The Tie-2 ligand Angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism

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Summary

The angiopoietins Ang-1 and Ang-2 have been identified as ligands of the endothelial receptor tyrosine kinase Tie-2, which controls vascular assembly and endothelial quiescence. The largely complementary phenotypes of Ang-1-deficient mice and Ang-2-overexpressing mice have led to an antagonistic model in which Ang-1 acts as Tie-2-activating agonist and Ang-2 acts as a Tie-2-inhibiting antagonist. To date, no mechanistic equivalent of the antagonistic Ang-1/Ang-2 model has been established and the mechanisms of Ang-2 function in particular remain mysterious. We have studied the effector functions of Ang-1 and Ang-2 on quiescent endothelial cells using a three-dimensional co-culture model of endothelial cells and smooth-muscle cells. Endothelial-cell monolayer integrity in this model is dependent on Tie-2 signaling, as evidenced by detaching endothelial cells following exposure to the small molecular weight Tie-2 inhibitor A-422885.66, which cannot be overcome by exogenous Ang-1. Accordingly, exogenous Ang-2 rapidly destabilizes the endothelial layer, which can be observed within 30-60 minutes and leads to prominent endothelial-cell detachment within 4 hours. Exogenous Ang-2-mediated endothelial-cell detachment can be rescued by Ang-1, soluble Tie-2 and vascular endothelial growth factor. Similar findings were obtained in an umbilical-vein explant model. Ang-2 is mainly produced by endothelial cells and therefore acts primarily in an autocrine manner. Thus, stimulated release of endogenous Ang-2 or overexpression of Ang-2 in endothelial cells perturbs co-culture spheroid integrity, which can be rescued by exogenous Ang-1 and vascular endothelial growth factor. However, autocrine Ang-2-mediated endothelial-cell detachment cannot be blocked by soluble Tie-2. Taken together, the data demonstrate for the first time the antagonistic Ang-1/Ang-2 concept in a defined cellular model and identify Ang-2 as a rapidly acting autocrine regulator of the endothelium that acts through an internal autocrine loop mechanism.

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Introduction

Angiogenesis, the formation of blood vessels from pre-existing vessels, is controlled by a hierarchically structured signaling cascade of endothelial-cell-specifically expressed receptor tyrosine kinases. The vascular endothelial growth factor (VEGF)/VEGF-receptor (VEGFR) system acts as a master switch of angiogenesis induction, orchestrating early events of the angiogenic cascade, such as directional sprouting and endothelial cell proliferation, indicated by the early embryonic lethality of VEGF- or VEGFR-deficient mice (Carmeliet, 2003). Moreover, bidirectional signaling through ephrin-B receptors (EphB) and their corresponding EphB ligands (ephrin-B) transduces propulsive and repulsive signals on angiogenic endothelial cells controlling arteriovenous differentiation and vascular network formation (Augustin and Reiss, 2003). In addition, interactions of angiopoietin ligands with the Tie-2 receptor regulate vascular maturation and control vessel quiescence (Davis et al., 1996; Jones et al., 2001; Maisonpierre et al., 1997). The phenotype of angiopoietin- and Tie-2-deficient mice has been analysed in great detail, yet the molecular mechanisms of action and functional consequences of endothelial-cell activation through the angiopoietin/Tie-2 system are poorly understood.

Angiopoietin-1 (Ang-1) has been identified as the agonistic ligand of Tie-2 (Suri et al., 1996). It regulates endothelial-cell survival and blood-vessel maturation (Suri et al., 1996). Ang-1 exerts a vessel-sealing effect (Thurston et al., 2000), has an anti-inflammatory action (Gamble et al., 2000; Jeon et al., 2003; Ramsauer and D'Amore, 2002) and protects against cardiac-allograft arteriosclerosis (Nykanen et al., 2003) and radiation-induced endothelial-cell damage (Cho et al., 2004). Low-level constitutive Tie-2 activation might be required in the adult to maintain the mature quiescent phenotype of the resting vascular endothelium (Wong et al., 1997). According to these findings, functional knockdown of Tie-2 has recently been shown to cause endothelial-cell apoptosis by decreasing Akt signaling (Niu et al., 2004). Ang-1 is produced by many cell types, acts in a paracrine manner on endothelial cells and has been described as a transcriptionally regulated molecule in some tumors (Stratmann et al., 1998; Sugimachi et al., 2003)

and during inflammation (Brown et al., 2004). However, its transcriptional regulation is rather moderate compared with other angiogenesis-regulating molecules such as VEGF and Angiopoietin-2 (Ang-2).

Ang-2 has been identified as a functional antagonist of Ang-1. It binds to Tie-2 without inducing signal transduction in Tie-2-expressing endothelial cells (Gale et al., 2002; Maisonpierre et al., 1997). The opposing effects of Ang-1 and Ang-2 support a model of constitutive Ang-1/Tie-2 interaction controlling vascular homeostasis as the default pathway (Wong et al., 1997) and with Ang-2 acting as a dynamically regulated antagonizing cytokine (Carmeliet, 2003; Hanahan, 1997; Korff et al., 2001).

Loss of the *Ang-2* gene and its function is compatible with life, as evidenced by the observation that Ang-2-deficient mice are born apparently normal (Gale et al., 2002). The functionally unaffected blood vascular system of Ang-2-deficient mice has only minor abnormalities (e.g. perturbed vessel regression phenotype of the eye's hyaloid blood vessels). However, depending on the genetic background of the mice, a significant proportion of newborn mice develop a lethal chylous ascites within the first 14 days as a consequence of a mechanistically unexplained lymphatic phenotype (Gale et al., 2002). In contrast to the mild phenotype of Ang-2-deficient mice, mice transgenically overexpressing Ang-2 have an embryonic lethal phenotype that essentially phenocopies the Ang-1-null and Tie-2-null phenotypes (Davis et al., 1996; Maisonpierre et al., 1997; Suri et al., 1996). The similarity of the Ang-1 loss-of-function phenotype with the Ang-2 gain-of-function phenotype strongly supports the antagonistic concept of Ang-1 and Ang-2 functions. Yet the embryonic lethal phenotype of mice systemically overexpressing Ang-2 also demonstrates that Ang-2 is a potentially dangerous molecule whose dosage and spatiotemporal appearance must be tightly regulated. The Ang-2 dosage concept is also supported by the observation that local overexpression of Ang-2 in the heart is compatible with life (Visconti et al., 2002), whereas strong overexpression of Ang-2 with systemic dissemination in a large organ such as the skin leads to an embryonic lethal phenotype similar to the systemic overexpression of Ang-2 (K. Alitalo, Molecular/Cancer Biology Laboratory, Helsinki, Finland; personal communication). Similarly, an activating Tie-2 mutation causes venous malformations that are composed of dilated endothelial channels covered by a variable amount of smooth-muscle cells, demonstrating that a precise balance of Tie-2 signals is crucial (Vikkula et al., 1996).

