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Angiogenic transforming capacity of IgG purified from plasma of type 1 diabetic patients

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Abstract

We previously demonstrated that plasma of type 1 diabetic patients contains antibodies complexed irreversibly with Grp94 that also display proteolytic activity. In this work, we wanted to test whether antibodies obtained from diabetic plasma may convey an inflammatory risk on vascular cells. To this aim, IgG were purified on the Protein-G column from individual plasma of eight type 1 diabetic patients, and then tested on HUVECs to measure effects on cell growth and morphologic changes at different incubation times. The purified fractions of IgG contained a significant amount of Fab/$(Fab)_2$, both free and in big aggregates, and anti-Grp94 antibodies, mostly irreversibly linked with, but also free of Grp94. The purified fractions of both Fab/$(Fab)_2$ and whole IgG stimulated the proliferation and sustained the angiogenic differentiation of human umbilical vein endothelial cells (HUVECs) at sub-nanomolar concentrations. IgG from normal plasma neither stimulated the cell growth nor induced any differentiation of HUVECs. The maximum cell growth stimulation occurred at 6–9 hrs and associated with the strong activation of the ERK1/2 pathway, whereas angiogenic transformation was completed later when the ERK1/2 activation was silenced and cell growth stimulation significantly reduced. Neither proteolytic activity of MMP-9 nor VEGF were apparently involved in mediating the angiogenic differentiation of HUVECs that mostly correlated with an increased expression of HSP70 closely coupled with cell membrane-bound inactive species of MMP-9. Results indicate that effects displayed on HUVECs by antibodies purified from diabetic plasma are likely sustained by immune complexes with Grp94 that may thus predict an increased risk of angiogenic transformation *in vivo*.

Keywords: diabetes mellitus • type 1 • heat shock proteins • immunoglobulins • immune complexes • antibodies • angiogenic proteins • cell physiology • endothelial cells

Introduction

Vascular complications are the most important cause of morbility and mortality in diabetic patients and represent an increasingly important medical and social problem. Although it is well established that alterations of metabolic control play a deleterious role in inducing and worsening vascular lesions, the unique etiopathogenetic role of hyperglycaemia in this respect has been questioned, and often hyperglycaemia does not account for both the severity and precocity of complications in diabetes [1, 2]. Thus, the identification of molecules that may be true indicators of early and stable alterations leading to open vascular pathologies is an urgent, still unmet goal for an effective preventive and/or curative intervention. Although a number of molecules has been found altered and variously associated with the development and/or progression of vascular complications in diabetes, no one has so far acquired eligibility for an effective biological marker.

Recently, we demonstrated that in the plasma of type 1 diabetic patients there is an increased concentration of the heat shock protein (HSP) Glucose-regulated protein94 (Grp94) [3], almost exclusively present in stable complexes with IgG, identified as anti-Grp94 antibodies (Abs) [4]. These findings indirectly revealed the immunogenic potential of Grp94 in human beings, supporting previous observations showing that Grp94 behaves like an inflammatory, cytokine-like molecule once it is expressed extra-cellularly [5, 6]. The unique immunomodulatory role played by Grp94 is further confirmed by the fact that, similarly to its cytoplasmic homologue HSP90 and to HSP70, Grp94 is capable of inducing

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intense inflammatory/immune reactions that ultimately lead to the development of autoimmune diseases [7, 8]. The additional finding that the level of Grp94 in the plasma of diabetic patients is apparently unrelated with the duration of diabetes and metabolic control [3, 4], raised the question of whether Grp94 in plasma may be a marker of disease associated with an increased risk of vascular inflammation [9]. To test this hypothesis, in this work we studied the effects of plasma-purified Ab fractions, containing immune complexes with Grp94, on HUVECs, taken as a model of vascular cells. Results indicate that Abs from any diabetic but not normal plasma stimulate the growth and induce a strong, time-dependent angiogenic transformation of HUVECs, thus representing a potentially useful marker of vascular lesion.

Materials and methods

Reagents and antibodies

Endothelial basal medium (EBM), foetal bovine serum (FBS), antibiotics, recombinant human endothelial growth factor (rHEGF), bovine brain extract and hydrocortisone were from Cambrex (Cambrex Bioscience Inc., Walkersville, MD, USA); gelatin and bovine serum albumin (BSA) were from Sigma (Sigma Chemicals, St. Louis, MO, USA). The following Abs were used: sheep anti-human whole IgG polyclonal (The Binding Site, Birmingham, UK); goat anti-Fab polyclonal (Sigma); mouse anti-Grp94, mouse anti-HSP70 monoclonal (Stress Gen Biotechnologies, Victoria, BC, Canada); mouse anti-β actin monoclonal (Cell Signaling & Neuroscience, St. Louis, MI, USA), rabbit anti-human vascular endothelial growth factor (VEGF) polyclonal (Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA) and mouse anti-Matrix Metalloprotease (MMP)-9 monoclonal Abs (Calbiochem Merk KGaA, Darmstadt, Germany). All other reagents were of analytical grade from Sigma.

Blood collection

Blood was collected from eight type 1 diabetic patients, matched for sex (4 males and 4 females), during routine control visits for diabetes. Patients gave their informed consent for blood sampling. Diabetic patients had mean (±S.D.) age of 32.8 years (±7.35), age at disease onset of 16.8 years (±7.15) and diabetes duration of 15 years (±7.5). They did not take any medication (other than insulin for diabetes) nor did they present any clinically detectable vascular complications. The mean (±S.D.) value of fasting blood glucose and HbA1c were 164.2 mg/100 ml (±47.84) and 8.42% (±1.41), respectively. Following extensive dialysis on Spectra-Por membranes (8000 MWCO, A.H. Thomas, Philadelphia, PA, USA), 1 ml plasma of each person was processed individually. One-millilitre pooled plasma of four normal persons (mean age ± S.D., 35.7 ± 6.7 years) was also analysed separately and served as control.

Antibody purification

Plasma proteins (6 mg) were loaded on a mono-Q HR 5/5 column (Amersham Biosciences, Uppsala, Sweden) applied on a fast liquid chromatography system (Pharmacia LKB, Uppsala, Sweden) and purification performed in two steps, as previously described [4]. Peak 2 from the mono-Q (0.6 mg proteins) was then loaded on to a 1-ml HiTrap Protein G (PG) HP column (Amersham Biosciences) for further purification of IgG. Proteins eluted at a flow rate of 0.5 ml/min. and fractions collected by monitoring absorbance at 280 nm. While Ab subunits eluted in the void volume with the eluent A (20mM phosphate buffer, pH 7.0), whole IgG were recovered with the eluent B (0.1M glycine buffer, pH 2.5) in the second peak. IgG fractions were collected in tubes with 200 μl of 1.0 M Tris–HCl pH 9.0 for preventing protein denaturation due to acidity of the eluent B. Peaks were ultra-filtered on Amicon Centriplus YM-3. Proteins were measured spectrophotometrically and by the micro BCA protein assay (Pierce, Rockford, IL, USA).

