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Decreased Expression of Heparin-Binding Epidermal Growth Factor–Like Growth Factor as a Newly Identified Pathogenic Mechanism of Antiphospholipid-Mediated Defective Placentation

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Objective. **Heparin-binding epidermal growth factor–like growth factor (HB-EGF) plays a role in blastocyst implantation and is down-regulated in preeclampsia and in hypertensive pregnancy disorders associated with defective extravillous trophoblast invasion. Defective placentation and severe preeclampsia are also features of the antiphospholipid syndrome (APS). The purpose of this study was to investigate whether abnormal HB-EGF expression plays a pathogenic role in antiphospholipid antibody (aPL)–mediated defective placentation.**

Methods. **HB-EGF expression in placental tissue was evaluated by Western blotting and messenger RNA analysis in normal and APS placentae. Polyclonal IgG fractions or monoclonal β_2-glycoprotein I–dependent aPL and their respective controls were investigated for the following 4 features: their binding to human trophoblast monolayers, as determined by cell enzyme-linked immunosorbent assay (ELISA); their effect on HB-EGF expression by Western blotting in trophoblast cell extracts as well as by ELISA as a protein secreted in the culture supernatants; their inhibitory effect on in vitro trophoblast invasiveness, as evaluated by Matrigel assay; and their inhibitory effect on matrix metalloproteinase (MMP) levels, as measured by gelatin zymography. Experiments were also performed in the presence of serial concentrations of heparin or recombinant HB-EGF.**

Results. **Placental APS tissue displayed reduced expression of HB-EGF. Polyclonal and monoclonal aPL bound to trophoblast monolayers and significantly reduced the in vitro synthesis and secretion of HB-EGF. Heparin inhibited aPL binding and restored HB-EGF expression in a dose-dependent manner. Addition of recombinant HB-EGF reduced the in vitro aPL-induced inhibition of Matrigel invasiveness as well as MMP-2 levels.**

Conclusion. **These preliminary findings suggest that the reduction of aPL-mediated HB-EGF represents an additional mechanism that is responsible for the defective placentation associated with APS and that heparin protects from aPL-induced damage by inhibiting antibody binding.**

Heparin-binding epidermal growth factor–like growth factor (HB-EGF) is a member of the EGF family (1–3). It is synthesized as a transmembrane protein of 208 amino acids. A small part of the membrane-anchored HB-EGF form, or pro form of HB-EGF (proHB-EGF), is cleaved from the cell surface to yield a soluble growth factor of 75–86 amino acids, while most of the molecule remains uncleaved on the cell surface (1). ProHB-EGF is not merely a precursor of the soluble form. It is also a biologically active molecule that is complexed with both CD9 and $\alpha 3\beta 1$ integrin. In this form, it actually has several biologic effects on different cell types (fibroblasts, endothelial cells, and smooth muscle cells) by increasing cell growth rate, migration,

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colony-forming ability, vascular endothelial growth factor (VEGF) expression, and activation of cyclin D1 promoter (2,4). Recently, a heparin-binding domain in the N-terminal portion of the EGF-like domain was identified as a sequence able to modulate EGF-like biologic activity (1,5).

Endometrial tissue expresses HB-EGF in response to sex steroids during the endometrial cycle and during early pregnancy in several species, suggesting a critical role in blastocyst implantation (6). HB-EGF is expressed in the human placenta during the first trimester, primarily within the villous trophoblast, but it also accumulates in the extravillous cytotrophoblast, in the basal plate, predominantly at the sites of cytotrophoblast extravillous invasion (7). Women with preeclampsia and infants who are small for gestational age display decreased expression of HB-EGF in placentae delivered as early as week 20 (8). This finding strongly suggests an association between HB-EGF down-regulation, poor trophoblast invasion, and failed physiologic transformation of the spiral arteries occurring in these disorders. Such a view is further supported by the evidence that HB-EGF favors trophoblast differentiation by inducing the invasive phenotype and stimulating cell motility (6).

Antiphospholipid syndrome (APS) is defined by the persistent presence of antiphospholipid antibodies (aPL) and by recurrent thrombotic events and/or fetal loss. The poor obstetric outcome in pregnant women with APS is also characterized by the occurrence of growth retardation and early and severe preeclampsia (9). There is evidence that aPL are pathogenic, but placental thrombosis cannot explain all the fetal losses, and aPL-mediated inhibition of trophoblast invasion has been suggested as one of the pathogenic mechanisms for APS pregnancy complications (10).

Owing to the putative physiologic role of HB-EGF during regular trophoblast development (6) and its alterations in pathologic conditions similar to APS (e.g., preeclampsia), we investigated whether HB-EGF expression is also affected in APS. To this end, we investigated HB-EGF expression in placental tissue obtained from women with APS and whether incubation with aPL may modulate its expression in human cytotrophoblast cells in vitro. Furthermore, we performed experiments to determine whether HB-EGF itself may interfere with the aPL-mediated effects on human trophoblast cell cultures, such as impairment of matrix metalloproteinases (MMPs).