Expression profiling studies have identified endothelial cells as the primary source of Ang-2 and a dramatic transcriptional regulation of Ang-2 production upon endothelial-cell activation (Gale et al., 2002; Hackett et al., 2000; Holash et al., 1999; Huang et al., 2002; Mandriota and Pepper, 1998; Stratmann et al., 1998; Zhang et al., 2003). Correspondingly, the *Ang-2* promoter contains unique positive and negative regulatory elements that control endothelial cell expression of *Ang-2* (Hegen et al., 2004). We have recently identified Ang-2 as a Weibel-Palade body-stored molecule that can be rapidly secreted upon stimulation (Fiedler et al., 2004). These findings, coupled with the strong transcriptional regulation of *Ang-2* expression at sites of endothelial-cell activation, suggest that Ang-2 might act as the dynamic antagonizing player of the constitutively acting blood-vessel-stabilizing Ang-1/Tie-2 axis.

The antagonistic Ang-1/Ang-2 model of Tie-2 signaling has been proposed on the basis of the corresponding phenotypes of Ang-1-deficient and Ang-2-overexpressing mice. However, to date, no mechanistic basis of this model has been provided and conclusive evidence for this model is still missing. We hypothesized that the established monolayer cell culture techniques of endothelial cells might not be suitable for studying the complex functions of the angiopoietins, relating as it does to the maintenance of the quiescent endothelial-cell phenotype, which involves intricate interactions with mural cells. We consequently used a recently established three-dimensional (3D) spheroidal co-culture model of endothelial cells and smooth-muscle cells that mimics the 3D assembly of the normal vessel wall in an inside-out orientation (Korff et al., 2001). Based on this unique model, we were able to demonstrate for the first time discrete antagonistic effector functions of Ang-2 on quiescent endothelial cells. Moreover, the data identify Ang-2 as a rapid autocrine regulator of vascular stability that acts through an internal autocrine loop.

Materials and Methods

Materials

Recombinant myc-tagged Ang-1, myc-tagged Ang-2 and an Fc fragment against soluble-Tie-2 fused to the Fc portion of human IgG (sTie-2-Fc) were produced as described previously (Fiedler et al., 2003). In brief, cDNA-containing plasmids were used for transfection of Sf9 cells along with linearized wild-type baculovirus DNA. Recombinant baculoviruses were obtained using the Baculo-Gold™ transfection kit following standard protocols (Pharmingen; http://www.bdbiosciences.com/pharmingen/). For protein production, Sf9 cells were grown in serum-free medium and infected with a multiplicity of infection of 10. Myc-tagged Ang-1 and myc-tagged Ang-2 were purified from Sf9 supernatants using a Tie-2-Fc Protein-A–Sepharose column as described previously (Davis et al., 2003). The angiopoietin-containing fractions were pooled and dialysed. Purified samples were stored, frozen and tested for activity prior to use in a 3D sprouting angiogenesis assay (Korff and Augustin, 1999).

Soluble Tie-2-Fc was purified from the supernatant of sTie-2[1-730]-Fc-expressing Sf9 cells as described previously (Fiedler et al., 2003). VEGF was purchased from R&D Systems (http://www.rndsystems.com/). The low-molecular-weight Tie-2 inhibitor was kindly provided by L. Arnold and X.-D. Qian (Abbott Bioresearch Center, Worcester, USA). Carboxymethylcellulose (4000 centipoises) was from Sigma (http://www.sigmaaldrich.com/). All other reagents were also from Sigma unless otherwise specified.

Cell culture

Human umbilical-vein endothelial cells (HUVECs), human umbilical-artery smooth-muscle cells (HUASMCs), endothelial-cell growth medium (ECGM), smooth-muscle-cell growth medium 2 (SMGM-2), endothelial-cell basal medium (EBM) and corresponding supplements were purchased from Promocell (http://www.promocell.com/). Cells were cultured at 37°C, 5% CO_2 in the appropriate growth medium containing 10% fetal calf serum (FCS) (Invitrogen; http://www.invitrogen.com/). HUVECs were used between passages 2 and 5. HUASMCs were used between passages 2 and 8.

Expression vectors and retroviral transduction

Retroviral transfer of *Ang-1* and *Ang-2* cDNAs was achieved using a modified pantropic retroviral expression system (Clontech; modified and kindly provided by R. Graeser, Tumour Biology Centre, Freiburg,

Germany). Briefly, human cDNAs encoding myc-tagged Ang-1 and Ang-2 (Fiedler et al., 2003) were cloned into pLIB-IRES-EGFPneo. The constructs were stably transfected into HEK ampho 293 cells and clones selected using 0.5 μg ml^{-1} G418 (PAA; http://www.paa.at/). For generation of pantropic retroviruses, clones were transfected with pVSVG (Clontech; http://www.clontech.com/) according to the manufacturer's instructions. Endothelial cells (6×10^4 each) were transduced in two cycles with 2 ml retrovirus containing supernatant from the HEK ampho 293 cells for 6 hours each. Transduction efficacy ranged from 20% to 60% for the Ang-1 and Ang-2 constructs, and between 85% and 100% for eGFPneo.

siRNA transfection of endothelial cells

HUVECs were seeded in six-well dishes at a density of 1.2×10^5 cells per well 24 hours before the experiment. Predesigned annealed small interfering RNAs (siRNAs) (100 nM each) directed to human Ang-2 (ANGPT2_1; ANGPT2_3; Ambion; http://www.ambion.com/) were transfected using oligofectamine (Invitrogen; http://www.invitrogen.com/) according to the manufacturer's instructions. The transfection was carried out in 1 ml OptiMEM (Invitrogen). 5 hours after transfection, cells were supplemented with additional 3 ml ECGM and cultured for another 2 days. Downregulation of *Ang-2* mRNA was analysed using reverse-transcription polymerase chain reaction (RT-PCR) as described previously (Hegen et al., 2004) and downregulation of Ang-2 protein was verified by western blotting.

Generation of co-culture spheroids of endothelial and smooth-muscle cells

Co-culture spheroids of endothelial and smooth-muscle cells were generated as described previously (Korff et al., 2001). In brief, equal amounts of HUVECs and HUASMCs (1500 cells each per spheroid) were suspended in endothelial-cell basal medium (Promocell) containing 10% FCS and 0.25% (w/v) carboxymethylcellulose and seeded in non-adherent round-bottom 96-well plates (Greiner; http://www.gbo.com/). After 48 hours, co-culture spheroids were used for the corresponding experiments.