Electrophoresis and Western blot analysis

SDS-PAGE was run on 10% acrylamide gel. Gels were stained with colloidal Coomassie brilliant blue. In the Western blot analysis, 5–10 μg proteins were transferred into a polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore, Bellerica, MA, USA) and treated in 20 ml TRIS-buffered saline (TBS) with anti-human Grp94 and IgG (both H&L and Fab-specific) Abs.

To detect specific immune reactions in Western blotting, secondary Abs comprised alkaline phosphatase conjugate, affinity-purified IgG (Sigma) and biotin conjugate affinity-purified IgG (Vector Laboratories, Burlingame, CA, USA). An affinity-purified egg white avidin conjugated to alkaline phosphatase (Sigma) was also used for detecting the immunoreaction (ABC system). Secondary Abs alone were used as control to exclude any false positive in the immune reaction.

Dot-blot analysis

In dot-blot experiments, strips of 2.0 × 2.5 cm of PVDF membrane were rinsed in methanol and de-ionized water and partially dried. Dots with 10 μl of native Grp94 (3 μg) purified from microsomal fraction of rat hepatocytes [10] and denatured by boiling (3 min.) were applied on each membrane strip. After incubation with a blocking solution of 1% BSA and Tween 0.2%, membranes were then incubated for 8 hrs with plasma-purified Ab-containing peaks (10 μg proteins/ml). Sheep anti-human IgG Abs were employed to detect anti-Grp94 Abs, and reaction revealed by means of alkaline phosphatase-conjugated anti-sheep IgG Abs. Control strips included dots of Grp94 incubated with TBS solution alone and then probed with both primary and secondary Abs, and with secondary Abs alone.

Cell cultures

HUVECs were obtained from freshly collected umbilical veins by collagenase treatment [11]. At least three different cords were employed for any cell preparation. Cells were maintained in 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 cells reached sub-confluence. Purity of the cell culture was assessed by microscopic examination of the typical cobblestone morphology and by PE mouse anti-human CD31 monoclonal Abs (BD Pharmingen™, San José, CA, USA). All experiments were performed with HUVECs at passages 3–5.

Cell proliferation assay

HUVECs (25×10^4/well) seeded in 12-well (2 ml each) plates in EBM supplemented with 10% FBS were allowed to attach to well plastics for 24 hrs in a humidified 95% air and 5% CO_2 atmosphere at 37°C. Cells were then starved in fresh, serum-free medium for 8–10 hrs. After this time, a fresh aliquot (2 ml) of serum-free medium was added without (control) and with plasma-purified Ab-containing peaks (10 ng/ml, final concentration), and with 10 ng/ml of native Grp94 as positive control. In each experiment, wells with both medium alone and medium supplemented with 1% FBS were included as additional controls. After time intervals of 6, 9 and 20 hrs, medium from duplicate wells was collected and stored at –20°C for further analysis. Cells were washed with PBS, detached from wells by 0.05% trypsin and 0.2% EDTA and counted in a haemocytometer. Cell viability was evaluated with the trypan blue dye exclusion method.

Analyses on cell lysates

After indicated incubation intervals, cells were washed with PBS, scraped and centrifuged for 15 min. at $1500 \times g$. After removal of the supernatant, cells were lysed in the Laemmli lysis buffer (50 mM Tris-HCl, pH 8.9, 5 mM EDTA, 380 mM glycine, 2% SDS with 7 mM β-mercaptoethanol) and the proteins measured by the method of micro-BCA (Pierce). In separate experiments, cell lysis was also performed in sterile de-ionized water followed by centrifugation at $100{,}000 \times g$ for 90 min. [12]. The supernatant was collected (cytosol fraction) and the pellet (membrane fraction) treated with the Laemmli buffer, as above without β-mercaptoethanol. Lysates were then analysed by SDS-PAGE on 10% polyacrylamide gel, followed by blotting on a nitrocellulose membrane and tested for ERK1/2 activity with total and phospho(P)-specific ERK1/2 polyclonal antibody (Cell Signaling & Neuroscience) (whole lysates), HSP70, MMP-9 and VEGF (membrane and cytosol fractions). Immunodetection was attained by both the Enhanced luminol-based ChemiLuminescent (ECL) system (Amersham Biosciences, Uppsala, Sweden) and the ABC system with biotin conjugated affinity-purified H&L IgG (Vector Laboratories) with affinity-purified egg white avidin (Sigma). Antibodies against β actin were also used as control for protein loading.

Treatment and analysis of media

Media from duplicate wells of both control and treated HUVECs were collected, centrifuged for 10 min. at $800 \times g$ to remove cell debris and dialysed overnight at 4°C against pure distilled water. The lyophilized material was re-suspended in 100 μl of sample buffer (0.125 M Tris-HCl, pH 6.8, glycerol 20% and SDS 4%) and analysed in SDS-PAGE followed by Western blotting with anti-HSP70, anti-MMP-9 monoclonal and anti-VEGF polyclonal Abs. Immuno-detection was performed with alkaline phosphatase conjugated affinity-purified H&L IgG (Sigma) and the ABC system.

Proteolytic activity of media was measured by zymogram gel analysis, in which samples were loaded on to the polyacrylamide gel (10%) co-polymerized with gelatin (0.8 mg/ml) in the presence of SDS. After repeated washings (15 min. each) with the renaturing solution of 2.5% Triton X-100, the gel was incubated overnight at 37°C in a solution of Tris buffer (50 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.4) under slow agitation. The gel was then submitted to staining with Coomassie brilliant blue followed by de-staining with a 5% methanol and 7.5% acetic acid solution (in de-ionized water) until clear bands appeared against the blue background.

Immunofluorescence microscopic analysis

For microscopic examination, cells (6×10^4 cells in 0.5 ml wells) were plated on 8-well tissue culture chamber slides with detachable upper structures. After a starving period of 9 hrs, cells were treated with 10 ng/ml proteins of both peak 1 and 2 from the PG column in the absence of FBS, and incubated for 20 hrs at 37°C. Cells were fixed with 4% formaldehyde in PBS for 15 min., washed and treated with 0.1% Triton X-100 at room temperature for 10 min. After repeated washings, cells were incubated for 30 min. with blocking buffer (1% BSA in PBS), and with both phalloidin (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA) (1:100, v:v ratio) for 2 hrs at 37°C, to evaluate the actin cytoskeleton, and rabbit anti-human HSP70 Abs (1:100, w:v ratio). Alexa Fluor 488 goat anti-rabbit IgG (1:350, v:v ratio, Molecular Probes) were added to detect fluorescent signals of HSP70. After incubation with specific Abs for 1 hr at room temperature, cells were treated with 21 μg/ml DNase-free Rnase, washed and treated with red-fluorescent Propidium Iodide for nuclear and chromosome counterstaining (Molecular Probes), added to Mowiol 40-88 at the final concentration of 0.5 μg/ml.