Low molecular weight heparin (LMWH) together with low-dose aspirin is now widely accepted as the standard therapy for preventing recurrent fetal loss in APS (10). However, the fine pharmacologic mechanisms responsible for the effects of this treatment are still a matter for research (10). In this regard, we investigated whether the therapeutic activity of LMWH may be related to its ability to bind β_2-glycoprotein I (β_2GPI) with high affinity, thus competing with β_2GPI for binding to cytotrophoblast cell membranes and eventually protecting them from the in vitro autoantibody-induced effects.

PATIENTS AND METHODS

Patients. Five patients with primary APS diagnosed according to the revised Sapporo criteria (9) and 5 aPL-negative women with no obstetric problems (controls) were studied. Control subjects were matched for age (mean ± SD 31.5 ± 4 years versus 31.2 ± 4 years in the APS patients), parity, and ethnicity, with no history of adverse pregnancy events, hypertension, diabetes, or systemic lupus erythematosus. Fetuses that were normotensive, nonproteinuric, not complicated by intrauterine growth retardation, and delivered after the thirty-seventh week were considered normal.

The characteristics of the patients are shown in Table 1. All of the study subjects provided written informed consent.

Anticardiolipin antibody (aCL), anti-β_2GPI antibody, and lupus anticoagulant were detected as previously described (11,12). Placentae from normal mothers and mothers with APS were obtained immediately after vaginal delivery and frozen in liquid nitrogen. The specimens included both the maternal and fetal part, but the decidual and amniochorial membranes were removed.

Human polyclonal β_2GPI-dependent antibodies. Whole IgG fractions from APS patients and from normal human sera were purified on protein G–Sepharose (MabTrap-GII; Pharmacia-Biotech) as previously described (13). The final protein IgG concentration was evaluated by nephelometry, and the specific reactivity with β_2GPI-coated plates was confirmed as previously described (13,14). The sterile-filtered IgG fractions were determined to be free of endotoxin contamination by the *Limulus* amebocyte lysate assay (E-Toxate; Sigma-Aldrich), which has a sensitivity of <0.03 IU/ml.

Human β_2GPI. Human β_2GPI was purified from human serum and was characterized as previously described (13).

Human monoclonal anti-β_2GPI antibodies. The human anti-β_2GPI monoclonal antibody (mAb) IS3 was obtained from an APS patient as previously described (15). The mAb was purified with the use of a protein G–Sepharose column from the culture supernatant of B cell clones. A human mAb IgG of irrelevant specificity was used as control. The protein content and endotoxin contamination of the preparations were evaluated as described above.

Trophoblast cell isolation and culture. Placentae were obtained from healthy women immediately after uncomplicated vaginal delivery at ≥36 weeks of gestation. Cytotrophoblast cells were isolated as detailed elsewhere (14,16,17). The enriched (95%) cytotrophoblast cells (5×10^5 cells/ml) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% fetal calf serum (FCS; Sigma-Aldrich) at

Table 1. Clinical and laboratory characteristics of the APS patients studied*

Characteristic	Patient				
	APS1	APS2	APS3	APS4	APS5
Age, years	25	36	30	32	33
Gestational week at delivery	38	38	36 + 3 days	37	38
Previous fetal loss					
At ≤10 weeks of gestation	2	3	0	0	1
At >10 weeks of gestation	1	1	2	2	2 (twins)
Preeclampsia at <34 weeks of gestation	0	1	2	2	0
Presence of arterial/venous thrombosis	Yes	No	Yes	Yes	Yes
Presence of thrombocytopenia	No	Yes	No	No	Yes
Anticardiolipin antibodies					
IgG, GPL units/ml	120	135	112	150	102
IgM, MPL units/ml	70	57	54	60	86
Anti-β_2GPI antibodies, OD units					
IgG	1.10	1.80	0.98	1.30	1.67
IgM	0.67	0.74	0.84	1.05	1.20
Presence of LAC	Yes	Yes	No	No	Yes

* Lupus anticoagulant (LAC) activity was determined according to the methods of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis (12). APS = antiphospholipid syndrome; GPL = IgG phospholipid; MPL = IgM phospholipid; anti-β_2GPI = anti–β_2-glycoprotein I; OD = optical density.

37°C in an atmosphere consisting of 5% CO_2 and 95% air. The purity and the maturation of the cell preparation were evaluated using a panel of antisera directed against fibroblasts, macrophages, cytokeratin, and human chorionic gonadotropin (HCG), as previously described (14,17). A total of 95% of the cell preparations tested positive for anticytokeratin antibodies. Cytotrophoblasts at different times of culture were further assayed for the cytoplasmic presence of HCG as a marker for syncytiotrophoblast.

