THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 314, No. 2

86553/3041602
JPET 314:738–744, 2005
Printed in U.S.A.

Angiopoietin-2 Causes Inflammation in Vivo by Promoting Vascular Leakage

Fiorentina Roviezzo, Stelios Tsigkos, Anastasia Kotanidou, Mariarosaria Bucci, Vincenzo Brancaleone, Giuseppe Cirino, and Andreas Papapetropoulos

Department of Experimental Pharmacology, Faculty of Pharmacy, University of Naples-Federico II, Naples, Italy (F.R., M.B., V.B., G.C.); "George P. Livanos-Marianthi Simou" Laboratories, Department of Critical Care and Pulmonary Services, Evangelismos Hospital, University of Athens, Athens, Greece (S.T., A.K., A.P.); and Laboratory for Molecular Pharmacology, School of Pharmacy, University of Patras, Patras, Greece (A.P.)

Received March 18, 2005; accepted April 28, 2005

ABSTRACT

Angiopoietins (Angs) are endothelium-selective ligands that exert most of their actions through the Tie-2 receptor. It is widely accepted that Ang-1 promotes the structural integrity of blood vessels and exhibits anti-inflammatory properties. In contrast, the role of Ang-2 remains less clear because it has been shown to behave as a Tie-2 agonist or antagonist under different experimental conditions. To define the role of Ang-2 in acute inflammation, we studied the effects of recombinant Ang-2 administration in vivo. We show herein that Ang-2, but not Ang-1, induces edema formation in the mouse paw in a dose-dependent manner; the edema seems to be fast-peaking (maximum at 30 min) and resolves within 4 h. The effect of Ang-2 is blocked by the coadministration with a soluble form of the Tie-2 receptor or Ang-1. NO and prostaglandin E_2 levels in mouse paw following the injection of Ang-2 remained unaltered, suggesting that the action of Ang-2 does not involve these mediators. In addition, Ang-2 exerted a weak stimulatory effect on leukocyte migration in the mouse paw. Similarly, Ang-2 injected into the mouse air pouch produced only a modest effect on cell extravasation that peaked at 30 min. However, when cell migration was elicited using zymosan, Ang-2 significantly inhibited leukocyte migration. We conclude that Ang-2 by itself stimulates the extravasation of cell-poor fluid, but in the presence of ongoing inflammation it reduces cellular infiltration in tissues.

The acute inflammatory response is associated with an increase in vascular permeability and cellular infiltration (Nathan, 2002). Both the extravasation of fluid and proteins and the accumulation of leukocytes at the inflammatory site contribute to edema formation. Several mediators involved in inflammation have been identified over the years. Histamine, serotonin, bradykinin, and prostaglandins (PGs), to name a few, trigger an increase in vascular permeability, whereas cytokines promote the expression of molecules responsible for rolling, firm adhesion, and diapedesis of circulating white blood cells (Malik and Lo, 1996; Nathan, 2002). The list of inflammatory mediators also includes a number of growth factors, such as vascular endothelial growth factor (VEGF), that increase both vascular permeability and leukocyte infiltration (Dvorak et al., 1995; Kim et al., 2001a).

Although VEGF was initially identified as a factor that induces vascular permeability, it has been studied most in the context of angiogenesis. VEGF is a receptor tyrosine kinase ligand that stimulates endothelial cell (EC) proliferation and migration and promotes EC organization into vessel structures (Ferrara et al., 2003; Zachary, 2003). Because angiogenesis and inflammation are two tightly linked processes, the search for factors that modify the inflammatory response among angiogenic growth factors seemed natural. The newly discovered growth factor angiopoietin-1 (Ang-1) has been shown not only to promote vessel stabilization during angiogenesis, but also to inhibit vascular permeability and exert anti-inflammatory effects (Davis et al., 1996; Thurston et al., 1999; Gamble et al., 2000). Ang-1 belongs to a family of proteins that bind to the Tie-2 receptors on EC and is a Tie-2 agonist (Davis et al., 1996). The second member of the Ang family, Ang-2, can inhibit Ang-1-induced Tie-2 receptor phosphorylation on EC but stimulates phosphorylation of ectopically expressed Tie-2 receptor in transfected fibroblasts (Maisonpierre et al., 1997). In addition, using

This work was supported by grants from the Greek Secretariat of Research and Technology (PENED 2001).

A.P. and G.C. contributed equally to this work.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.086553.

ABBREVIATIONS: PG, prostaglandin; VEGF, vascular endothelial growth factor; EC, endothelial cell(s); Ang, angiopoietin; MPO, myeloperoxidase; PKC, protein kinase C.

high concentrations or prolonged incubation times with Ang-2 led to the phosphorylation of Tie-2 receptors on some types of EC (Kim et al., 2000; Teichert-Kuliszewska et al., 2001); thus, Ang-2 is referred to as a context-dependent antagonist. The least studied angiopoietin so far is Ang-4. Ang-4 has been shown to evoke an agonist response upon binding to the Tie-2 receptor, sharing many of the actions of Ang-1 on cultured cells (Valenzuela et al., 1999; Lee et al., 2004).

If Ang-2 truly acts as a Tie-2 antagonist on the endothelium, one would expect this angiopoietin to promote vascular permeability and aggravate inflammation; however, the action of Ang-2 in these phenomena has not been examined to date. Because of the conflicting results on the action of Ang-2 on Tie-2 receptor activation in vitro, valid conclusions regarding the ability of Ang-2 to affect vascular leakage can only be drawn using in vivo models. To this end, we used two in vivo inflammatory mouse models of acute inflammation: the mouse hind paw and air pouch. We have found that Ang-2 administered alone promotes vascular leakage that is characterized by restricted migration of leukocytes, whereas it acts as an inhibitor of zymosan-induced cell migration.