Stimulation of co-culture spheroids

Co-culture spheroids were harvested (25 spheroids per experimental setting) and collected in 2 ml Eppendorf tubes. Spheroids were allowed to sediment and supernatants were aspirated. Basal medium (1 ml) containing 10% FCS, 0.25% (w/v) carboxymethylcellulose and the test substances was added. Spheroids were incubated for the indicated time points in spinner culture at 37°C. After stimulation, supernatants were aspirated and spheroids were fixed overnight with 4% paraformaldehyde (PFA) in PBS. Spheroids were stimulated with 200 ng ml^{-1} Ang-1, 200 ng ml^{-1} Ang-2, 25 ng ml^{-1} VEGF, 50 ng ml^{-1} phorbyl myristate (PMA), 5 μg ml^{-1} sTie-2-Fc and 10 nM A-422883.66 (a Tie-2 inhibitor).

CD31 whole-mount staining

Staining of co-culture spheroids was performed in 2 ml Eppendorf tubes. The fixative was removed and spheroids were washed three times with 1 ml PBS and blocked with 300 μl 3% FCS in PBS. Spheroids were then incubated for 1 hour with an anti-human-CD31 antibody (Dako; http://www.dako.com/) diluted 1:100 in 150 μl blocking solution. After three washing steps, co-culture spheroids were incubated for 30 minutes with biotinylated secondary antibody (goat anti-mouse; Zymed; http://www.zymed.com/) followed by three washings with PBS. Spheroids were then exposed to Alexa Fluor 546 conjugated streptavidin (Molecular Probes; http://www.probes.com/) and Hoechst dye 33258. After 15 minutes, spheroids were washed twice and supernatants were removed. Then, one drop of Dako fluorescent mounting medium (Dako) was added and spheroids were transferred to glass slides. Short pieces of human hair were used as spacers in order not to destroy the 3D structure of the spheroids.

Preparation of umbilical-cord veins

Human umbilical cords were cut into slices and small fragments of the umbilical vein were trimmed with a razor blade. Explants were washed and incubated overnight in 2 ml endothelial-cell basal medium containing 10% FCS and 0.25% (w/v) carboxymethylcellulose in non-adherent 24-well plates (Greiner). Explants were then stimulated with the test substances for 4 hours and fixed overnight in 4% PFA in PBS. Fragments of umbilical veins were processed for paraffin embedding by a graded series of alcohol (70% ethanol, 85% ethanol, 99% ethanol-isopropanol at 1.5 hours each). Specimen were then immersed in paraffin I (melting temperature 42°C) for 12 hours at 70°C followed by a 24 hour incubation in paraffin II (melting temperature 56°C) at 70°C.

Immunhistochemistry

Sections of umbilical-vein fragments were deparaffinised in a graded series of descending alcohol concentrations and rehydrated. Endogenous peroxidase activity was inhibited with 3% (v/v) H_2O_2. Unspecific binding sites were blocked with 10% goat serum in PBS followed by incubation with the first antibody (monoclonal mouse anti-human CD34, Chemicon; http://www.chemicon.com/) in a humid chamber overnight at 4°C. Antibody binding was detected with a biotinylated goat anti-mouse IgG (Zymed). Sections were then incubated with a streptavidin-peroxidase conjugate (Zymed), developed with diaminobenzidine and counterstained with hemalaun.

Image analysis

Endothelial-cell detachment was quantified using an automated image-analysis system with an Olympus IX50 inverted microscope and Olympus image-analysis software. The surface area of individual spheroids was determined and set to 100%. The area of detached endothelial cells was then quantified by automated planimetric analysis. The ratio of denuded surface area to total surface area was calculated and expressed as the percentage denudation.

Statistical analysis

All results are expressed as means ± s.d. Differences between experimental groups were analysed using the Student's *t* test and $P<0.05$ was considered to be statistically significant.

Results

Endothelial-cell monolayer integrity in a 3D co-culture requires Tie-2 signaling

The physiological assembly of the blood-vessel wall, with a quiescent monolayer of endothelial cells covering a multilayered assembly of smooth-muscle cells, can be mimicked in culture by a 3D co-culture system of endothelial cells and smooth-muscle cells (Korff et al., 2001). In this model, smooth-muscle cells form the core of a spheroid that is covered by a monolayer of endothelial cells. The phenotype of the endothelial-cell monolayer is regulated by the underlying smooth-muscle cells and mimics many of the quiescent phenotypic properties of endothelial cells in vivo (Korff et al., 2001). The surface endothelial monolayer can be visualized by whole-mount CD31 or ICAM-1 immunocytochemistry (Fig. 1A; see Fig. S1 in supplementary material).

We have taken advantage of this unique cell-culture model to

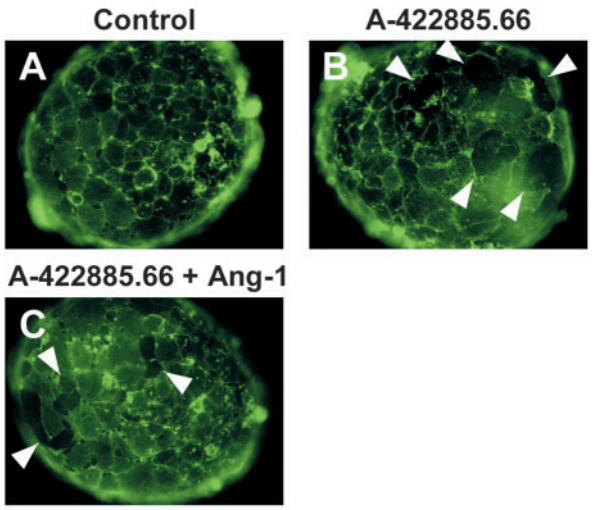


Fig. 1. Induction of endothelial-cell detachment from endothelial-cell/smooth-muscle-cell co-culture spheroids by pharmacological Tie-2 inhibition. Co-culture spheroids of HUVECs and HUASMCs were treated for 4 hours with 10 nM A-422885.66 and, after fixation, whole-mount stained for CD31. Control co-culture spheroids form an intact monolayer of CD31-positive endothelial cells (A). Inhibition of Tie-2 phosphorylation by A-422885.66 leads to detachment of endothelial cells from the endothelial-cell monolayer, as indicated by holes in the monolayer (B, arrowheads). This process cannot be antagonized by exogenous recombinant Ang-1 (C, arrowheads).

study the regulation of the quiescent endothelial-cell phenotype by the angiopoietin/Tie-2 system. Low-level constitutive Tie-2 phosphorylation has been reported in vivo and might contribute to maintenance of the quiescent endothelial-cell phenotype (Wong et al., 1997). Correspondingly, the smooth-muscle cells in the co-culture model act as a source of Ang-1 (Fiedler et al., 2003; Korff et al., 2001). We consequently assessed the involvement of Tie-2 signaling in maintenance of the quiescent endothelial-cell phenotype by treating co-culture spheroids of HUVECs and HUASMCs with the low-molecular-weight Tie-2 inhibitor A-422885.66. A-422885.66 leads to pronounced disintegration of the surface endothelial monolayer, with individual endothelial cells detaching from the spheroid (Fig. 1B). This effect cannot be rescued by excess exogenous Ang-1 (Fig. 1C) indicating that Tie-2 signaling is required to maintain the quiescent endothelial phenotype in EC/SMC co-culture spheroids.