Western blot analysis. HB-EGF expression was investigated by Western blotting of APS and control placenta tissues, as well as trophoblast cell extracts obtained after treatment with IS3 mAb (polyclonal APS IgG) and the respective controls for 24 hours, in the presence or absence of serial concentrations of LMWH (0.1, 1, and 10 IU/ml), as previously described (18). Eighty micrograms of each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels. After electroblotting onto a polyvinylidene fluoride, the membranes (Millipore) were incubated with 5% nonfat dry milk in 1 mole/liter of Trizma/base, 1.54 moles/liter NaCl, and 0.05% Tween 20 (TBST; pH 7.4) and then exposed overnight at 4°C to TBST containing 0.4 μg/ml of primary antibody (polyclonal goat IgG anti-human HB-EGF; Oncogene). After further washes, the membranes were incubated with peroxidase-conjugated rabbit anti-goat IgG antiserum (Cappel/MP Biomedicals). Bands were analyzed using a Gel Doc 200 Image Analysis System and quantified using Quantity One Quantitation Software (both from Bio-Rad). The level of HB-EGF was estimated versus the constant level of a 42-kd protein present in the cytosolic extract (β-actin), which was identified with the use of a mouse monoclonal anti-human β-actin antibody (Sigma-Aldrich).

Enzyme-linked immunosorbent assay (ELISA) for HB-EGF. Microtiter plates (Costar; Corning) were coated for 24 hours at 4°C with 100 μl of 0.5 μg/ml of anti–HB-EGF mAb (R&D Systems) and blocked overnight at 4°C with 1% bovine serum albumin in phosphate buffered saline (PBS). A standard curve was prepared by diluting recombinant human HB-EGF (R&D Systems) in culture medium (0–1,000 pg/ml). Samples (100 μl of trophoblast cell culture supernatants in the presence of IS3 mAb, APS IgG, or the respective controls, with or without serial concentrations of LMWH) were incubated for 2 hours at 25°C in duplicate. After 4 washes with 300 μl of PBS–0.05% Tween 20, 100 μl of biotin-labeled affinity-purified polyclonal anti-human HB-EGF (200 ng/ml; R&D Systems) was added to each well for 30 minutes at 25°C. The wells were washed again and then incubated for 30 minutes at 25°C with 100 μl of streptavidin-conjugated horseradish peroxidase (1:200 dilution; R&D Systems). The wells were washed, and peroxidase activity was detected using a kit from R&D Systems.

The optical density (OD) at 450 nm was determined with a spectrophotometer (Titertek Multiscan Plus; ICN Flow). The calculated interassay and intraassay coefficients of variation of the immunoassay were 8% and 6%, respectively. Standard curves using recombinant HB-EGF were generated for each run and used to extrapolate the concentration of detectable HB-EGF in placenta-conditioned medium. No cross-reactivity was found with recombinant human EGF, recombinant fibroblast growth factor, or recombinant VEGF, respectively.

Total RNA extraction. Total cellular RNA was extracted using a QuickPrep Total RNA Extraction kit (GE Healthcare) according to the manufacturer's protocol. Briefly, cell pellets were suspended in lithium chloride solution containing β-mercaptoethanol and extraction buffer. Samples were homogenized and incubated for 10 minutes on ice with cesium trifluoroacetate solution. After centrifugation at 14,000 revolutions per minute for 15 minutes, the RNA pellets were washed and dissolved in 50 μl of diethyl pyrocarbonate–treated water. RNA quality was evaluated according to the absorbance ratio at 260 nm to 280 nm. The total RNA concentration was determined by measuring the absorbance at 260 nm.

Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR). Analyses of the quantitative expression of the HB-EGF and MMP-2 genes were performed by real-time RT-PCR using an iCycler iQ system (Bio-Rad) as previously described (19). For the target genes and the endogenous housekeeping gene encoding for GAPDH, a primer pair and TaqMan probe, which hybridizes to the region between primers, were designed using Beacon Designer 2 version 3.00 software (Premier Biosoft) and synthesized by MWG Biotech.

Binding assay. Cells were cultured for 72 hours in standard medium, washed 3 times with Hanks' balanced salt solution (Sigma-Aldrich), and cultured in serum-free medium to remove adherent β_2GPI (14,17). On day 3 (after 72 hours), the medium was removed, and the cells were washed and cultured in the same medium. Trophoblast cells in serum-free medium were incubated for 1 hour with exogenous human β_2GPI (5 μg/ml). Parallel cultures were also performed in the presence of serial concentrations of LMWH (0.1–10 IU/ml) in order to investigate the possible role of LMWH in interfering with β_2GPI/anti-β_2GPI antibody binding. Polyclonal or monoclonal anti-β_2GPI antibodies were added to the wells. After a 2-hour incubation followed by 3 washes, the plates were incubated with alkaline phosphatase–conjugated goat anti-human IgG (Sigma-Aldrich) for 90 minutes. After 2 further washes, *p*-nitrophenylphosphate (1 mg/ml) in 10% diethanolamine buffer, pH 9.8, was added to each well and incubated for 30 minutes. The OD was read at 405 nm with a microplate photometer (PlateReader; Bio-Rad).

Invasion assay. To assess the invasive potential of cytotrophoblasts, cells were plated on Transwell inserts (Millipore) as previously described (19). Cultures were performed in the presence of aPL or controls (50 μg/ml) with or without recombinant HB-EGF (0.1–10 ng/ml). In the Cell Invasion kit (Chemicon), invasive cells migrate through the extracellular matrix (ECM) layer and cling to the bottom of the polycarbonate membrane. The ECM layer is a reconstituted basement membrane matrix of proteins derived from the Engelbreth-Holm-Swarm mouse tumor (Matrigel; BD Bioscience). The membrane size was 6.4 mm, and the membrane surface area was 0.3 cm^2.