Statistical analysis

All data examined were presented as mean ± S.D. unless otherwise stated. Statistical analysis of data was performed by means of GraphPad Prism 3 (GraphPad Software, Inc. San Diego, CA, USA). Comparison between groups was made using the two-way ANOVA and unpaired Student's t-test. A P value <0.05 was considered statistically significant.

Results

Grp94 is stably associated with Fab of both fragmented and whole IgG

By processing any individual plasma on the mono-Q column, strikingly overlapping elution profiles were obtained (Fig. 1A) that also duplicated those previously reported with pooled plasma [4]. Small inter-individual differences were only noted for the amount of proteins eluted in each peak. For this reason, results obtained on plasma of a single person (A.M., male of 26 yrs) are shown as representative of all others.

IgG that eluted from the mono-Q column mostly concentrated in peaks 1-3, as revealed by Western blotting with anti-IgG (data not shown) and anti-Fab specific Abs (Fig. 1B). It was observed that, in addition to whole IgG with mass of 150 kD, also subunits of Fab and $(Fab)_2$, with masses of 50 and 110 kD, respectively, and monomeric chains of 25 kD were present in peaks 1-3a (Fig. 1B), as previously noted with pooled plasma [4]. Since in peak 3 also transferrin elutes together with IgG (Fig. 1B), purification of Abs was carried out only on peak 2, that was the purest and richest source of IgG, as proved by the specific Western blot analysis.

The elution of peak 2 mono-Q from the PG column led to the separation of IgG subunits of Fab/$(Fab)_2$ (in the flow-through

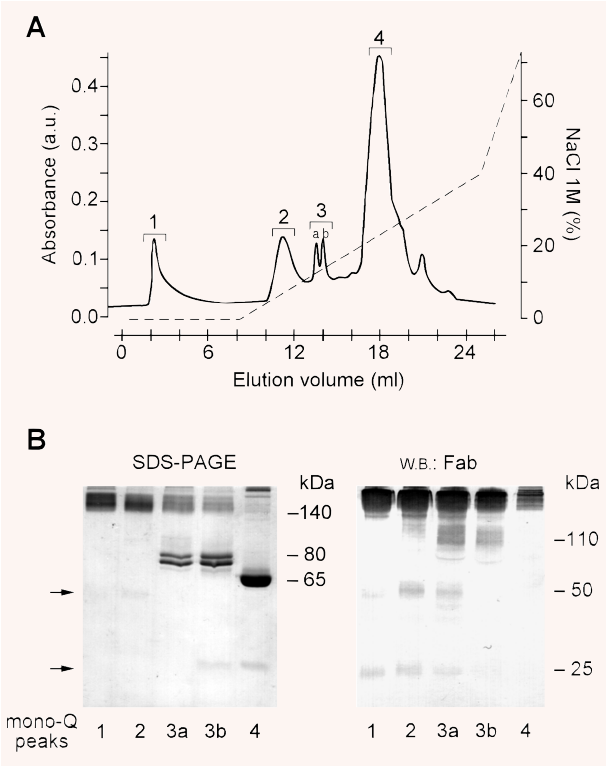

Fig. 1 Purification of IgG from plasma. The elution pattern from the mono-Q column of 6 mg proteins of a single dialysed plasma (A.M.) as representative of other diabetic plasma samples is shown in (**A**). Each peak (5 μg proteins) was then analysed in SDS-PAGE in absence of reducing and boiling conditions, followed by Western blotting with anti-human, Fab-specific polyclonal Abs (**B**), as specified in Materials and Methods. The bands at about 80 kD and the 65-kD band in SDS-PAGE are, respectively, transferrin and albumin. Arrows mark Fab and single chains of IgG in bands at 50 kD and 25 kD, respectively, mostly visible in peak 2 following Western blotting. The pattern is representative of three Western blot analyses made on separate occasions.

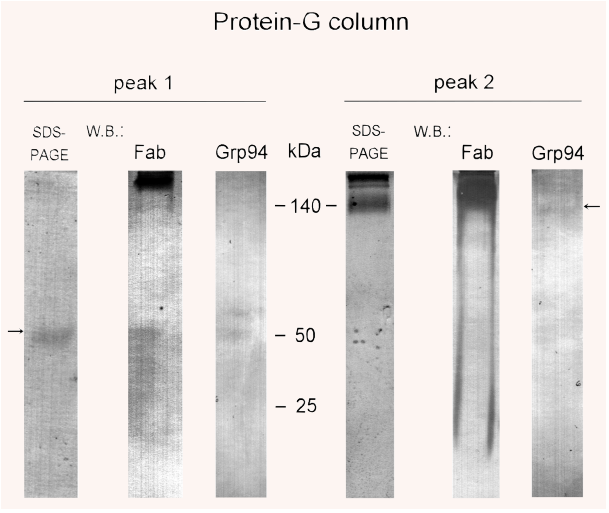

Fig. 2 Grp94 linked to Fab in both fragmented and whole Abs. Peak 2 from the mono-Q column was loaded (0.6 mg proteins) on to the PG column, as specified in Materials and Methods, obtaining two peaks, submitted to SDS-PAGE in non-reducing and boiling conditions, followed by Western blotting with both anti-human, Fab-specific and anti-Grp94 monoclonal Abs. Seven μg proteins were loaded in each lane. Arrows mark on left (peak 1) the positivity with both anti-Fab and anti-Grp94 Abs in the band at 50 kD, and on right (peak 2) the band at about 140 kD, positive for Grp94 linked to Fab in the whole IgG.

peak 1) from whole IgG that remained bound to the column with their intact Fc portion, thus eluting in peak 2. Western blotting with anti-Fab-specific Abs, performed in absence of reducing and boiling conditions of samples in SDS-PAGE, revealed in peak 1 Fab, both free (50 kD) and in big aggregates (at 140 kD and near the cathode), whereas in peak 2 Fab were exclusively detected in the band of whole IgG (150 kD) (Fig. 2). After samples were submitted to reducing conditions in SDS-PAGE, aggregates of Fab disappeared, while Fab at 50 kD were reduced to monomers of 25 kD (data not shown).

To confirm our previous results on pooled plasma showing that stable complexes form between IgG and Grp94 [4], we performed blot analysis on peaks 1 and 2 from the PG column to detect Grp94 linked to Fab in both Fab/$(Fab)_2$ and whole IgG. In both non-reducing and reducing conditions of samples in SDS-PAGE, it was observed that Fab at 50 kD in peak 1 also reacted with anti-Grp94 Abs. Similarly, in peak 2, the positivity for Grp94 was visible in the diffuse Fab-positive band at 140 kD (Fig. 2, arrows). In neither peak Grp94 was detected at its expected mass of 95-100 kD, consistent with its free form, thus confirming that in diabetic plasma Grp94 was exclusively and closely associated with Fab.