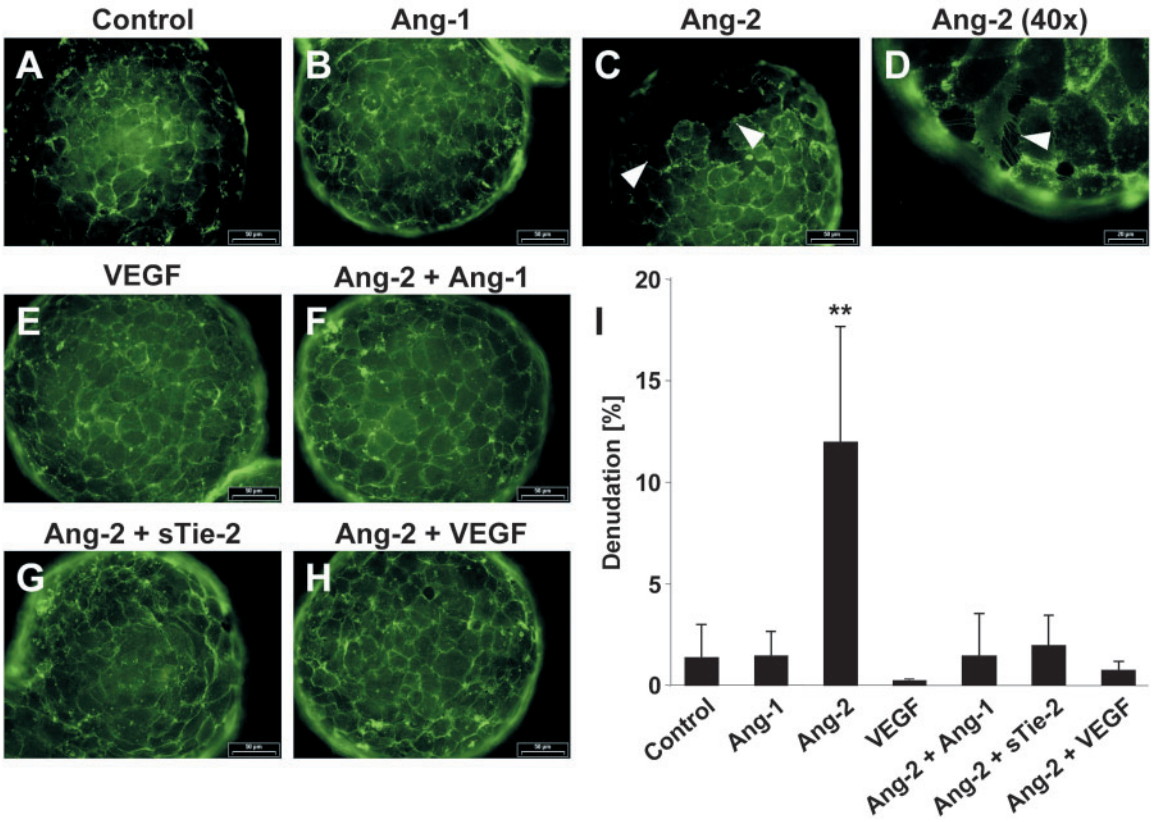


Fig. 2. Destabilization of endothelial-cell-monolayer integrity in co-culture spheroids of HUVECs and HUASMCs by exogenous Ang-2. Control co-culture spheroids have an intact CD31-positive endothelial-cell monolayer (A). Treatment of co-culture spheroids with Ang-1 (B) or VEGF (E) has no effect on the integrity of the surface endothelial-cell monolayer. Treatment of co-culture spheroids with Ang-2 leads to destabilization of the endothelial-cell monolayer within 4 hours, as evidenced by intense detachment of individual and groups of endothelial cells (C; high magnification in D, arrowheads). Monolayer destabilization elicited by exogenous Ang-2 is rescued by Ang-1 (F), sTie-2 (G) and VEGF (H). (I) Quantitative assessment of the Ang-2-mediated denudation by image analysis of the denuded spheroid surface area. Results are expressed as means ± s.d. of three independent experiments quantifying ten spheroids per experiment. **, $P<0.001$ compared with all other treatment groups.

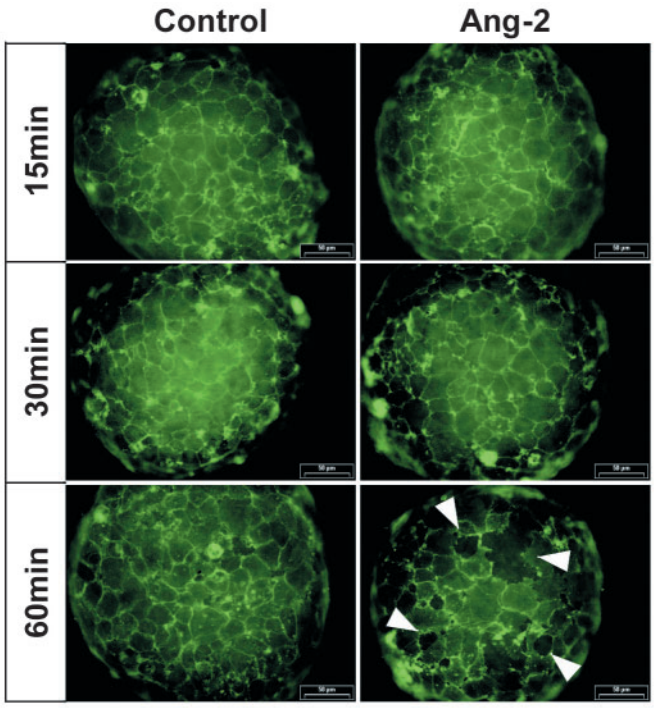


Fig. 3. Time course of Ang-2-mediated endothelial-cell-monolayer destabilization in HUVEC/HUASMC co-culture spheroids. Gaps in the endothelial-cell monolayer are first observed after 30 minutes. Individual endothelial cells have detached from the monolayer within 60 minutes of Ang-2 exposure (arrowheads).

Angiopoietin-2 rapidly destabilizes quiescent endothelium in vitro and in situ

Stimulation of co-culture spheroids of HUVECs and HUASMCs with either Ang-1 or VEGF has no obvious effect on the integrity of the quiescent endothelial-cell monolayer (Fig. 2B,E,I). In turn, stimulation of co-culture spheroids with Ang-2 perturbs endothelial-cell-monolayer integrity, as evidenced by the detachment of single and groups of endothelial cells from the surface monolayer within 4 hours of Ang-2 exposure (Fig. 2C,D,I). Ang-2-mediated disruption of endothelial-cell-monolayer integrity occurs rapidly and can first be observed after 30-60 minutes (Fig. 3). Based on these observations, we studied the effect of exogenously added Ang-2 on in situ explants of human umbilical vein. Exposure of umbilical-vein explants to Ang-2 for 4 hours similarly led to massive disintegration of the endothelial-cell surface monolayer as evidenced by detachment of CD34-positive surface-lining endothelial cells (Fig. 4B,D). Control samples incubated with medium alone have an intact CD34-positive surface monolayer (Fig. 4A). Collectively, these in vitro and in situ experiments demonstrate that exogenous Ang-2 is potently capable of destabilizing the integrity of the surface endothelial-cell monolayer that is in contact with smooth-muscle cells.