Briefly, 300 μl of warm serum-free medium was added to the interior of the invasion chamber inserts to allow the rehydration of the Matrigel (1–2 hours at room temperature). Media were then carefully removed from the inserts without disturbing the membrane. Media containing 10% FCS and/or chemoattractant were dispensed into the lower chamber (500 μl final volume). A total of 3×10^5 cells were plated in 300 μl of serum-free medium. After 24 hours of culture, noninvading cells as well as Matrigel from the interior of the insert were gently removed using a cotton-tipped swab. Invasive cells on the lower surface of the membrane were stained by dipping the inserts in the staining solution supplied with the kit for 20 minutes. Then, inserts were rinsed and air-dried, stained cells were dissolved in 10% acetic acid, and 100 μl was transferred to 96-well plates for colorimetric reading at 560 nm using a microplate photometer.

Gelatin zymography. Levels of MMP-2 and MMP-9 in the supernatants from trophoblast cell cultures were measured by gelatin zymography. Samples were subjected to electrophoresis on SDS–polyacrylamide gels containing 0.3% gelatin.

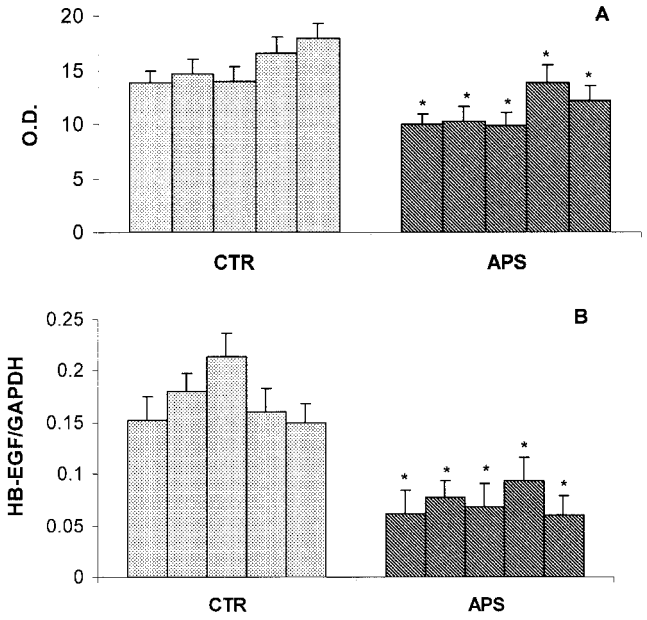

Figure 1. Expression of heparin-binding epidermal growth factor–like growth factor (HB-EGF) in placental tissue from 5 patients with antiphospholipid syndrome (APS) and 5 healthy control (CTR) subjects. **A,** HB-EGF protein expression as determined by Western blot analysis. Placental levels of HB-EGF are expressed as the optical density (OD). **B,** HB-EGF mRNA expression as determined by real-time reverse transcription–polymerase chain reaction. Placental mRNA levels of HB-EGF are expressed as the ratio of HB-EGF to GAPDH. Values are the mean and SEM of 5 independent experiments. $* = P < 0.05$ versus healthy tissue.

Following electrophoresis, gels were washed 3 times for 10 minutes at room temperature in 2.5% Triton X-100 to remove SDS. After overnight incubation at 37°C in 50 m*M* Tris HCl (pH 7.4, containing 5 m*M* $CaCl_2$, 0.15*M* NaCl, and 0.02% NaN_3), gels were stained with 0.5% Coomassie brilliant blue for 30 minutes, and then destained in 20% methanol and 10% acetic acid. Gelatinolytic activity was observed as a clear band of digested gelatin on a blue background. Images were acquired with a digital camera (Nikon), and bands were analyzed with the Gel Doc 200 Image Analysis System using Quantity One Quantitation Software.

Statistical analysis. The results are presented as the mean ± SEM. The data were analyzed using one-way analysis of variance, followed by a post hoc test (the Bonferroni test). *P* values less than 0.05 were considered significant.

RESULTS

HB-EGF expression in placental tissue. Five placentae obtained from healthy control subjects and 5 placentae obtained from women with APS were examined. Western blots showed that expression of HB-EGF was significantly reduced in placentae from women with

Table 2. Quantitative expression of HB-EGF protein in antiphospholipid antibody–treated human trophoblast cells, as measured by Western blotting*

Experiment	Untreated cells	IS3 mAb, 50 μg/ml	APS patient IgG			NHS control IgG, 50 μg/ml
			12 μg/ml	25 μg/ml	50 μg/ml	
1	5.05 ± 0.23	3.53 ± 0.12†	4.04 ± 0.99 (APS1)	3.94 ± 0.20† (APS1)	3.63 ± 0.46† (APS1)	4.97 ± 0.19 (CTR1)
2	4.16 ± 0.29	3.00 ± 0.14†	3.81 ± 0.30 (APS2)	3.33 ± 0.10† (APS2)	3.12 ± 0.52† (APS2)	4.21 ± 0.24 (CTR2)
3	6.89 ± 0.11	4.69 ± 0.20†	5.44 ± 0.82 (APS3)	5.17 ± 0.33† (APS3)	4.82 ± 0.29† (APS3)	6.81 ± 0.16 (CTR3)
4	5.35 ± 0.59	3.74 ± 0.14†	4.33 ± 0.69† (APS4)	4.01 ± 0.39† (APS4)	3.85 ± 0.56† (APS4)	5.22 ± 0.37 (CTR4)
5	4.01 ± 0.34	2.61 ± 0.33†	3.21 ± 0.29† (APS5)	3.05 ± 0.41† (APS5)	2.81 ± 0.12† (APS5)	4.16 ± 0.45 (CTR5)