Anti-Grp94 Abs free of antigen in the pool of IgG

We then analysed any diabetic plasma to see whether anti-Grp94 Abs were also present free of Grp94. To this aim, experiments of dot-blot were carried out in which native Grp94, purified from the microsomal fraction of rat hepatocytes, was used as antigen and incubated with plasma-purified Ab-containing peaks (Fig. 3). Only anti-Grp94 Abs having free antigen-binding site(s) could react with Grp94. An intense immune reaction was observed with peaks 2, from both mono-Q and PG columns, but not with peak 1 from the PG column. The result indicated that free anti-Grp94 Abs were originally present in peak 2 from the mono-Q column as whole Abs that could be then recovered in peak 2 from the PG column. The lack of immune reaction with peak 1

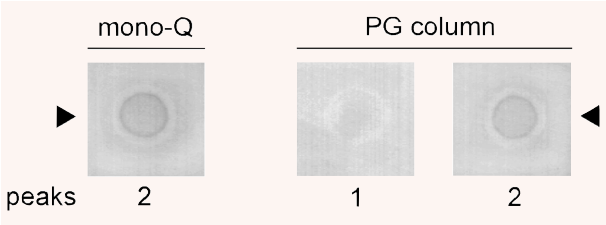

Fig. 3 Antigen-free anti-Grp94 Abs present in diabetic plasma. Dot-blot experiments were performed by incubating membranes containing the dot of native Grp94 (10 μl, 2.5 μg protein) with Ab-containing peaks (protein concentration, 12 μg/ml) purified from any plasma, as specified in Materials and Methods. To detect the immune reaction, anti-human IgG (whole molecule) polyclonal Abs were used. As negative controls, dots of native Grp94 were incubated with TBS alone and probed with both primary (anti-IgG) and secondary (anti-sheep, affinity-purified IgG) Abs, both alone and together. In the figure, a representative dot-blot (of three other experiments) with peaks purified from single plasma is shown; overlapping pictures were obtained with any other plasma. Arrowheads mark dots with positive immune reactions due to anti-Grp94 Abs in peaks 2, from both the mono-Q and PG column.

containing only Fab/$(Fab)_2$ may be explained by considering that goat anti-sheep IgG used as secondary Abs reacted only weakly with IgG subunits that lack the Fc portion of the IgG molecule. It cannot also be excluded that Fab/$(Fab)_2$ do not react with Grp94 because antigen-binding sites have already been engaged in binding circulating Grp94.

Cell growth stimulation and angiogenic differentiation of HUVECs by plasma-purified antibody fractions

To test the effects on HUVECs of Ab-containing plasma-purified fractions, we first incubated cells for 20 hrs and noted that after this time peak 2 from the mono-Q column did not stimulate the cell growth at all, whereas peaks 1 and 2 from the PG column caused average stimulations of 15% and 24.4%, respectively (Fig. 4A). To rule out that also non-immune Abs contributed to this effect, IgG purified from normal plasma were used as additional control in cell cultures. In this case, the cell count overlapped that of control in absence of FBS (Fig. 4A). In both absence and presence of IgG from normal plasma, control HUVECs showed typical cobblestone morphology, whereas after the addition of Ab-containing peaks – especially peak 2 from the PG column – cells underwent morphologic changes consistent with angiogenic transformation. To mimic the condition found in Ab-containing fractions from diabetic plasma, we also used native Grp94 as further control in cell culture experiments. Native Grp94 (10 ng/ml) caused an average cell growth stimulation of 32% also displaying strikingly similar angiogenic effects on HUVECs after 20 hrs of incubation (Fig. 4B).

Since peaks 1 and 2 from the PG column were the most active Ab-containing fractions, these two peaks were used in subsequent experiments in which the proliferative and differentiation effect on HUVECs were also measured at shorter incubation times (6 and 9 hrs). It was thus observed that the maximum cell growth stimulation of 35% and 31% occurred at 9 hrs, with peaks 1 and 2, respectively, (unpaired t-test: $P = 0.003$ and $P = 0.001$, for peak 1 and 2, respectively, $n = 4$) (Fig. 5A). At 6 hrs, peak 2 turned out to be more effective than peak 1, causing an average increase in the cell growth of 26% (unpaired t-test: $P = 0.014$, $n = 4$). Apparently thus, cell growth stimulation by peak 2 started earlier and lasted for a longer time (up to 20 hrs) than that caused by peak 1. Overlapping morphologic changes of HUVECs, consistent with a time-dependent progression of angiogenic transformation, were instead shown with both peaks 1 and 2 at any incubation time (Fig. 5B).

The ERK1/2 pathway and the MMP-9 expression are differently involved in the mechanism of cell proliferation and differentiation by antibody-containing fractions

To see whether the ERK1/2 pathway was involved in the cell growth stimulation and angiogenic differentiation induced by peaks 1 and 2 from the PG column, total and P-ERK1/2 were measured in whole cell lysates. In control, serum-free HUVECs, a slight stimulation of P-ERK1/2 was detected only in first hours, disappearing completely after 20 hrs (Fig. 6). With both peaks (mostly peak 2) there was an intense activation, especially of P-ERK2 at 6 and 9 hrs, which decreased in time, remaining however still significantly elevated with respect to the control even after 20 hrs.

Since MMPs are specifically involved in the development and the finest modulation of angiogenesis [13], we investigated whether effects on HUVECs were mediated by an increase in activity and/or expression of MMP-9, the secreted MMP capable of degrading the extra-cellular matrix in various physiological and pathological conditions [13–15]. Proteolytic activity of conditioned media, measured on gel of gelatin, unexpectedly showed that both peaks caused a significant reduction in the secretion of inactive MMP-9 (the 92 kD form that is activated artificially by zymography) at 6 and 9 hrs (Fig. 7). Reductions were significantly different at 6 hrs (unpaired t-test: $P = 0.005$ and $P < 0.0001$ for peak 1 and 2, respectively, with respect to control), being still significantly different at 9 hrs for peak 2 only ($P = 0.044$, $n = 4$). The secretion of inactive MMP-9 started to increase after 9 hrs of incubation, reaching values similar to those of control at 20 hrs (Fig. 7). Also the secretion of the 90-kD active form of MMP-9, only slightly detectable at 6 and 9 hrs, increased significantly after 20 hrs independently of the treatment with peaks 1 and 2 (two-way ANOVA, $P < 0.0001$ for both control and treated HUVECs compared to respective values at 6 and 9 hrs, $n = 4$). Apparently,