Exogenous Ang-2-mediated perturbation of the endothelium is rescued by Ang-1, VEGF and sTie-2

We next performed cytokine combination experiments in order to elucidate the mechanism of the Ang-2-mediated perturbation of endothelial-cell-monolayer integrity. Ang-2-

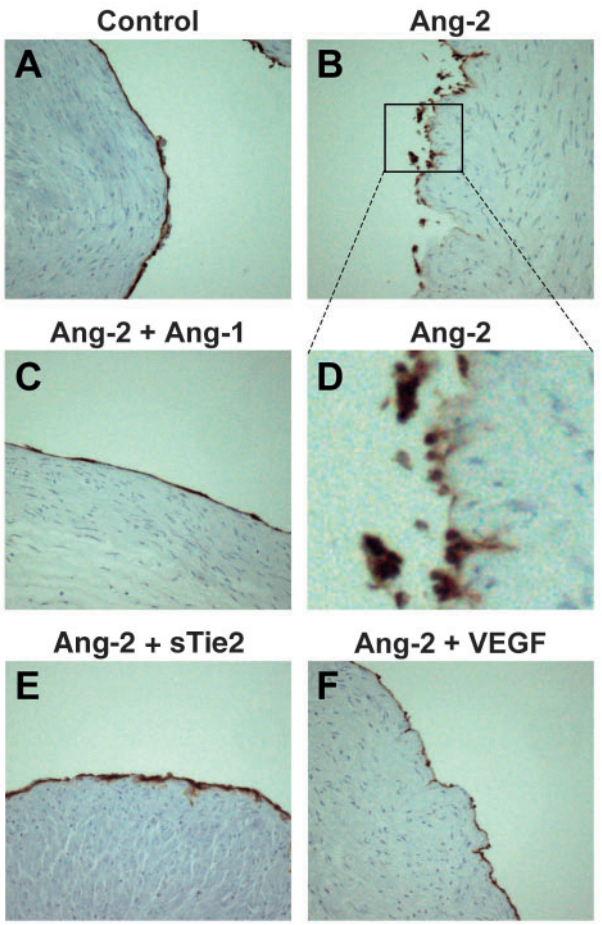


Fig. 4. Ang-2-mediated endothelial-cell-monolayer destabilization in explanted fragments of human umbilical vein. Fragments of umbilical vein were cultured overnight and exposed to different combinations of cytokines for 4 hours. Samples were then fixed, embedded and processed for CD34 immunohistochemistry. Control umbilical-cord fragments have an intact CD34-positive surface monolayer (A). Treatment with exogenous Ang-2 leads to vascular destabilization, as evidenced by detachment of the endothelial cells (B, higher magnification in D). Ang-1 (C), sTie-2 (E) and VEGF (F) are all able to rescue vascular destabilization elicited by exogenous Ang-2.

mediated detachment of endothelial cells can be inhibited by competition with exogenous Ang-1 (Fig. 2F,I). Similarly, soluble Tie-2 (sTie-2) can neutralize Ang-2-induced endothelial-cell detachment (Fig. 2G,I). Soluble Tie-2 binds with similar affinity both Ang-1 and Ang-2. Ang-1 is constitutively produced in the co-culture model by smooth-muscle cells (Fiedler et al., 2004; Korff et al., 2001), suggesting that the luminally applied exogenous sTie-2 is not able to interfere effectively with Ang-1 that is abluminally presented by smooth-muscle cells. Finally, VEGF proved to be similarly effective in rescuing Ang-2-induced endothelial-cell detachment (Fig. 2H,I). Rescue of endothelial-cell-monolayer integrity was also observed in the in situ model of explanted umbilical-vein fragments in which Ang-1 (Fig. 4C), sTie-2 (Fig. 4E) and VEGF (Fig. 4F) were all found to be able to inhibit Ang-2-mediated endothelial-cell detachment. Taken together, the in vitro and in situ rescue experiments confirm the