* Cell cultures were left untreated or were treated with IS3, an anti–β_2-glycoprotein I (anti-β_2GPI) monoclonal antibody (mAb) obtained from a patient with antiphospholipid syndrome (APS), or were treated with polyclonal IgG fractions from APS patients 1–5, or from normal human serum (NHS) obtained from healthy control subjects (CTR) 1–5. The data for untreated cells and for the anti-β_2GPI mAb–treated cells were obtained from 5 replicate experiments. The mean ± SEM level of expression of heparin-binding epidermal growth factor–like growth factor (HB-EGF) in the presence of irrelevant mAb (50 μg/ml) was 5.77 ± 0.26 optical density (OD) units, where the OD values represent the ratio between HB-EGF and a constant level of β-actin. Values are the mean ± SEM relative expression in triplicate experiments.
† $P < 0.05$ versus untreated cells.

APS as compared with that in placentae from controls (Figure 1A). The reduced HB-EGF expression in APS placentae was confirmed at the messenger RNA (mRNA) level by real-time RT-PCR (Figure 1B).

Reduced HB-EGF protein expression in trophoblast cell monolayers by aPL antibodies. To demonstrate the role of β_2GPI-dependent aPL on placental expression of HB-EGF, human trophoblast cells were cultured in the presence of a β_2GPI-dependent aPL mAb (IS3; 50 μg/ml) or β_2GPI-dependent polyclonal aPL IgG (12–50 μg/ml). HB-EGF protein levels were analyzed in cell extracts and in conditioned medium by Western blotting and ELISA, respectively. To rule out any variability related to the donors' trophoblasts, the experiments were performed using samples from 3 different donors.

After 24 hours of culture, polyclonal aPL IgG fractions from all of the APS patients and the anti-β_2GPI mAb (IS3) induced a significant decrease in trophoblast HB-EGF expression as compared with the effects of normal control IgG and irrelevant mAb, respectively, as evaluated by Western blotting (Table 2). Consistent with the Western blot data, incubation of cells with anti-β_2GPI IS3 mAb (50 μg/ml) or polyclonal aPL IgG (50 μg/ml) significantly reduced HB-EGF protein secretion in culture supernatants, as determined by ELISA (mean ± SEM 3.00 ± 0.22 OD units in untreated cells, 0.65 ± 0.15 OD units in IS3 mAb–treated cells [$P < 0.001$ versus untreated cells], and 1.30 ± 0.15 OD units in polyclonal aPL–treated cells [$P < 0.01$ versus untreated cells]).

Effect of LMWH on aPL trophoblast binding and enhancement of HB-EGF expression. Owing to the well-known beneficial effects of heparin on the obstetric manifestations of APS, we performed experiments to demonstrate the effect of LMWH on aPL binding and on the in vitro–mediated biologic effects on trophoblasts at concentrations comparable with those used in vivo. Cell cultures were extensively washed and cultures were performed using serum-free medium to remove adherent β_2GPI. Consistent with previous findings (17), anti-β_2GPI IS3 mAb displayed background binding values comparable with those found with the irrelevant control mAb. Addition of exogenous human β_2GPI (5 μg/ml) restored IS3 mAb binding (data not shown) (17). When trophoblast cell cultures were incubated in the presence

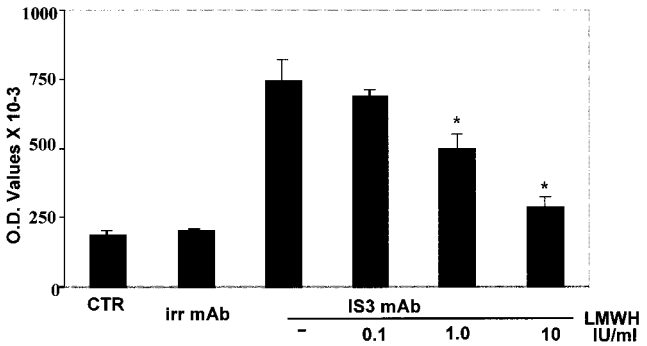

Figure 2. In vitro effect of low molecular weight heparin (LMWH) on the binding of the IS3 monoclonal antibody (mAb) to trophoblast cells. Cells were left untreated (control [CTR]) or were incubated in the presence of irrelevant (irr) mAb (50 μg/ml) or in the presence of the human anti–β_2-glycoprotein I mAb IS3 obtained from a patient with antiphospholipid syndrome (50 μg/ml). Serial concentrations of LMWH (0.1, 1.0, or 10 IU/ml) were then added to the cultures. Values are the mean and SEM optical density (OD) of 6 independent experiments. * = $P < 0.01$ versus IS3 mAb–treated cells in the absence of LMWH.