Materials and Methods

Mouse Paw Edema. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals and were approved by the local committee. Male Swiss mice (CD-1; Charles River Italica, Calco, Italy) weighing 30 ± 2 g were divided into groups (n = 8/group) and lightly anesthetized with 4% enflurane mixed with 0.5 l/min O_2 and 0.5 l/min N_2O. Each group of animals received subplantar administration of 50 μl of saline that contained either bovine serum albumin (0.1%, vehicle), Ang-2 (30–300 ng), Ang-1 (300 ng), Ang-4 (300 ng), or VEGF (1–10 ng). In a separate set of experiments, a soluble form of the Tie-2 receptor (3000 ng; 1:10 ratio with Ang-2) was coadministered with Ang-2 in the same final volume (50 μl). To verify a possible interplay between VEGF, Ang-1, and Ang-2, mice were injected with the combination of VEGF plus Ang-1 or VEGF plus Ang-2. The volume was measured using a hydroplethysmometer specially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before subplantar injection and 0.5, 1, 2, 3, and 4 h thereafter. The same operator always performed the double-blind assessment of paw volume. The increase in paw volume was calculated by subtracting the initial paw volume (basal) from the paw volume measured at each time point.

Mouse Air Pouch. To generate air pouches, mice were anesthetized (ketamine/xylazine) on day 0 and received an injection of 5 ml of sterile air into the back (n = 6/group). Three days later, the patency of the pouch formed was maintained by injecting 2.5 ml of sterile air at the same site. On day 6, 1 ml of vehicle (bovine serum albumin plus saline) or Ang-2 (300 ng) was injected into the air pouch. Mice were sacrificed 0.5, 1, or 2 h following injection of either Ang-2 or vehicle by cervical dislocation, and the exudates in the pouch were collected by gently washing the pouch with 1 ml of sterile saline. The liquid collected was centrifuged, and the pellet obtained was resuspended in 500 μl of saline. Leukocyte counts were performed by diluting an aliquot of the cell suspension in Turk's solution using a microscope. The person scoring the samples was unaware of the treatment.

In another set of experiments, mice that had the pouch formed as described above were injected on day 6 with zymosan (1 ml of 1% w/v). Ang-2 was administered in the pouch 30 min before injection of zymosan. Four hours later, the animals were sacrificed, and the leukocyte number was determined as described above.

Myeloperoxidase Measurement. Mice were killed with carbon dioxide at 0.5, 1, or 2 h after Ang-2 administration, and the paws were weighed, cut, and homogenized in 1 ml of hexadecyltrimethylammonium bromide buffer containing 5 g of hexadecyltrimethylammonium bromide in 1 l of potassium phosphate buffer (50 mM, pH 6.0) using a Polytron homogenizer (two cycles of 10 s at maximum speed). After centrifugation at 10,000 rpm for 2 min, supernatant fractions were assayed for myeloperoxidase (MPO) activity as an estimate of the presence of neutrophils in the tissues. Briefly, samples (20 μl) were mixed with phosphate buffer (180 μl) containing 1 mM O-dianisidine dihydrochloride and 0.001% hydrogen peroxide in a microtiter plate. Absorbance was measured at 450 nm, performing three readings at 30-s intervals. The calculation of MPO units was based on the fact that 1 U MPO equals 1 μmol H_2O_2 generated per min and that 1 μmol H_2O_2 gives a change in absorbance of 1.13×10^{-2} (change in absorbance = nanometers per minute).

NO_x and PGE_2 Exudate Levels. Mice from different groups were killed with carbon dioxide 0.5, 1, or 2 h after Ang-2 administration. Paws were cut and centrifuged at 4000 rpm for 30 min. Exudates (supernatants) were collected with 100 μl of saline and used for NO_x (nitrite plus nitrate) and PGE_2 quantification. To determine NO_x levels, proteins were removed from the exudates with 30% $ZnSO_4$. Supernatants and a standard curve of sodium nitrate were incubated in a microplate with cadmium for 1 h to convert NO_3^- to NO_2^-. After centrifugation at 14,000 rpm for 15 min, total nitrite (NO_x) content was determined fluorometrically in microtiter plates using a standard curve of sodium nitrite. NO concentration in the samples was calculated using the internal standard curve. PGE_2 levels were determined in deproteinized exudates by radioimmunoassay.

Drugs and Reagents. Bradford reagent was obtained from Bio-Rad (Segrate, Italy). [^{3}H]PGE_2 was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Recombinant human angiopoietins, $VEGF_{1-165}$, and soluble Tie-2 were purchased from R&D Systems (Minneapolis, MN). The purity for each of the recombinant growth factors or proteins used was as follows: Ang-1, >90%; Ang-2, >97%; Ang-4, >85%; $VEGF_{1-165}$, >97%; and soluble Tie-2, >90%, as determined by SDS- polyacrylamide gel electrophoresis and visualized by silver staining. Professor Ciabattoni (University of Chieti, Chieti, Italy) provided the antibody against PGE_2. All the other reagents and compounds used were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. Data were expressed as mean ± S.E.M. The level of statistical significance was determined by one-way analysis of variance, followed by Bonferroni post test for multiple comparisons using Prism software (GraphPad Software Inc., San Diego, CA).

