mass of the complex. An aliquot (8 μg proteins) of the incubated solutions was also directly submitted to Western blotting with anti-Grp94 monoclonal and anti-human whole IgG polyclonal Abs before analysis by glycerol density gradient centrifugation.

### 2.4. Cell cultures

HUVECs were isolated from freshly collected umbilical veins by collagenase treatment (Jaffe et al., 1973). At least three different cords were employed for any cell preparation. Cells were maintained in endothelial basal medium (EBM) supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 10 μg/ml streptomycin, 0.1% (v/v) rHEGF, 0.1% (v/v) hydrocortisone and 0.4% bovine brain extract, at 37 °C in a humidified 95% air, 5% $CO_2$ atmosphere, until the cells reached sub-confluence. Cell culture purity was assessed by microscopic examination of the typical cobblestone morphology and by PE mouse anti-human CD31 monoclonal Abs (BD Pharmingen™, San Josè, CA, USA). All experiments were performed with HUVECs at passages 3–5.

### 2.5. Cell proliferation assay

HUVECs ($12 \times 10^4$/well) were seeded in 12-well (2 ml each) plates in EBM supplemented with 10% FBS, and allowed to attach to well plastics for 24 h in a humidified 95% air and 5% $CO_2$ atmosphere