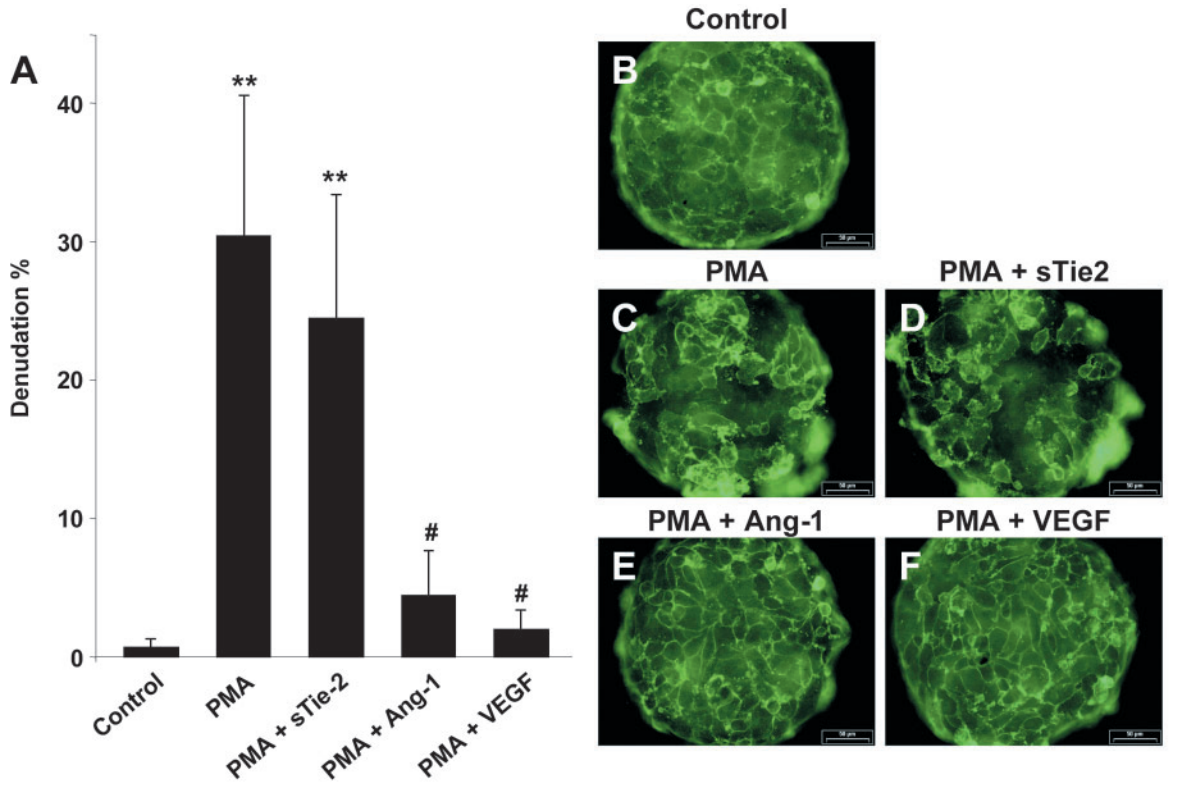


Fig. 5. Rescue of PMA-mediated disintegration of surface endothelial cells co-cultured with smooth-muscle cells in three-dimensional spheroids. Co-culture spheroids of HUVECs and HUASMCs were incubated in spinner culture with the indicated combinations of reagents for 4 hours and endothelial-cell detachment was quantified by automated image analysis of CD31 whole-mount-stained spheroids. PMA treatment leads to massive denudation of the surface endothelial-cell monolayer within 4 hours (A,C). Both Ang-1 (A,E) and VEGF (A,F) inhibit the PMA-mediated destabilization of the endothelial-cell monolayer. In turn, sTie-2 cannot block PMA-mediated perturbation of endothelial-cell-monolayer integrity (A,D). **, $P<0.001$ compared with control; #, $P<0.001$ compared with PMA treatment.

specificity of the Ang-2-mediated endothelial-cell detachment experiments and identify Ang-1, sTie-2 and VEGF as molecules that antagonize the effects of exogenous Ang-2.

Endogenous endothelial Ang-2 acts through an internal autocrine-loop mechanism

Endothelial cells have been identified as the primary source for Ang-2, suggesting that it might act as an autocrine regulator of endothelial-cell functions (Gale et al., 2002; Hackett et al., 2000; Holash et al., 1999; Huang et al., 2002; Mandriota and Pepper, 1998; Stratmann et al., 1998; Zhang et al., 2003). We have recently identified Ang-2 as a Weibel/Palade-body-stored molecule that can be rapidly released upon stimulation (Fiedler et al., 2004). PMA stimulation leads within minutes to release of Ang-2 from the endothelial-cell storage compartment (Fiedler et al., 2004). Treatment of co-culture spheroids of endothelial cells and smooth-muscle cells with PMA leads to rapid disintegration of the surface endothelial-cell monolayer (Fig. 5A,C), as evidenced by massive detachment of endothelial cells. PMA stimulation leads to global protein-kinase-C activation, which affects many other endothelial-cell functions in addition to the release of Ang-2. However, Ang-1 expression in smooth-muscle cells is not affected by PMA treatment, demonstrating that Ang-1 downregulation is not responsible for the observed disintegration of the co-culture spheroid (see Fig. S2 in supplementary material). However, both Ang-1 (Fig. 5A,E) and VEGF (Fig. 5A,F) are able to rescue the PMA-mediated perturbation of the endothelial-cell-monolayer integrity. Surprisingly, sTie-2 is not able to rescue PMA-mediated endothelial-cell detachment (Fig. 5A,D). This suggests that PMA either causes endothelial-cell detachment by mechanisms that go beyond its role as a stimulator of Ang-2 release and/or that PMA-released Ang-2 is presented in a way that makes it inaccessible for sTie-2-mediated neutralization of Ang-2.

To demonstrate that the effect of PMA on the integrity of the co-culture spheroid is mediated by released Ang-2, we silenced Ang-2 expression in endothelial cells using siRNA and verified the downregulation by RT-PCR and western blotting (see Fig. S3 in supplementary material). Ang-2 levels were downregulated to 20% in the siRNA-transfected HUVECs compared with HUVECs that had been treated with oligofectamine alone (data not shown). Endothelial cells with low Ang-2 levels do not detach from the surface of co-culture spheroids upon treatment with PMA (Fig. 6A,E), indicating that endothelial-cell-released Ang-2 causes the observed PMA-mediated disintegration of the endothelial-cell monolayer in endothelial-cell/smooth-muscle-cell co-culture spheroids.

The PMA stimulation experiments in combination with the rescue by Ang-1 and VEGF provided strong indirect evidence that PMA-mediated Ang-2 release might be responsible for the

observed endothelial-cell detachment following PMA stimulation. We therefore studied the properties of constitutively Ang-2-overexpressing endothelial cells. Control (Fig. 7A,B) and Ang-1-overexpressing (Fig. 7A,C) HUVECs form regular co-culture spheroids with HUASMCs and are resistant to mechanical challenge in spinner culture. Similarly, Ang-2-overexpressing HUVECs and HUASMCs form regular differentiated co-culture spheroids. However, these spheroids are sensitive to mechanical challenge, as evidenced by intense detachment of endothelial cells upon transfer in spinner culture (Fig. 7A,D). Detachment of Ang-2-overexpressing HUVECs in spinner culture is completely rescued by exogenous Ang-1 and VEGF (Fig. 7A,F,G). However, sTie-2 [which blocked the activities of exogenous Ang-2 (Fig. 2G, Fig. 4E)] has no effect on the endothelial-cell detachment of Ang-2-overexpressing HUVECs (Fig. 7A,E). This indicates that endogenously released Ang-2 cannot be inhibited by exogenously provided soluble receptor and demonstrates that endogenous Ang-2 acts through an internal autocrine-loop mechanism.

Discussion

The functional consequences of angiopoietin/Tie-2 signaling have been well established through genetic loss-of-function and gain-of-function experiments (Carmeliet, 2003; Maisonpierre et al., 1997; Suri et al., 1996; Yancopoulos et al., 2000). The phenotypes of Ang-1- and Ang-2-deficient and -overexpressing mice have led to an agonistic Ang-1/Tie-2 model and an antagonistic Ang-2/Tie-2 model (Hanahan, 1997). According to these, Ang-1 activates Tie-2 and induces subsequent signal transduction promoting endothelial-cell survival, endothelial quiescence and vessel assembly. Conversely, Ang-2 is believed to act as a non-signal-transducing Tie-2 ligand that binds to endothelial Tie-2 and thereby negatively interferes with agonistic Ang-1/Tie-2 functions. As such, it does not exert functions of its own but rather acts as a facilitator of other vascular cytokines. The net outcome of Ang-2 functions is therefore considered to be contextually determined by the presence of other cytokines. For example, Ang-2 has a pro-angiogenic effect in the presence of angiogenic factors such as VEGF and a induces vessel regression in the absence of angiogenic cytokines.