Results

Ang-2 Induces Edema Formation in Vivo. Given that Ang-2 blocks phosphorylation of Tie-2 by Ang-1 on vascular EC, exerting a destabilizing effect, and that transgenic overexpression of Ang-2 in vivo results in discontinuous and leaky vessels (Maisonpierre et al., 1997), we postulated that acute administration of recombinant Ang-2 protein in vivo could alter endothelial integrity and increase vascular leakage. To determine the ability of Ang-2 to stimulate edema formation, we assessed changes in paw volume after subplantar injection of Ang-2. Ang-2 administration in the hind paw of CD1 Swiss mice resulted in a dose- and time-dependent increase in paw volume that was fast in onset, peaking at 30 min and lasting at least 3 h (Fig. 1A). To determine whether Tie-2 agonists have a similar effect to that observed with Ang-2 on edema formation, we used 300 ng/paw (equivalent to the maximal dose of Ang-2 used) of Ang-1 or Ang-4. Unlike Ang-2, Ang-1 did not trigger edema formation in the hind paw, whereas Ang-4 caused an inflammatory response that peaked at 1 h (Fig. 1B).

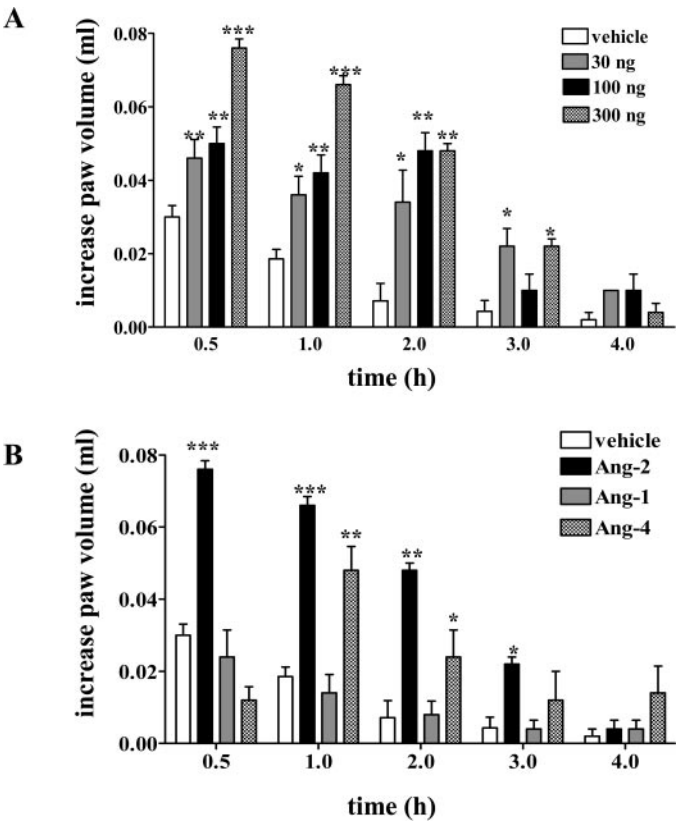

Fig. 1. Ang-2, but not Ang-1, promotes edema formation. A, CD1 mice injected subplantarly with Ang-2 (30–300 ng/paw), and edema formation measured at the indicated times. B, angiopoietins administered at 300 ng/paw, and edema measured up to 4 h. Values are mean + S.E.M.; n = 8 mice; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus vehicle.

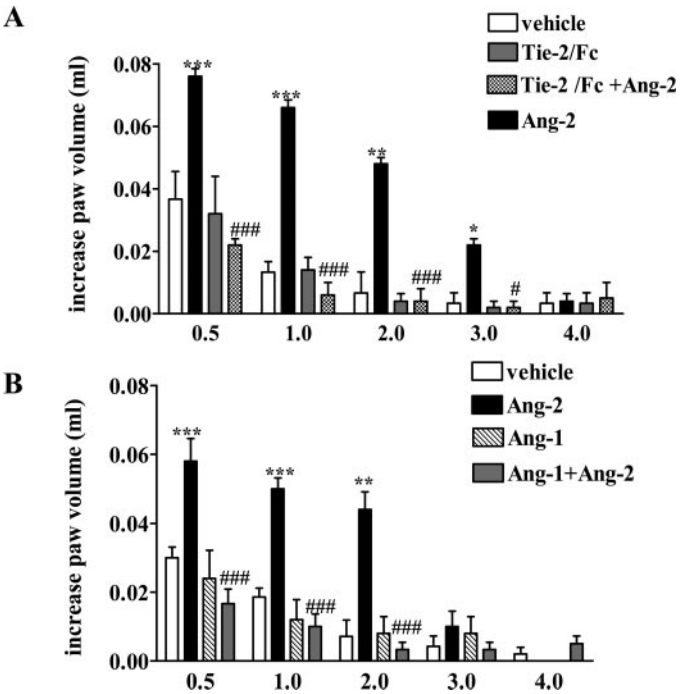

Fig. 2. Soluble Tie-2 and Ang-1 prevent Ang-2-stimulated edema. A, Ang-2 (300 ng) administered either alone or in combination with 10-fold excess of soluble Tie-2 (Tie-2/Fc; 3000 ng). Values are mean + S.E.M.; n = 8 animals. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus vehicle; #, $p < 0.05$ and ##, $p < 0.01$ versus Ang-2. B, Ang-2 or Ang-1 (300 ng each) administered alone or in combination, and edema formation measured at the indicated time. Values are mean + S.E.M.; n = 8 mice. **, $p < 0.01$ and ***, $p < 0.001$ versus vehicle; ##, $p < 0.01$ versus Ang-2.