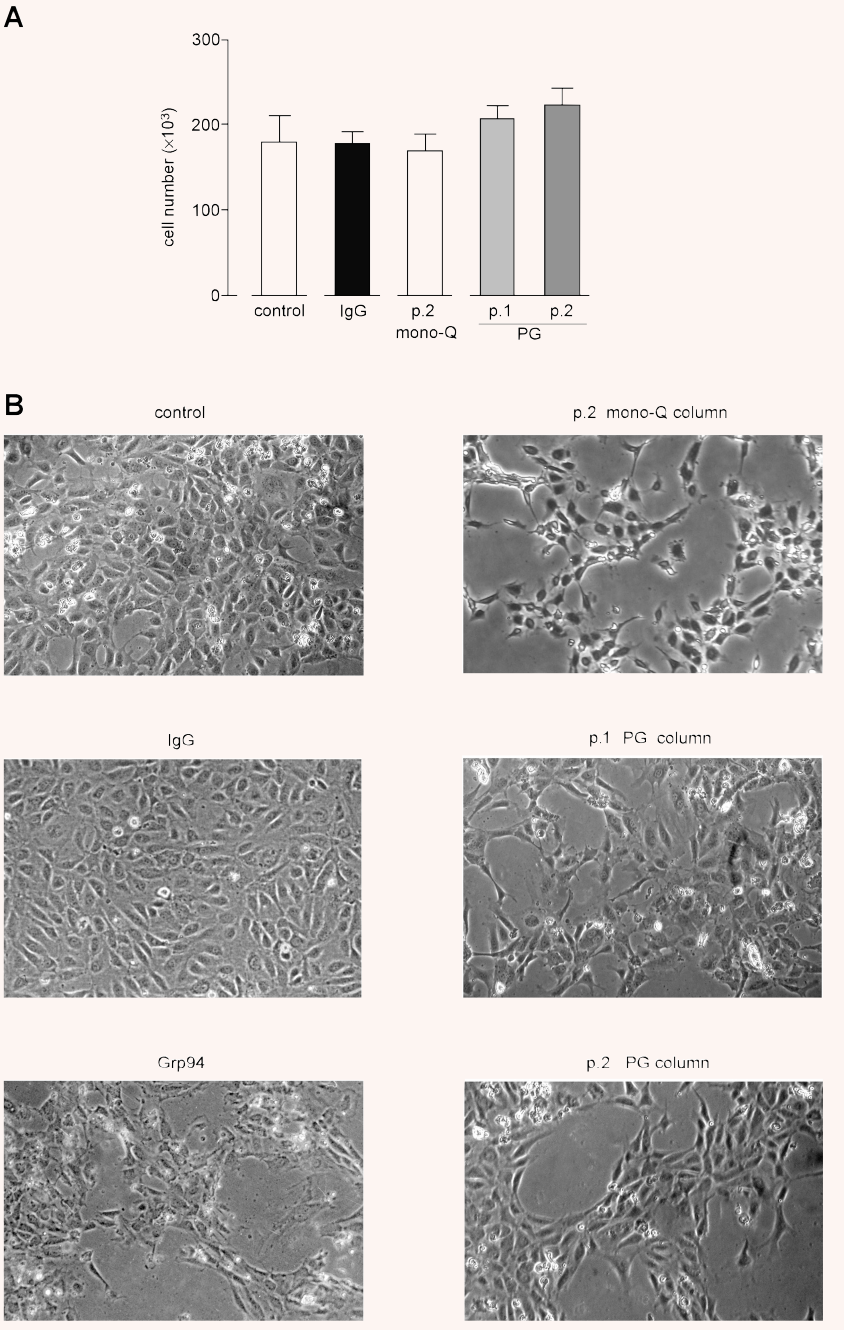

Fig. 4 Cell-growth stimulation and angiogenic transformation of HUVECs. Cells were grown as specified in Materials and Methods and after starvation they were challenged with serum-free medium, in the absence (control) and presence of 10 ng/ml of the indicated plasma-purified peaks, control human IgG and native Grp94. After incubation for 20 hrs, cells from duplicate wells were counted. (**A**) Histograms are means (±S.D.) of at least three separate experiments. The mean (±S.D.) cell number in the control and after incubation with peaks 1 and 2 from the PG column was 180 (±51) $\times$ 10^3, 207 (±38) $\times$ 10^3 and 224 (±52.4) $\times$ 10^3, respectively. No significant differences were observed in multiple comparisons between control and treatments. (**B**) Microscopic evaluation of the morphogenic response of HUVECs to the indicated treatments (10 ng/ml each). Representative pictures (of several other made with any plasma) are shown. Original magnification of 10×.

thus, the mechanisms leading to complete angiogenic transformation did not involve an increased activity of MMP-9. However, since the measurement of proteolytic activity does not give precise information about the protein expression, we also measured in Western blotting the level of MMP-9 in both conditioned media and cell lysates after 20 hrs of incubation (Fig. 8). The expression of both active and inactive MMP-9 in the media of control and treated HUVECs did not differ at all, nor there was any difference in MMP-9 species of 50 and 40 kD, mostly representing inactive products of MMP-9, not visible in zymography (Fig. 8, lower panels, arrows). Instead, in the membrane fraction of cell lysates, the bands of MMP-9 were more numerous and more intensely stained