This early and far-sighted model of the contextual facilitating role of Ang-2 in the vessel wall is essentially compatible with all subsequently published genetic and manipulatory in vivo experiments (Gale et al., 2002; Hammes et al., 2004; Holash et al., 1999; Lobov et al., 2002; Maisonpierre et al., 1997; Suri et al., 1996). However, to date, no defined mechanistic experiments have been performed that unambiguously demonstrate cellular effector functions for Ang-2 that are compatible with an antagonistic mode of action towards Tie-2. Instead, increasing evidence supports the notion that long-term stimulation of cultured endothelial cells with Ang-2 exerts agonistic Tie-2-activating functions, as evidenced by Tie-2 receptor phosphorylation (Teichert-Kuliszewska et al., 2001), sprouting angiogenesis (Korff et al., 2001; Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001), increased MMP-9 expression (Das et al., 2003) and endothelial-cell survival (Kim et al., 2000).

We hypothesized that limitations of the routinely used cellular models might be responsible for the apparent discrepancy between the in vivo and in vitro findings, and thought to explore experimental systems that better mimic the context of the cells of the vessel wall as it occurs in vivo. We have towards this end established a co-culture model of endothelial cells and smooth-muscle cells that mimics the 3D assembly of the vessel wall in an inside-out configuration (Korff et al., 2001). In this model, smooth-muscle cells form a multicellular spheroid core that is lined by a surface monolayer of polarized endothelial cells. Most notably, in contrast to standard monolayer culture, the surface spheroid endothelial cell layer is perfectly growth arrested and the quiescent endothelial-cell phenotype is controlled by contact with the underlying Ang-1-expressing smooth-muscle cells (Korff et al., 2001). Furthermore, we used fragments of explanted

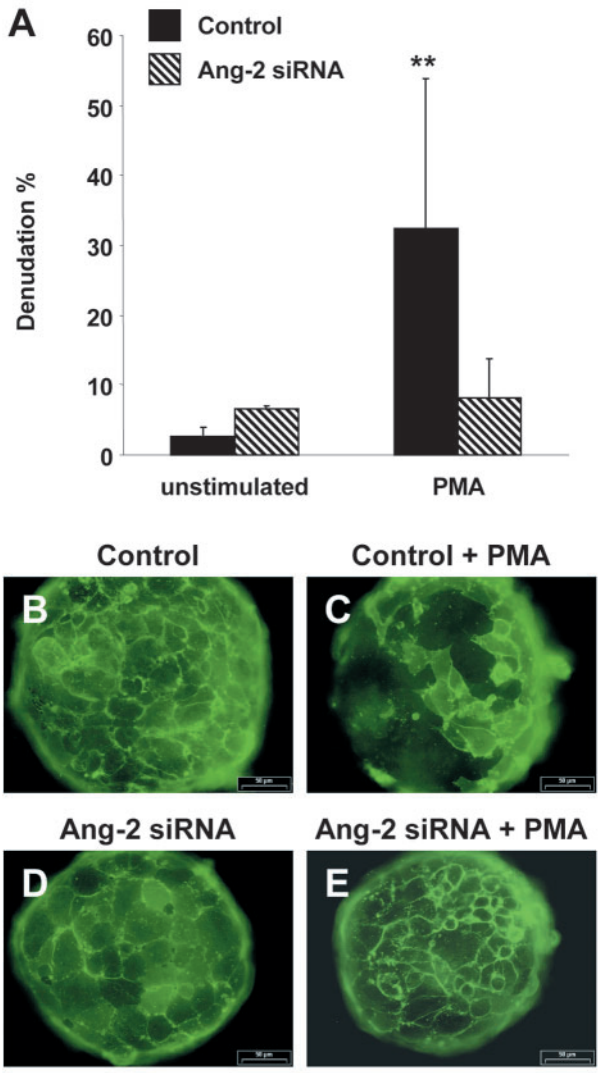
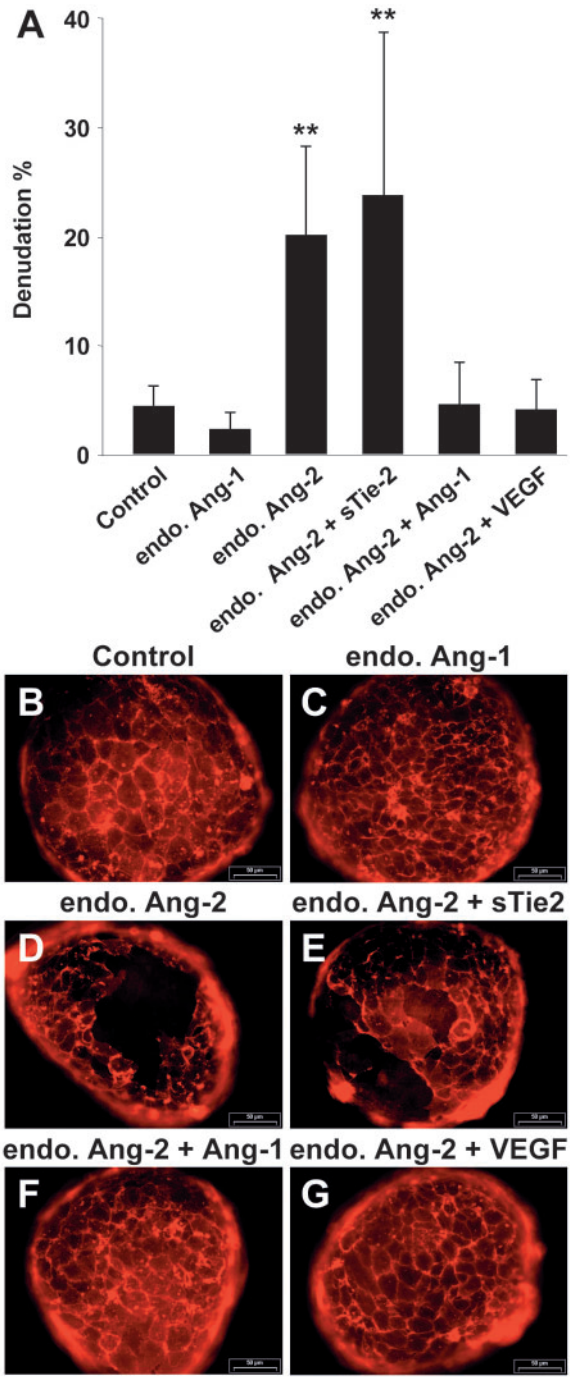


Fig. 6. Ang-2-dependent PMA-mediated destabilization of co-culture spheroids. Co-culture spheroids of siRNA-treated or untreated HUVECs with HUASMCs were incubated in spinner culture with the indicated combination of reagents for 4 hours and endothelial-cell detachment was quantified by automated image analysis of CD31 whole-mount-stained spheroids. PMA treatment leads to massive denudation of the surface monolayer of the untreated endothelial cells within 4 hours (A,C) but not of the monolayer of *Ang-2*-siRNA-silenced endothelial cells (A,E). **, $P<0.001$ compared with control.

umbilical veins as an in situ model of the vessel wall. Applying these unique models, we were for the first time able formally to establish rapid Ang-2 antagonistic functions on endothelial cells and demonstrate: (1) that endothelial-cell stability in the co-culture model is dependent on Tie-2 signaling; (2) that exogenous Ang-2 rapidly destabilizes endothelial-cell-monolayer integrity within 30 minutes to 4 hours, leading to detachment of endothelial cells; (3) that Ang-1, sTie-2 and VEGF rescue the endothelial-cell-destabilizing effect of exogenous Ang-2; (4) that endogenous Ang-2 similarly destabilizes endothelial-cell monolayers that are in contact with smooth-muscle cells; and (5) that endogenous endothelial-cell-derived Ang-2 acts through an internal autocrine-loop mechanism that cannot be blocked by exogenous sTie-2.