Soluble Tie-2 and Ang-1 Inhibit Ang-2-Induced Edema. Angiopoietins bind a common receptor on the EC surface, the Tie-2 receptor (Yancopoulos et al., 2000). To verify that the effects of Ang-2 were specific, we repeated the Ang-2 injections mixed with 10-fold excess of a soluble form of Tie-2 (Tie-2/Fc; 3000 ng). Whereas Tie-2/Fc alone did not have any effect, coinjection with Ang-2 abrogated changes in paw volume stimulated by the latter (Fig. 2A). To determine the ability of Ang-1 to protect against Ang-2-induced vascular leakage, mice were coinjected with an Ang-1/Ang-2 mixture at a 1:1 ratio. Under these conditions, Ang-1 abolished Ang-2-stimulated edema (Fig. 2B).

Ang-2-Induced Edema Is Independent of NO and PGE_2 Generation and Characterized by Only Minor Cellular Infiltration. To determine the mechanisms that mediate Ang-2 edema, we measured NO and PGE_2 (common mediators produced by EC that alter EC permeability) in vehicle- and Ang-2-treated tissues (Fig. 3, A and B). After subplantar injections of the dose of Ang-2 that caused maximal edema, no significant increase in both mediators was noted after 0.5, 1, or 2 h. To investigate whether the edema consisted mainly of fluid or was also leukocyte-rich, we measured tissue MPO activity, an enzyme found in high amounts in phagocytes. Treatment of mice with Ang-2 resulted in only a small increase in MPO activity in the paw that was significant 2 h postinjection (Fig. 3C). Histological sections from animals at several different time points after Ang-2 treatment confirmed that the edema observed in response to Ang-2 consisted mainly of fluid with only very few infiltrating cells (unpublished data).

To better evaluate the effect of Ang-2 on cellular infiltration, we switched to a model that allows quantitation of the number of cells migrated, the air pouch model. The injection of Ang-2 alone into the pouch triggered the migration of a few cells (mainly neutrophils), which was similar to what was observed in the hind paw. However, when Ang-2 was given in the presence of ongoing inflammation, it reduced the number of infiltrating cells in response to zymosan by approximately 50% (Fig. 4). In the same model, Ang-1 had no effect on cell migration.

Effect of Angiopoietins on VEGF-Induced Increase in Paw Volume. VEGF is a well-documented permeability-inducing growth factor (Dvorak et al., 1995). Because of its interaction with the angiopoietins during angiogenesis, we sought to determine whether angiopoietins modify VEGF-induced vascular permeability. VEGF produced a time- and dose-dependent increase in paw volume (Fig. 5A), which exhibited similar kinetics with Ang-2, reaching a maximum 0.5 h after the injection and receding after 4 h. We proceeded to investigate whether the coinjection of these two factors would have an additive effect. Administration of a submaximal Ang-2 dose (30 ng) along with a submaximal VEGF dose (3 ng) resulted in additive edema formation after 0.5 h (Fig. 5B). In contrast, coadministration of maximal Ang-2 and VEGF doses resulted in an increase in paw volume that was not greater than the increase observed when either growth factor was used alone (Fig. 5C). When Ang-1 was used in combination with VEGF, it prolonged the duration of edema

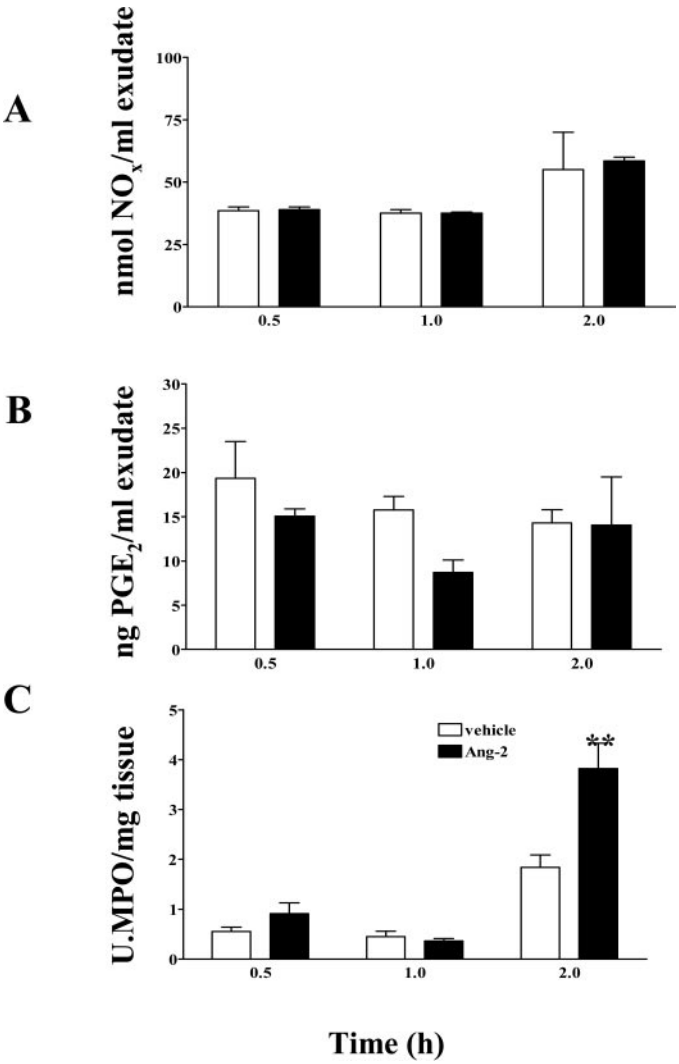

Fig. 3. MPO, NO_x, and PGE_2 production after Ang-2 administration. Mouse paws were injected with vehicle or Ang-2 (300 ng); No_x levels (A), PGE_2 levels (B), and MPO activity (C) were measured in the exudates after 0.5, 1, or 2 h. Values are mean + S.E.M.; $n = 6$ mice. **, $p < 0.01$ versus vehicle.