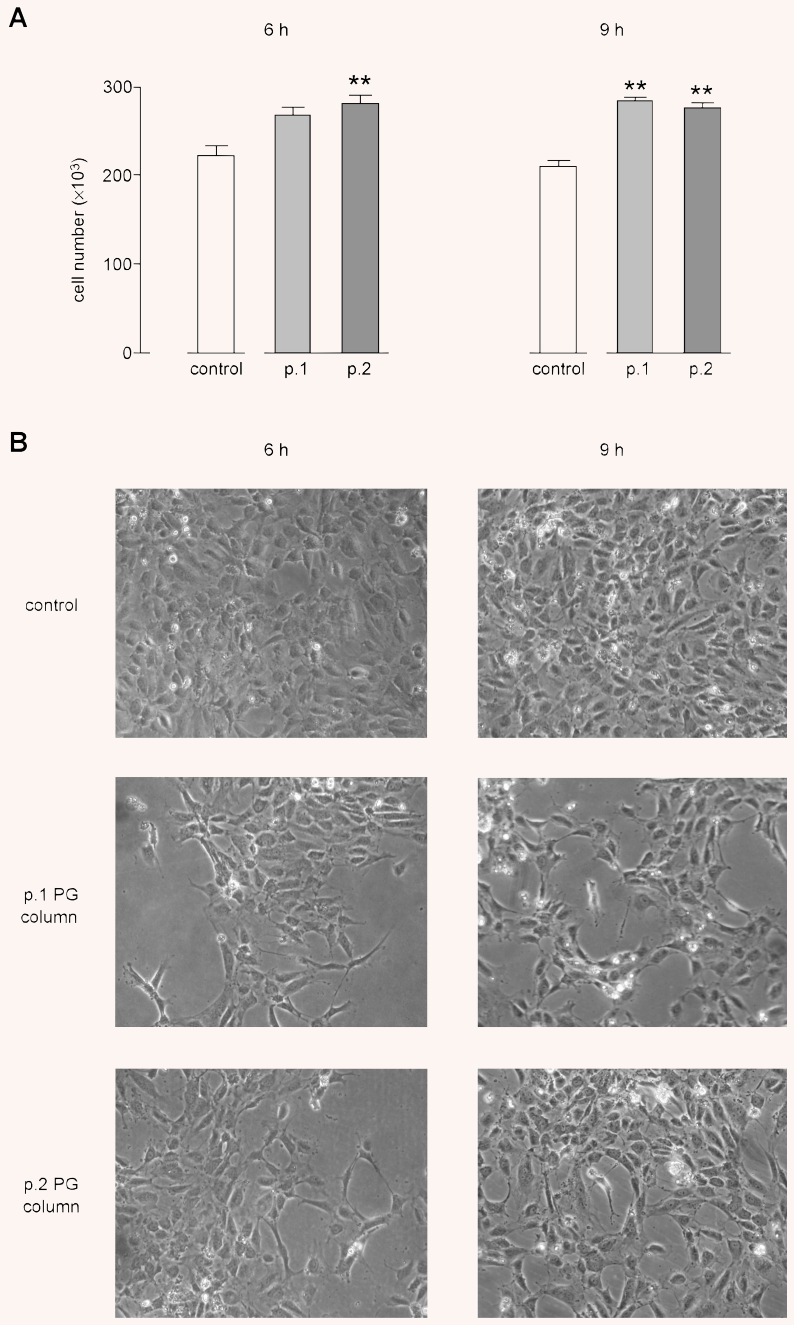

Fig. 5 Stimulation of cell growth and induction of angiogenic transformation by peaks from the PG column. Cells were grown and treated as specified in the legend to Figure 4 and in Materials and Methods, with the exception that incubation was stopped at 6 and 9 hrs. (**A**) Histograms are means (±S.D.) of at least three separate experiments. The cell number in the control HUVECs at 6 and 9 hrs was 222.2 (±19.5) $\times$ 10^3 and 210 (±10) $\times$ 10^3, respectively. Asterisks mark highly significant (**) differences with respect to the control (unpaired t-test). (**B**) Morphogenic transformation of HUVECs at 6 and 9 hrs following indicated treatments, as evidenced at the optical microscopy. Representative pictures of each condition are shown with original magnification of 10×.

in treated HUVECs than in the control (Fig. 8, upper panels). This pattern strongly resembled that described by others who showed that a series of inactive and active species of MMP-9 with various molecular weight, form intra-cellularly in response to various growth factors [16, 17]. Consistent with the result that membrane-bound species of MMP-9 were expressed at a higher level in HUVECs treated with both peaks than in the control, in the corresponding cytosol fraction of cell lysates after treatment, MMP-9 was only weakly detected in two bands at 67 and 60 kD, whereas in control HUVECs the pattern of MMP-9 bands was similar to that seen in the membrane fraction (data not shown). Thus, after challenging HUVECs with Ab-containing peaks, MMP-9 was almost exclusively confined to cell membranes, whereas an insignificant part of it was in soluble form in the cytosol.

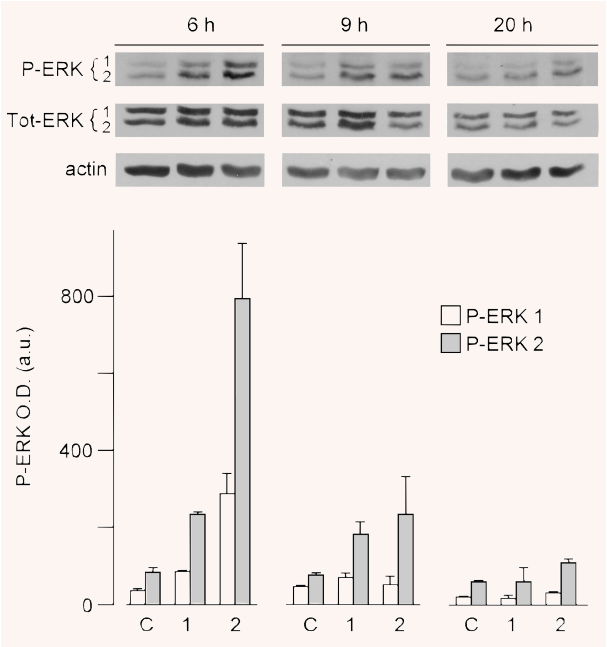

Fig. 6 Time-dependent stimulation of P-ERK1/2 in HUVECs treated with peaks eluting from the PG column. Whole lysates of cells grown in serum-free medium and treated with peaks from the PG column were analysed in Western blotting with anti-total and anti-P-ERK1/2 polyclonal Abs, as specified in Materials and Methods. The same membranes were then stripped and tested with anti-β actin Abs for normalization of the protein content. In the figure are shown: a representative blotting of two separate experiments (above), and the corresponding graphical representation of the intensity (means ± S.D., $n = 2$) of the P-ERK1/2 bands measured by the densitometric analysis (below).

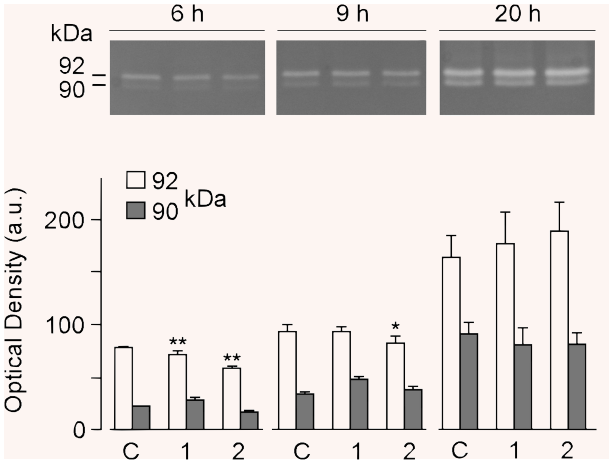

Fig. 7 Proteolytic activity of MMP-9 in the medium of HUVECs treated with peaks from the PG column. After the indicated incubation times, supernatant of cell cultures, both in absence (control) and presence of peaks 1 and 2 from the PG column (10 ng/ml) were collected and probed in zymogram gel analysis, as described in Materials and Methods. A representative gelatin zymography (of four, made on different occasions) is shown. Bands of digestion of the 92-kD inactive species of MMP-9 (artificially activated by zymography) and of the active form at 90 kD are visible. Below is the graph representing the intensity (in arbitrary units) of the digestion area of the two bands of MMP-9, measured by densitometric analysis. The height of histograms is the mean (±S.D.) of the digestion area measured in at least three independent experiments. Asterisks indicate statistically significant (*) and highly significant (**) differences among means (see text for details).