The findings of the present study have several important implications for our understanding of vascular angiopoietin biology. First, the established models may serve as relevant tools for further rational studies of endothelial cell angiopoietin functions, including the biochemical analysis of the competitive interactions of the angiopoietin ligands with the Tie-2 receptor and the molecular study of the junctional and cell-matrix changes that lead to endothelial-cell destabilization and, subsequently, to endothelial-cell detachment. Second, the study has also yielded important novel mechanistic insight into endothelial Ang-2 functions. sTie-2 was effective at blocking the endothelial-cell-destabilizing functions of exogenous Ang-2 but not of endogenous endothelial-cell-derived Ang-2. This suggests that Ang-2 might act through an internal autocrine-loop mechanism that is not accessible to extracellular inhibitors. Intracrine signaling mechanisms have been described for a number of molecules (Re, 2003), including interleukin 1 (Werman et al., 2004), chemokines (Gortz et al., 2002) and platelet-derived growth factor (Keating and Williams, 1988). Likewise, VEGF has been identified as an intracrine regulator of hematopoietic-stem-cell survival (Gerber et al., 2002). Mechanistically, the identification of a functional internal autocrine loop mechanism for endothelial-cell-derived Ang-2 corresponds to the recently identified intracrine Tie-2 signaling within endothelial-cell caveolae (Yoon et al., 2003). The biological relevance of the identified intracrine Ang-2 loop is also highlighted by the observations that endothelial cells serve as the primary source of Ang-2 and that Ang-2 is dramatically transcriptionally upregulated in activated endothelial cells (Gale et al., 2002; Hackett et al., 2000; Holash et al., 1999; Huang et al., 2002; Mandriota and Pepper, 1998; Stratmann et al., 1998; Zhang et al., 2003). Correspondingly, we have identified unique positive and negative regulatory elements in the Ang-2 promoter that control its endothelial-cell-specific expression (Hegen et al., 2004).

Interestingly, we found that Ang-2-mediated endothelial-cell detachment can be rescued not only by Ang-1 but also by VEGF. Ang-1 is the natural agonist of Tie-2, mediating anti-apoptotic, anti-inflammatory and vessel-sealing functions. Equal amounts of Ang-1 can rescue Ang-2-mediated effects,

Fig. 7. Perturbation of endothelial-cell-monolayer integrity in co-culture spheroids of Ang-1 or Ang-2 overexpressing HUVECs and HUASMCs. Control HUVECs (B) or HUVECs retrovirally transduced with *Ang-1* (C, endo. Ang-1) or *Ang-2* (D, endo. Ang-2) were mixed with HUASMCs and allowed to form co-culture spheroids for 48 hours. Established co-culture spheroids were then kept in spinner culture for 4 hours and endothelial-cell surface coverage was quantified by automated image analysis following whole-mount staining for CD31 using Alexa Fluor 546 as fluorescent dye. Control HUVECs (B) and *Ang-1* expressing HUVECs (C) form stable, differentiated co-culture spheroids with HUASMCs. By contrast, *Ang-2* overexpressing HUVECs form co-culture spheroids with HUASMCs, with endothelial cells detaching upon mechanical challenge in spinner culture (A,D). Co-culture spheroids of *Ang-2*-overexpressing HUVECs and HUASMCs can be stabilized by exogenous Ang-1 (F) or VEGF (G) but not by sTie-2 (A,E). **, $P<0.01$ compared with control.

indicating that a balance between the two factors regulates the quiescent and responsive endothelial-cell phenotypes. Both Ang-1 and VEGF are known to induce overlapping signaling pathways, mainly the protein-kinase-B/Akt and the mitogen-activated-protein-kinase pathways. The protein-kinase-B/Akt pathway mediates endothelial-cell survival and quiescence, suggesting that activation of this pathway rescues the Ang-2-induced endothelial-cell detachment (Kim et al., 2000; Gerber et al., 1998).

Ang-2 is not just produced by endothelial cells. It is also stored in endothelial-cell Weibel-Palade bodies, from where it can be made rapidly available within minutes of stimulation (Fiedler et al., 2004). As such, it appeared likely that Ang-2 controls rapid vascular reactions. Correspondingly, among the most intriguing findings of the present study is the observation that Ang-2 is able to perturb rapidly endothelial-cell-monolayer integrity. The first signs of endothelial-cell-monolayer disintegration could be observed within 30 minutes and massive endothelial-cell detachment was seen within 4 hours. The storage of Ang-2 in endothelial cells in combination with the identified rapid endothelial-cell functions of Ang-2 might, in fact, establish a new model in angiopoietin biology, and suggest that the angiopoietins do not just act as regulators of long-term morphogenic events as they occur during angiogenesis and vessel assembly. Instead, the primary function of Ang-2 in the adult might turn out to be a gatekeeper role of vascular homeostasis, controlling rapid vascular responses as they occur during, for example, inflammation and coagulation.

We have in the present study explored the detachment of endothelial cells as readout of Ang-2-mediated cellular effector functions. Detachment of endothelial cells denotes an exaggerated cellular reaction that surely does not correspond to physiological responses of endothelial cells in vivo. However, the dramatic cellular Ang-2 effects have their equivalent in vivo, as evidenced by the observation that mice that systemically overexpress Ang-2 have an embryonic lethal phenotype (Maisonpierre et al., 1997). Likewise, Ang-2 in the absence of angiogenic activity leads to vessel regression in the corpus luteum (Goede et al., 1998; Maisonpierre et al., 1997) and ocular models of angiogenesis (Hammes et al., 2004; Lobov et al., 2002). Conversely, Ang-2-deficient mice have a disturbance of vessel regression as it occurs developmentally in the eye's hyaloid vessels (Gale et al., 2002). The complementarity of the in vitro and in vivo observation supports the relevance of the models. However, they also support the notion that Ang-2 might, like VEGF, be a spatially and temporally tightly dosage-regulated vascular cytokine. High concentrations in vitro and in vivo lead to the described endothelial-cell detachment and vessel-regression phenotype. Future work based on manipulatory in vivo models and cellular models described in this study will have to establish the role of the low concentrations of physiologically stored endogenous endothelial-cell Ang-2 in controlling rapid vascular homeostatic reactions as they occur during, for example, inflammation and coagulation.

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