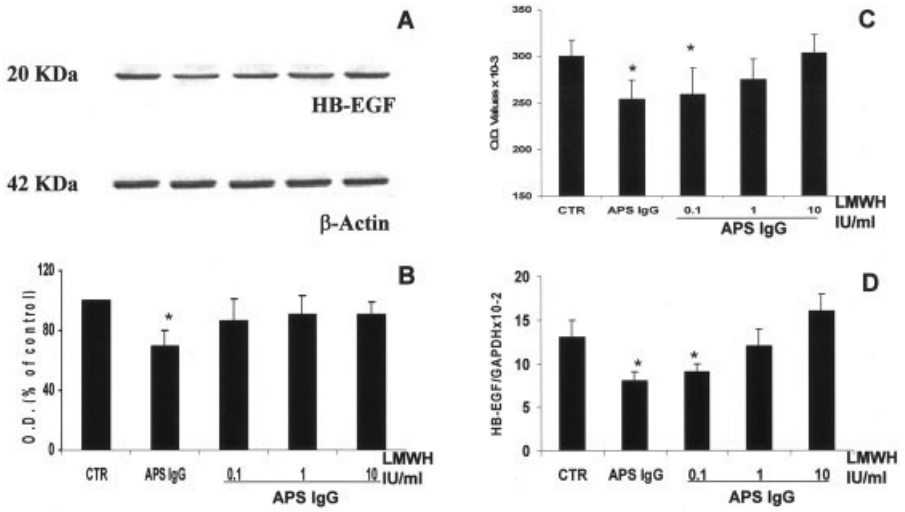

Figure 3. Effect of low molecular weight heparin (LMWH) on the expression and secretion of heparin-binding epidermal growth factor–like growth factor (HB-EGF) from trophoblast cells in the presence of IgG from antiphospholipid syndrome (APS) patient 2. **A,** Representative results of Western blot analysis. β-actin was used as a loading control. **B,** Results of densitometric analyses. The levels of HB-EGF in trophoblast cells were estimated in comparison with a constant level of β-actin and are expressed as a percentage of the values obtained in untreated cells. **C,** Secretion of HB-EGF protein in trophoblast culture supernatants, as quantified by enzyme-linked immunosorbent assay. **D,** HB-EGF mRNA expression in trophoblast cells as determined by real-time reverse transcription–polymerase chain reaction. Results are presented as the ratio of HB-EGF to GAPDH. In **B–D**, cells were left untreated (control [CTR]) or were incubated in the presence of the polyclonal IgG fraction (50 μg/ml) from APS patient 2. Serial concentrations of LMWH (0.1, 1.0, or 10 IU/ml) were then added to the cultures. Values are the mean and SEM of 5 independent experiments in **B** and **C** and of 3 independent experiments in **D**. $* = P < 0.05$ versus control cultures and versus cultures performed in the presence of LMWH. OD = optical density.

of serial concentrations of LMWH (0.1, 1, and 10 IU/ml), a reduction of IS3 mAb binding was seen (Figure 2). Comparable results were found when polyclonal IgG fractions with β_2GPI-dependent aPL activity were used (12–50 μg/ml), whereas NHS control IgG gave background values (data not shown).

Treatment with LMWH (0.1–10 IU/ml) greatly restored trophoblast cell expression of HB-EGF protein and secretion of HB-EGF in culture supernatants in the presence of polyclonal aPL IgG (Figures 3A–C). A comparable effect on the expression of mRNA for HB-EGF was found, as evaluated by real-time RT-PCR (Figure 3D).

Moreover, comparable results were obtained by incubating the cells with anti-β_2GPI mAb (data not shown). Control polyclonal IgG as well as the irrelevant mAb did not modify the levels of HB-EGF protein or mRNA at any of the concentrations tested (data not shown). No changes in the findings of the TUNEL assay for cell death were observed in the presence of polyclonal aPL or the IS3 mAb (data not shown).

Exogenous HB-EGF restoration of in vitro trophoblast invasiveness inhibited by aPL. Trophoblast differentiation is characterized by the development of extravillous trophoblasts that migrate into the maternal myometrium. Matrigel cultures have been reported to be useful in vitro assays for the evaluation of trophoblast invasiveness (19). Consistent with previous findings (17), incubation with polyclonal β_2GPI-dependent aPL IgG, but not with normal IgG, significantly reduced trophoblast cell invasiveness (Figure 4A). The addition of exogenous recombinant HB-EGF (0.1–10 ng/ml) to the cultures restored the cytotrophoblast invasion inhibited by aPL, with a plateau effect at 1 ng/ml (Figure 4B). Comparable results were found with the use of IS3 mAb (data not shown). Incubation of trophoblast cells with recombinant HB-EGF alone induced a comparable increase in invasiveness (mean $\pm$ SEM 730 $\pm$ 42 OD units, 918 $\pm$ 72 OD units, 963 $\pm$ 79 OD units, and 911 $\pm$ 39 OD units in untreated cells and cells treated with 0.1, 1, and 10 ng/ml of recombinant HB-EGF, respectively). This finding suggests that there is direct HB-EGF stim-