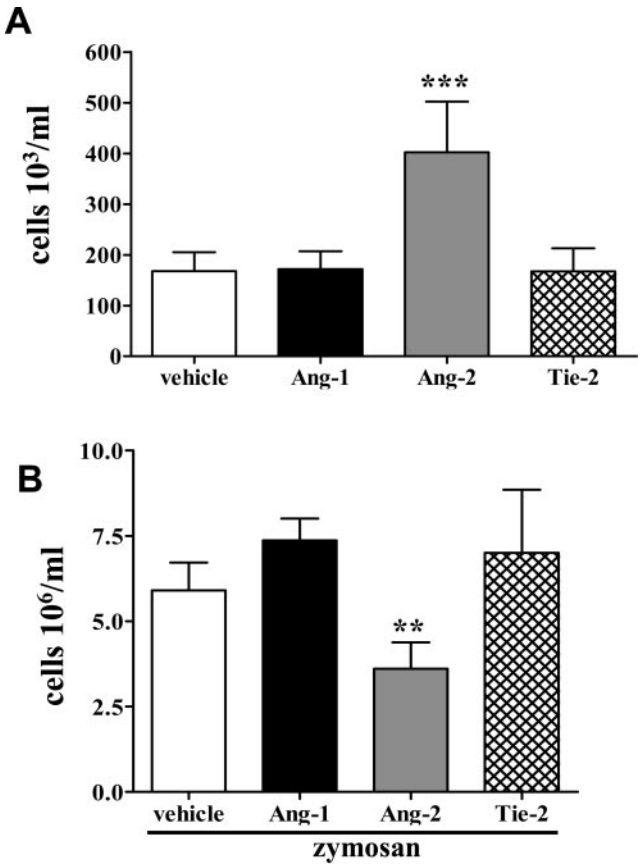

Fig. 4. Effects of Ang-2 on leukocyte migration in the air pouch model. A, Ang-2 (300 ng), Ang-1 (300 ng), or soluble Tie-2 (3000 ng) administered into the air pouch, and leukocyte migration evaluated after 30 min. B, Ang-2, Ang-1, or Tie-2 administered locally in the pouch 0.5 h before zymosan administration. Values are mean + S.E.M.; $n = 6$ animals. **, $p < 0.01$ and ***, $p < 0.001$ versus vehicle.

without affecting the maximal response obtained in the presence of VEGF (Fig. 5D).

Discussion

The angiopoietin family of proteins includes four different angiopoietins termed Ang-1 through Ang-4 (Yancopoulos et al., 2000). Ang-1 is vital for neovascularization during development because Ang-1-deficient mice exhibit embryonic lethality (Suri et al., 1996). Ang-1 lacks growth-stimulating properties but promotes EC sprouting, migration, and survival (Koblizek et al., 1998; Witzenbichler et al., 1998; Papapetropoulos et al., 2000). Ang-4 shares many of the properties of Ang-1 and, together with Ang-1, is classified as an agonist of the Tie-2 receptor based on its ability to promote Tie-2 autophosphorylation (Valenzuela et al., 1999; Lee et al., 2004). On the other hand, Ang-2 exhibits context-dependent behavior because it can inhibit or stimulate Tie-2 receptor phosphorylation under different conditions (Maisonpierre et al., 1997; Kim et al., 2000; Papapetropoulos et al., 2000). This is also reflected in the biological responses brought about by Ang-2; for example, Ang-2 has been shown to both promote EC migration (Mochizuki et al., 2002) and inhibit Ang-1-stimulated EC migration (Witzenbichler et al., 1998). More recently, based on observations of the ability of Ang-1 to rescue the phenotype of Ang-2 knockouts, Gale et al. (2002) suggested that Ang-2 in vivo acts a Tie-2 antagonist on vascular EC, whereas it acts as a Tie-2 agonist on lymphatic vessels. The varying behavior of Ang-2 in the different experimental systems in vitro and its differential effects on vascular versus lymphatic endothelium make it difficult to predict the actions of this growth factor in vivo on vascular leakage. To determine the role of Ang-2 in vascular leakage, we used recombinant Ang-2 and measured edema formation in the mouse hind paw. Unlike what had been reported for Ang-2 in vitro, where it has no effect on vascular permeability (Wang et al., 2004), we observed that Ang-2 promoted edema formation in a time- and dose-dependent manner. Edema formation in response to Ang-2 was prevented by neutralizing its action using a soluble form of the Tie-2 receptor. It should be mentioned that targeted disruption of the Ang-2 locus results in ascites formation and lethality soon after birth in mice (Gale et al., 2002). This observation is not in conflict with the present data because the increased leakage in Ang-2 knockout mice is caused by defects in lymphatic patterning during development.