Enhanced co-expression of MMP-9 species and HSP70 in HUVECs treated with plasma-purified antibody fractions

It is known that growth factors and cytokines that stimulate the expression of MMP-9 [17–19] also mutually affect the expression and cellular secretion of HSPs [9], the inflammatory response being thus activated and expanded by autocrine-paracrine mechanisms [20, 21]. In particular, an increased expression of MMP-9 has been shown to correlate positively with up-regulation of HSP70 [22], an event known to occur in the process of capillary formation and maturation [23]. In order to investigate whether HSP70 was involved in the process of angiogenesis by peaks 1 and 2, we measured the amount of HSP70 in both media and membrane fraction of cell lysates after 20 hrs of incubation, *i.e.* when the maximum cell differentiation was observed. An intense staining for HSP70 was noted in both control and treated HUVECs, although in the presence of peak 2, bands at masses lower than 70 kD were more numerous and intensely stained than in the control (Fig. 8, upper panels). Interestingly, the pattern of positivity for HSP70 almost completely overlapped that of MMP-9, with the only exception of HSP70 bands at 86 and 24 kD, a result that strongly suggested a close association of the two proteins. As noted for MMP-9, also HSP70 was barely detectable in conditioned media, where it was present exclusively at masses consistent with a complex, in both control and treated HUVECs (Fig. 8, lower panels, arrow). The increased expression of HSP70 following incubation with plasma-purified, Ab-containing fractions was further confirmed in immunofluorescence experiments in which an intense and homogeneously diffuse cellular fluorescence for HSP70, in some parts also fused with that of actin, was similarly observed with both peaks (Fig. 9).

Since VEGF is a potent mitogenic and angiogenic factor [24, 25], and its effects on endothelial cells are known to be also mediated by HSP90 [26], it was of interest to see whether VEGF was involved in sustaining angiogenic transformation by Abs. In Western blotting of conditioned media, VEGF was visible in two bands near the cathode and at 88 and 70 kD in both control and treated HUVECs (Fig. 8, lower panels, arrows). The bands of VEGF at molecular masses higher than the expected mass of the soluble form likely represent

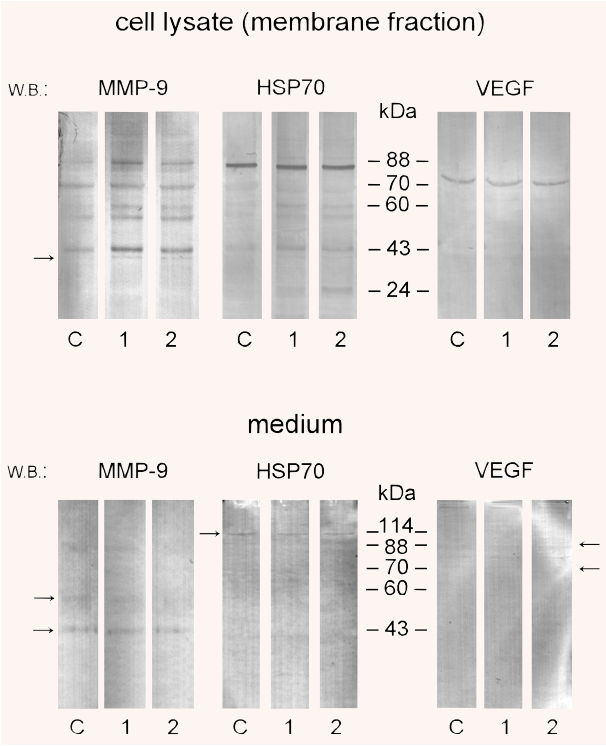

Fig. 8 MMP-9 and HSP70 are closely associated in the membrane fraction of HUVECs. After the incubation time of 20 hrs, cell cultures in both absence (control) and presence of peaks 1 and 2 from the PG column (10 ng/ml) were lysed to obtain cytosol and membrane fractions, as specified in Materials and Methods. In the figure, representative Western blot analyses with anti-MMP-9 (reacting with both inactive and active species of the protease), anti-HSP70 and anti-VEGF monoclonal Abs on both the membrane fraction of cell lysates and corresponding conditioned media are presented. The same quantity of proteins was loaded in each lane in SDS-PAGE in non-reducing conditions of samples. Upper panel: the arrow on left marks the band of MMP-9 at 40 kD visible in HUVECs treated with peaks 1 and 2 but not in the control. Arrows in the lower panel mark the bands of interest (see text).

complexes that VEGF efficiently forms with receptors and with molecules present in both the cell membrane and extra-cellular matrix [27]. In the membrane fraction of cell lysates, VEGF was detected in a sharp band at 70 kD, shared by all samples (Fig. 8, upper panel). Appar-ently thus, neither the expression nor the secretion of VEGF was significantly affected by plasma-purified Ab-containing fractions.

Discussion

In this work, we present experimental evidence showing that Abs, purified from diabetic but not normal plasma, are able to induce the proliferation and angiogenic transformation of HUVECs.

We observed that any diabetic plasma shared both a significant amount of free Fab/$(Fab)_2$ that were purified separately from whole IgG, and the presence of Grp94, exclusively and steadily bound to IgG in immune complexes (Fig. 2). Free Fab/$(Fab)_2$ likely originate from the fragmentation of whole Abs driven by the catalytic activity of Grp94 itself [3, 28] that is similar to that displayed by serine proteases on the IgG molecule [4, 29]. Although less probable, the possibility also exists that the increased plasma concentration of IgG subunits is due to an accelerated extracellular liberation of unassembled Ig chains, known to represent a common condition in various autoimmune diseases [30, 31] including type 1 diabetes [32]. Irreversibility of Grp94 binding to Fab in the IgG molecule explains why Grp94 could be recovered in both the antigen (peak 1) and Ab fractions (peak 2) eluting from the PG column during the purification process of IgG. In agreement with our previous results, obtained on a pool of diabetic plasmas [4], also here, on individual plasma, we demonstrate that Grp94 behaves as an immunogen capable of triggering a specific immune response. In dot blot analysis, we thus detected the presence of anti-Grp94 Abs free of antigen that may represent those not yet bound to the antigen or in excess over the plasma concentration of antigen, probably dependent on an exaggerated immune response. Both free and Grp94-complexed anti-Grp94 Abs in the plasma of any diabetic patient are the expression of a prolonged exposure to the common immunogen probably linked to disease itself. The immunoblot analysis does not permit to establish what is the concentration of anti-Grp94 Abs, both free and in complexes with Grp94, in the pool of IgG. The proportion of these immune Abs may also vary from one person to another, depending on variables related to disease itself and to the individual immune response [4].