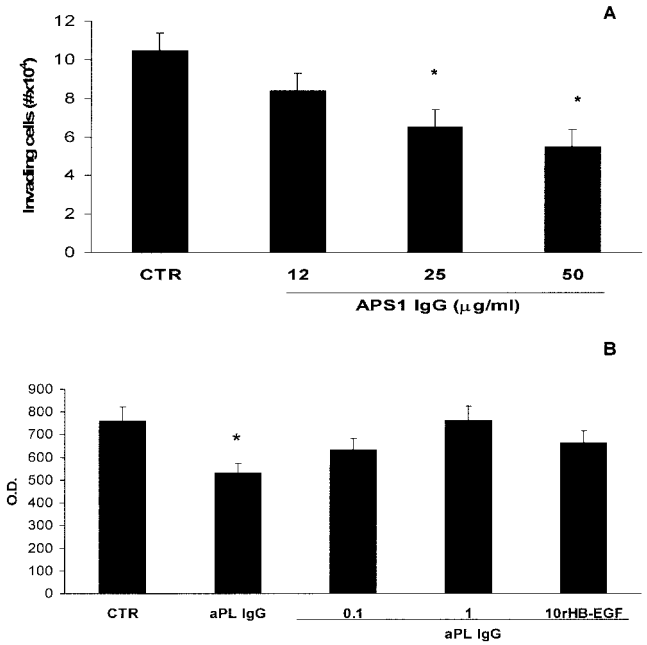

Figure 4. Effect of recombinant heparin-binding epidermal growth factor–like growth factor (rHB-EGF) on trophoblast invasiveness. **A,** Matrigel invasion assay in the absence or presence of the polyclonal IgG fraction from a patient with antiphospholipid syndrome (APS). Cells were left untreated (control [CTR]) or were incubated in the presence of 12, 25, or 50 μg/ml of IgG fraction from a representative patient with APS (APS1). Results are expressed as the absolute number of transmigrating trophoblast cells, as determined by microscopy. Values are the mean and SEM of 3 experiments. **B,** Matrigel invasion assay in the presence of the polyclonal IgG fraction from APS patients and recombinant HB-EGF. Cells were left untreated or were incubated in the presence of antiphospholipid antibody (aPL) IgG (mean of IgG fractions from 5 APS patients), in the absence or presence of 0.1, 1, or 10 ng/ml of recombinant HB-EGF. Results are expressed as the mean and SEM optical density (OD) values. $* = P <$ 0.05 versus untreated cells in **A** and **B** and versus cells cultured in the presence of recombinant HB-EGF in **B**.

ulation on invasiveness that does not necessarily involve a rescuing activity on aPL-induced inhibition.

Exogenous HB-EGF restoration of in vitro trophoblast MMP levels inhibited by aPL. MMP-2 levels were higher than MMP-9 levels in human trophoblast cells (data not shown). Treatment with recombinant HB-EGF (0.1–10 ng/ml) increased the levels of proMMP-2 (gelatinase A; 72 kd) and active MMP-2 (62 kd) in trophoblasts cultured in medium alone. The effect was significant only at the highest concentration used (mean ± SEM 660 ± 14 versus 500 ± 48 OD units for proMMP-2 [$P < 0.05$; n = 6 experiments] and 400 ± 20 versus 280 ± 18 OD units for active MMP-2 [$P < 0.05$; n = 6 experiments]).

In order to investigate whether aPL can affect trophoblast MMPs, we performed cell cultures in the presence of polyclonal and monoclonal aPL, and we evaluated MMP-2 levels. Incubation with polyclonal β_2GPI-dependent aPL IgG (from APS patient 3; 50 μg/ml) significantly reduced the levels of both proMMP-2 and active MMP-2 (mean ± SEM 399 ± 37 OD units versus 500 ± 48 OD units without aPL for proMMP-2 [$P < 0.05$; n = 6 experiments] and 170 ± 13 OD units versus 280 ± 18 OD units without aPL for active MMP-2 [$P < 0.05$; n = 6 experiments]). This inhibition was restored by the addition of exogenous recombinant HB-EGF (mean ± SEM 429 ± 32, 481 ± 21, and 499 ± 18 OD units at 0.1, 1, and 10 ng/ml, respectively, for proMMP-2 and 180 ± 20, 220 ± 15, 290 ± 28 OD units, respectively, for active MMP-2 [n = 6 experiments]). Comparable results were observed after incubation with IS3 mAb (50 μg/ml) (mean ± SEM 387 ± 33, 433 ± 25, 493 ± 30, and 548 ± 39 OD units for proMMP-2 and 130 ± 10, 180 ± 20, 240 ± 15, and 290 ± 30 OD units for active MMP-2 in untreated cells and cells treated with 0.1, 1, and 10 ng/ml of recombinant HB-EGF, respectively [n = 6 experiments]). Levels of MMP-2 in the presence of IgG from healthy subjects or the irrelevant mAb were the same as those observed in untreated trophoblast cells (data not shown).