One of the cardinal features of inflammation is the leuko-

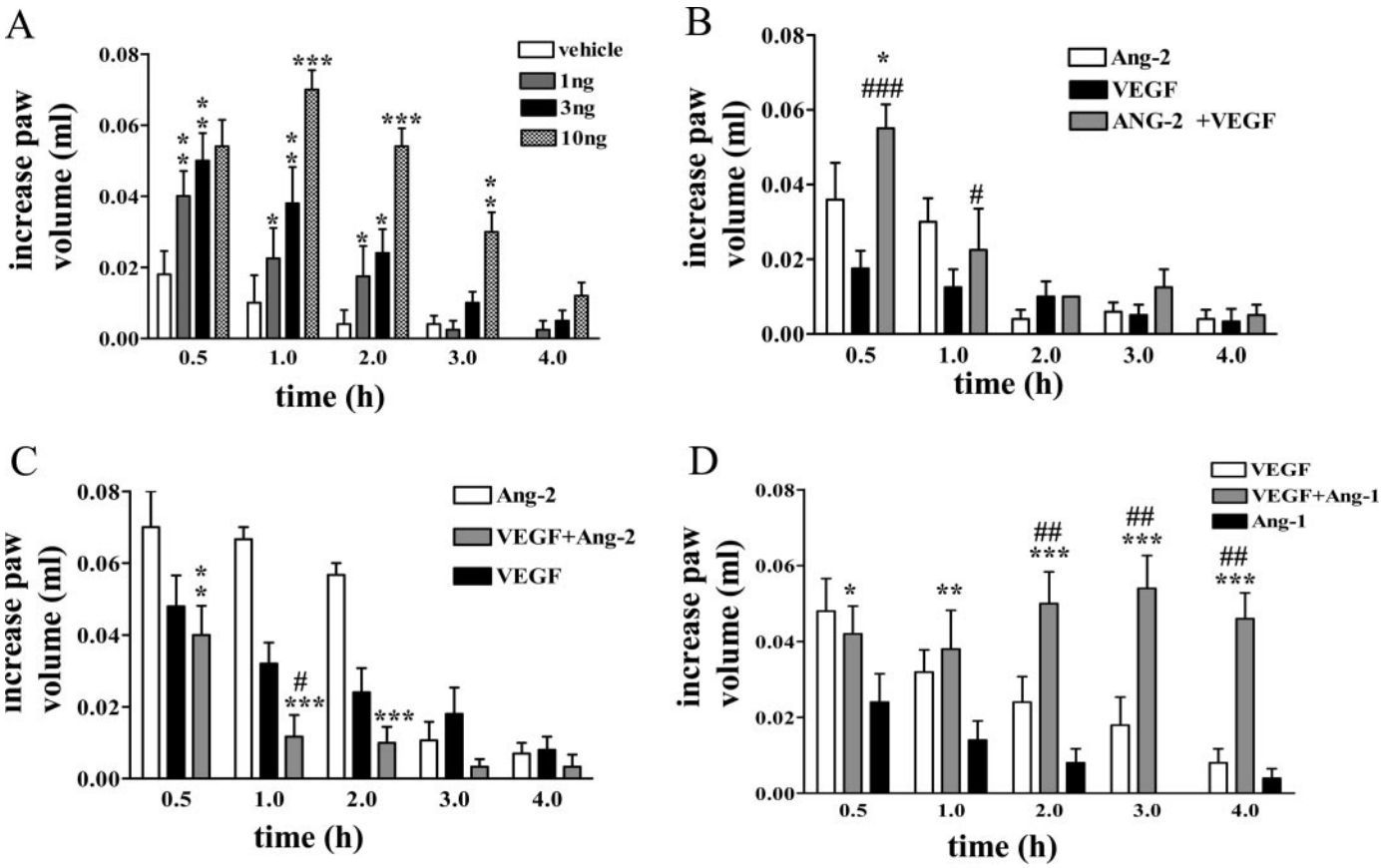

Fig. 5. Interaction of VEGF and angiopoietins in edema formation. A, VEGF (1–10 ng/paw) administered subplantarly, and edema formation measured at the indicated times. Values are mean + S.E.M.; $n = 6$ animals. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus vehicle. B, mice injected subplantarly with Ang-2 (30 ng) alone, VEGF (3 ng) alone, or a combination of Ang-2 (30 ng) and VEGF (3 ng) before evaluation of edema formation. Values are mean + S.E.M.; $n = 8$ animals/group. *, $p < 0.05$ versus Ang-2; #, $p < 0.05$; and ###, $p < 0.001$ versus VEGF. C, mice injected subplantarly with Ang-2 (300 ng) alone, VEGF (10 ng) alone, or a combination of Ang-2 (300 ng) and VEGF (10 ng), and edema formation determined. **, $p < 0.01$ and ***, $p < 0.001$ versus Ang-2; #, $p < 0.05$ versus VEGF. D, Ang-1 (300 ng) or VEGF (1 ng) injected alone or in combination, and edema formation measured at the indicated times. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus Ang-1; ##, $p < 0.001$ versus VEGF.

cyte migration into tissues. To determine whether Ang-2 stimulates transmigration of circulating leukocytes in addition to promoting fluid passage, we measured tissue MPO activity and cell number after exposure to Ang-2 in the mouse paw and air pouch model, respectively. We observed that Ang-2 promoted a small but significant increase in neutrophil accumulation in tissues. Ang-2 could promote neutrophil margination by acting either on the endothelium or on the leukocytes themselves. A recent report by Lemieux et al. (2005) showed that Ang-2 promotes a rapid translocation of P-selectin on the EC surface and stimulates neutrophil adherence to the endothelium. In the same report, neutrophils were also shown to express functional Tie-2 receptors. Tie-2 activation by Ang-1 or Ang-2 stimulates platelet-activating factor synthesis, whereas treatment with a combination of Ang-1 and Ang-2 also triggers the functional up-regulation of the β2-integrin complex to facilitate binding of polymorphonuclear leukocytes to EC. Interestingly, in the present study, Ang-2 blocked zymosan-induced leukocyte infiltration in the air pouch, indicating that in the presence of this inflammatory stimulus Ang-2 reduces excessive leukocyte mobilization. Collectively, our data indicate that Ang-2 is efficient in promoting vascular leakage when used by itself and has a small effect on leukocyte trafficking; however, in the presence of ongoing inflammation, it inhibits leukocyte migration.