Although the separation of immune complexes with Grp94 from the bulk of non-immune Abs cannot be obtained due to unsurmountable technical difficulties – a condition that prevents us from demonstrating the direct involvement of immune complexes with Grp94 in eliciting effects on HUVECs – our results nevertheless indicate that this specific immune portion of IgG may be responsible for both cell growth stimulation and angiogenic differentiation of HUVECs. First, plasma-purified fractions containing both Fab/$(Fab)_2$ and whole IgG yielded overlapping effects at subnanomolar concentrations (10 ng/ml), *i.e.* concentrations similar or even lower than those requested to elicit effects on the cell by growth factors and cytokines. Second, non-immune IgG from normal persons did not display any proliferative and differentiation effects on HUVECs. Considering also that in diabetic plasma the concentration of non-immune IgG is expected to be in large excess over that of immune ones, the result further supports the possibility that a small percentage of immune complexes with Grp94 is sufficient to activate the cellular mechanism(s) leading to cell proliferation and differentiation. Third, since the same angiogenic transformation of HUVECs was obtained with native Grp94 (Fig. 4B), Grp94 itself, rather than anti-Grp94 Ab steadily associated with it in the immune complex, is likely responsible for the observed effects on HUVECs.

In support of the possibility that effects driven on HUVECs by plasma-purified, Ab-containing fractions may involve cellular

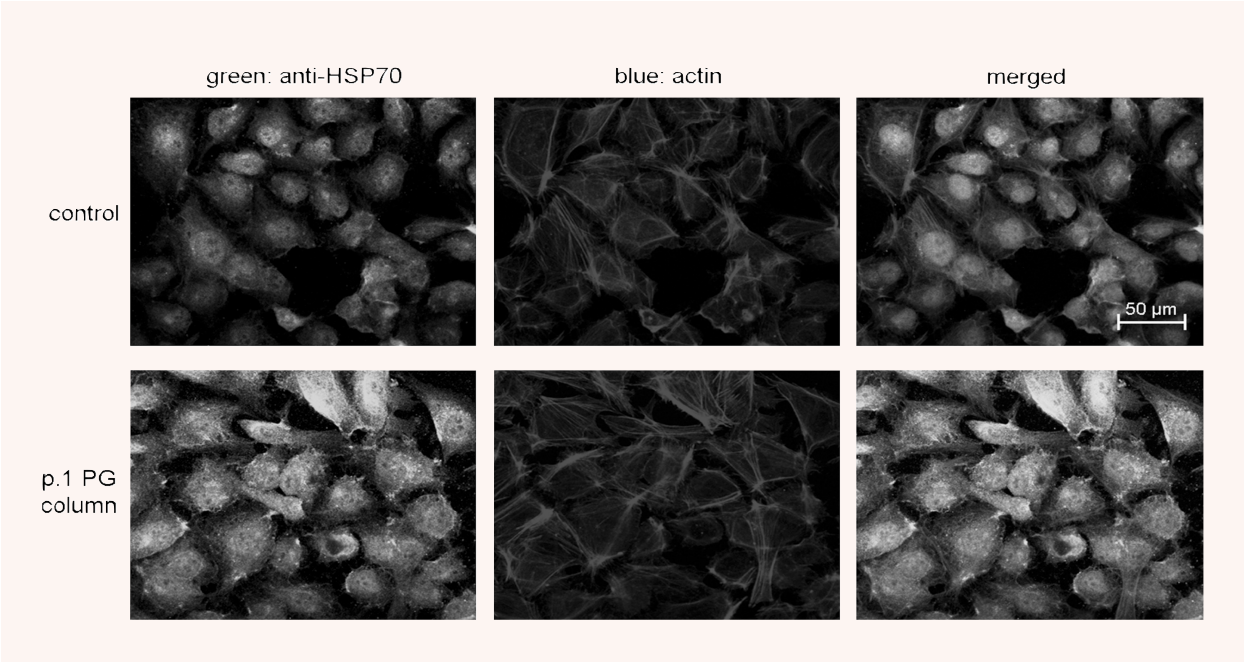

Fig. 9 Over-expression of HSP70 in HUVECs treated with peaks 1 and 2 from the PG column. Cells were cultured in serum-free medium in the absence (control) and presence of both peak 1 and 2 (10 ng/ml) from the PG column, as described in Materials and Methods. Images with peak 1 are shown as representative also of those obtained with peak 2. Individual fluorescence for HSP70 (green) and actin (blue) is represented for both control and treated HUVECs, while the merged light-blue fluorescence appears when the two proteins co-locate in the cell.

metabolic pathways that are also activated by diverse mitogenic stimuli, we showed that an intense stimulation of P-ERK1/2 correlated positively with cell growth stimulation, whereas negatively with angiogenic transformation that picked when P-ERK1/2 activation was silenced and cell growth stimulation significantly reduced (Fig. 6). These findings are in accord with separate reports indicating that an intense and/or prolonged activation of P-ERK1/2 is necessary to promote differentiation in various cell types [33, 34], and that cell differentiation in the angiogenic process takes place after silencing of proliferation, involving the expression of proteins aimed at displaying specific differentiation functions [23]. Consistent with this mechanism are also our results showing that the expression and activity of MMP-9 induced by plasma-purified fractions containing whole Abs correlated negatively with the proliferative effect, whereas a direct relationship was noted later in the differentiation process (Fig. 7). The proteolytic activity of MMP-9 appeared to be dispensable in the mechanism of angiogenesis promoted by Abs purified from diabetic plasma, in accord with others showing that the role of MMP-9 in angiogenesis is independent of its proteolytic activity [35, 36].

Our results collectively indicate that the process of angiogenic transformation promoted by plasma-purified fractions of Abs is associated with a significant increase in the expression of different, membrane-bound inactive species of MMP-9. It is known that MMP-9, although mainly secreted, can also be found closely associated with cell membranes [18, 37], and that species of MMP-9 with different masses – like those identified in our work – can be generated intra-cellularly in response to mitogenic stimuli by still unknown proteolytic mechanisms [37–39]. The binding to cell membranes of inactive species of MMP-9 suggests the involvement of this protease in the differentiation process of HUVECs. This is also supported by our finding showing an enhanced expression of HSP70 overlapping that of MMP-9 in the membrane fraction of HUVECs (Fig. 8). HSP70 may function as chaperone of MMP-9, favouring its folding and controlling biological activity (Fig. 8). Up-regulation of HSP70 has been observed during processes of cell differentiation [23, 40], in which HSP70 co-operates with proteins involved in the formation and stabilization of tubes at late stages of angiogenesis [23]. The overexpression of HSP70 in the experiments of immunofluorescence following treatment with plasma-purified fractions of Abs further supports the role of HSP70 in the process of angiogenic differentiation (Fig. 9).

Although further studies are necessary to confirm the role of Grp94 as the shared immunogen in type 1 diabetes, our results nevertheless indicate that immune complexes with Grp94 may be responsible for cell growth stimulation and angiogenic differentiation

induced by Ab-containing fractions purified from diabetic plasma. The effects observed on HUVECs may be thus of physiopathological relevance for predicting the inflammatory risk occurring *in vivo* on vascular cells.

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