The reduced MMP-2 expression in the presence of β_2GPI-dependent aPL and the restorative effect of HB-EGF were confirmed at the mRNA level by real-time RT-PCR (data not shown).

DISCUSSION

This pilot study is the first to show decreased placental expression of HB-EGF in patients with obstetric manifestations of APS. Although a limited number of samples have been collected, a homogenous reduction of HB-EGF expression was found in all the samples, both at the protein and the mRNA levels. Accordingly, in vitro studies suggest that aPL are responsible for a comparable reduction in HB-EGF trophoblast expression.

Consistent with the findings of previous studies, we confirmed that β_2GPI-dependent aPL bind human trophoblasts and affect in vitro invasiveness (14,17). We extended this observation to show that the inhibition is at least partly related to an aPL-mediated inhibitory effect on trophoblast MMP-2 activity. Addition of exogenous recombinant HB-EGF to the cultures restored both the MMP-2 activity and the impaired trophoblast in vitro invasiveness.

As a whole, these findings suggest that decreased expression of HB-EGF at the level of the placenta may represent an important pathogenic mechanism in the defective placentation mediated by aPL.

HB-EGF expression is induced by sex steroids during the secretory phase of the endometrial cycle, and it persists during normal pregnancy. It has been suggested that accumulation of HB-EGF at sites of implantation may activate the downstream signaling of its receptors (human epidermal growth factor receptor 1 [HER-1] and HER-4) for cytotrophoblast survival (20). Accordingly, HB-EGF down-regulation was reported in patients with preeclampsia, in association with impaired extravillous trophoblast invasion, spiral artery remodeling, and increased apoptosis (20).

HB-EGF cell invasion–promoting and antiapoptotic activities support its potential role in APS-associated trophoblast abnormalities characterized by increased cell death and inadequate invasion. In fact, there is evidence that sera from APS patients inhibit trophoblast proliferation and increase cell apoptosis (21,22). We also demonstrated that both human polyclonal and monoclonal β_2GPI-dependent aPL may inhibit in vitro models of trophoblast invasion (17).

This study is the first to demonstrate that the pathogenic effect of aPL on trophoblasts may be mediated by a decrease in the expression of HB-EGF and by a reduction in the activity of specific proteases, such as MMP-2.

MMPs are a group of degradative enzymes that are secreted as inactive zymogen and become active after cleavage (23–26). Among the members of the MMP family, MMP-2 (also known as gelatinase A, type IV collagenase, or 72-kd gelatinase) was reported to be involved in trophoblast invasion of endometrial tissue (24,25). MMP up-regulation results in proteolytic degradation of the ECM and of the basement membrane, promoting cellular growth, angiogenesis, and cellular migration (26). We found reduced MMP-2 levels in trophoblast cell cultures incubated in the presence of aPL. The addition of HB-EGF to the cultures restored the MMP-2 levels. It is possible that the addition of EGF favors trophoblast syncytialization with subsequent release of MMP-2, rather than being responsible for a direct effect on MMP-2 as was previously suggested (27). Whatever the fine mechanism is, this finding suggests that aPL may induce defective placentation by inhibiting MMPs and that such inhibition is at least partly related to the decreased expression of HB-EGF.

There is sound clinical evidence that LMWH is effective in preventing fetal loss in women with APS (28). However, its mechanisms of action are still a matter for research. Since thrombotic events can explain only part of the aPL-mediated fetal loss, pharmacologic mechanisms in addition to the well-known anticoagulant activity have been suggested (29).

We report that LMWH decreases aPL binding to human trophoblast monolayers and, as a consequence, prevents the antibody-mediated biologic effects. Beta$_2$-glycoprotein I displays high affinity for heparin, and the heparin-binding site was shown to be located in the so-called phospholipid-binding site in the fifth domain of the molecule (30). The same part of the molecule is also involved in the binding to the syncytiotrophoblast surface membrane (14). We speculate that LMWH interacts with the phospholipid-binding site, thus inhibiting the adherence of β_2GPI to the cell membranes. Such an event may explain the ability of LMWH to decrease antibody binding and, eventually, the protection from aPL-mediated functional effects. This activity may represent an additional pharmacologic mechanism to explain its protective effect on aPL-mediated recurrent fetal loss.

In conclusion, our preliminary ex vivo finding suggests that there is impaired expression of HB-EGF in placental tissue from women with APS that, apparently, is aPL-mediated, as supported by the results of the in vitro experiments with trophoblast cultures. The ability of exogenous recombinant HB-EGF to reduce the aPL-mediated effects on trophoblast cells supports a key pathogenic role of this molecule. The experimental conditions do not involve complement activation, indicating that aPL may also affect placental tissue through direct, complement-independent effects, as previously suggested (10). On the other hand, the ability of heparin to inhibit the in vitro aPL binding to human trophoblast cell monolayers, as well as the impaired invasiveness, represent an additional explanation for the therapeutic effect of heparin.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Meroni had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Di Simone, Marana, D'Alessio, Raschi, Borghi, Chen, Caruso, Meroni.

Acquisition of data. Castellani, Di Nicuolo.

Analysis and interpretation of data. Raschi, Borghi, Sanguinetti, Meroni.

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