Although some observations have suggested that NO reduces permeability, recent in vivo observations and in vitro studies using microvascular EC are consistent with a permeability-promoting effect of NO (Yuan, 2002). We have confirmed in the mouse paw that NO derived from the endothelium is critical for vascular leakage during acute inflammation using endothelial NO synthase knockout mice (Bucci et al., 2005). A link between Tie-2 receptor activation and NO has also been proposed: NO was reported to be increased following Ang-1 exposure and to contribute to the angiogenic actions of Ang-1 (Babaei et al., 2003; Chen et al., 2004). However, it should also be mentioned that we have been unable to detect NO release from cultured human umbilical vein EC, as measured by its surrogate marker cGMP (Papapetropoulos et al., 1999). In the present study, no change in NO_x in response to Ang-2 administration was noted, suggesting that vascular leakage in response to Ang-2 does not result from increased NO production. To further investigate the mechanism through which Ang-2 promotes vascular leakage, we measured the levels of PGE_2 in tissue homogenates of vehicle- and Ang-2-treated mice. The data obtained ruled out the possibility that this autacoid mediates the action of Ang-2.

The working hypothesis in the field of angiogenesis is currently that neovessel formation requires the temporal and spatial integration of signals originating from both the Tie-2

and VEGF receptors. When the need for new blood vessels arises, Ang-2 expression is up-regulated, blocking the vessel-stabilizing action of Ang-1; this allows loosening of existent vascular structures, which in turn enables VEGF (and other growth factors) to promote EC migration, proliferation, and organization of EC into networks (Gale and Yancopoulos, 1999). Once the new vessels have been formed, a concomitant increase in Ang-1 levels and a decrease in Ang-2 levels are observed, securing the structure of the newly formed vasculature (Gale and Yancopoulos, 1999; Holash et al., 1999). Because of the importance of the interaction of VEGF and the angiopoietins in angiogenesis, we tested the effect of Ang-2 on the VEGF-induced increase in vascular permeability. Similar to what has been shown in other vascular beds and species, VEGF administration promoted dose-dependent edema formation in the mouse paw. This edema had similar kinetics and was of a comparable magnitude to the one observed with Ang-2. When submaximal doses of both Ang-2 and VEGF were used, there was an additive effect of the two growth factors after 0.5 h, whereas when maximal Ang-2 and VEGF doses were used, no additivity was observed, suggesting that Ang-2 and VEGF could be acting through similar pathways. A recent review reported 46 different signaling pathways that can be activated by VEGF in cultured EC (Zachary and Gliki, 2001). However, only phospholipase C and mitogen-activated protein kinase cascades have been shown to mediate the increase in vascular permeability stimulated by VEGF in microvessel preparations (Bates and Harper, 2002). We observed that Ang-2 stimulated extracellular signal-regulated kinase 1/2 phosphorylation (unpublished data); experiments are underway to determine whether the mitogen-activated protein kinase pathway mediates the increase in permeability brought about by Ang-2.

Ang-1, in addition to being important for angiogenesis, also possesses anti-inflammatory properties. Genetic overexpression or overexpression following infection with adenovirus carrying the Ang-1 gene protects the vasculature from VEGF- and irritant-induced leakage (Thurston et al., 1999, 2000). Ang-1 also blocks the increase in permeability brought about by a variety of agents (Gamble et al., 2000; Pizurki et al., 2003), inhibits endothelial interleukin 8 production (Pizurki et al., 2003), blocks VEGF-induced expression of adhesion molecules, and reduces leukocyte adhesion and transmigration in vitro (Gamble et al., 2000; Kim et al., 2001b; Pizurki et al., 2003). The inhibition of VEGF-induced permeability afforded by Ang-1 in vitro has been attributed to a reduction in protein kinase Cβ (PKCβ) activation, inhibition of dissociation of β-catenin from vascular endothelial cadherin, and stabilization of EC junctional complexes (Gamble et al., 2000; Li et al., 2004; Wang et al., 2004). A different PKC isoform (PKCζ) has been implicated in the inhibitory action of Ang-1 on thrombin-induced permeability (Li et al., 2004). Contrary to what we expected, administration of recombinant Ang-1 did not inhibit VEGF-induced vascular permeability but instead delayed edema resolution. The discrepancy between in vivo and in vitro results can be easily explained by the presence of additional types of cells that also express angiopoietin receptors and contribute/modify the Ang-1 response. The prolonged action of VEGF on permeability in the presence of Ang-1 could result from the activation of the neutrophil Tie-2 receptor, leading to increased adherence of neutrophils to the endothelium and platelet-activating factor release (Lemieux et al., 2005). On the other hand, the low levels of locally injected Ang-1 compared with the levels achieved after overexpression, as well as the fact that different vascular beds were studied, could account for the differences between the present report and earlier in vivo studies. In any case, our observations suggest that Ang-1 is not a universal inhibitor of EC permeability as previously thought.

In conclusion, we have shown that Ang-2 can act as a modulator of the inflammatory response by promoting vascular leakage. However, it does not exhibit the full features of a classic inflammatory substance because it mainly stimulates fluid passage without strongly promoting leukocyte migration. This effect of Ang-2 on endothelial barrier function could be relevant for phenomena related to angiogenesis and inflammation.

References

Babaei S, Teichert-Kuliszewska K, Zhang Q, Jones N, Dumont DJ, and Stewart DJ (2003) Angiogenic actions of angiopoietin-1 require endothelium-derived nitric oxide. *Am J Pathol* **162:**1927–1936.

Bates DO and Harper SJ (2002) Regulation of vascular permeability by vascular endothelial growth factors. *Vascul Pharmacol* **39:**225–237.

Bucci M, Roviezzo F, Posadas I, Yu J, Parente L, Sessa WC, Ignarro LJ, and Cirino G (2005) Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo. *Proc Natl Acad Sci USA* **102:**904–908.

Chen JX, Lawrence ML, Cunningham G, Christman BW, and Meyrick B (2004) HSP90 and Akt modulate Ang-1-induced angiogenesis via NO in coronary artery endothelium. *J Appl Physiol* **96:**612–620.

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, et al. (1996) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87:**1161–1169.

Dvorak HF, Brown LF, Detmar M, and Dvorak AM (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability and angiogenesis. *Am J Pathol* **146:**1029–1039.

Ferrara N, Gerber H-P, and LeCouter J (2003) The biology of VEGF and its receptors. *Nat Med* **9:**669–676.

Gale N, Thurston G, Hackett S, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte M, Jackson D, et al. (2002) Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning and only the latter role is rescued by angiopoietin-1. *Dev Cell* **3:**411–423.

Gale NW and Yancopoulos GD (1999) Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins and ephrins in vascular development. *Genes Dev* **13:**1055–1066.

Gamble J, Drew J, Tresize L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, and Vadas M (2000) Angiopoietin-1 is an anti-permeability and anti-inflammatory agent in vitro and targets cells junctions. *Circ Res* **87:**603–607.

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, and Wiegand SJ (1999) Vessel cooption, regression and growth in tumors mediated by angiopoietins and VEGF. *Science (Wash DC)* **284:**1994–1998.

Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, and Koh GY (2000) Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. *Oncogene* **19:**4549–4552.

Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, and Koh GY (2001a) Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem* **276:**7614–7620.

Kim I, Moon SO, Park SK, Chae SW, and Koh GY (2001b) Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1 and E-selectin expression. *Circ Res* **89:**477–479.

Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, and Risau W (1998) Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr Biol* **8:**529–532.

Lee HI, Cho C-H, Hwang S-J, Choi H-H, Kim K-T, Ahn SY, Kim J-H, Oh J-L, Lee GM, and Koh GY (2004) Biological characterization of angiopoietin-3 and angiopoietin-4. *FASEB J* **18:**1200–1208.

Lemieux C, Maliba R, Favier J, Theoret J-F, Merhi Y, and Sirois MG (2005) Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses. *Blood* **105:**1523–1530.

Li X, Hahn CN, Parsons M, Drew J, Vadas MA, and Gamble JR (2004) Role of protein kinase Czeta in thrombin-induced endothelial permeability changes: inhibition by angiopoietin-1. *Blood* **104:**1716–1724.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, et al. (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science (Wash DC)* **277:**55–60.

Malik A and Lo S (1996) Vascular endothelial adhesion molecules and tissue inflammation. *Pharmacol Rev* **48:**213–229.

Mochizuki Y, Nakamura T, Kanetake H, and Kanda S (2002) Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. *J Cell Sci* **115:**175–183.

Nathan C (2002) Points of control in inflammation. *Nature (Lond)* **420:**846–852.

Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, Altieri DC,

and Sessa WC (2000) Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem* **275:**9102–9405.

Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonpierre PC, Yancopoulos GD, and Sessa WC (1999) Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival and interaction with other angiogenic growth factors. *Lab Investig* **79:**213–223.

Pizurki L, Zhou Z, Glynos K, Roussos C, and Papapetropoulos A (2003) Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production. *Br J Pharmacol* **139:**329–336.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, and Yancopoulos GD (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87:**1171–1180.

Teichert-Kuliszewska K, Maisonpierre PC, Jones N, Campbell AI, Master Z, Bendeck MP, Alitalo K, Dumont DJ, Yancopoulos GD, and Stewart DJ (2001) Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. *Cardiovasc Res* **49:**659–670.

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, and Yancopoulos GD (2000) Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med* **6:**460–463.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, and McDonald DM (1999) Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science (Wash DC)* **286:**2511–2514.

Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ, Jenkins NA, et al. (1999) Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. *Proc Natl Acad Sci USA* **96:** 1904–1909.

Wang Y, Pampou S, Fujikawa K, and Varticovski L (2004) Opposing effect of angiopoietin-1 on VEGF-mediated disruption of endothelial cell-cell interactions requires activation of PKC beta. *J Cell Physiol* **198:**53–61.

Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD, and Isner JM (1998) Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J Biol Chem* **273:**18514–18521.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, and Holash J (2000) Vascular-specific growth factors and blood vessel formation. *Nature (Lond)* **407:** 242–248.

Yuan SY (2002) Protein kinase signaling in the modulation of microvascular permeability. *Vascul Pharmacol* **39:**213–223.

Zachary I (2003) VEGF signalling: integration and multi-tasking in endothelial cell biology. *Biochem Soc Trans* **31:**1171–1177.

Zachary I and Gliki G (2001) Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res* **49:**568–581.

Address correspondence to: Dr. Andreas Papapetropoulos, Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras, Greece 26504. E-mail: apapapet@upatras.gr