Activation of Flk-1/KDR mediates angiogenesis but not hypotension

Bernard Malavaud a,b, Ivan Tack c, Frédéric Jonca a,b, Françoise Praddaude c, Françoise Moro a, Jean-Louis Ader c, Jean Plouet a

a Laboratoire de Biologie Moléculaire Eucaryote / UPR CNRS 9006, 118 route de Narbonne, 31062 Toulouse Cedex, France
b Service d'Urologie, CHU Purpan — Faculté de Médecine, 31062 Toulouse Cedex, France
c Laboratoire de Physiologie / Unité INSERM 388 31062 Toulouse Cedex, France

Received 12 December 1996; accepted 26 June 1997

Abstract

Objective: The concept of therapeutic angiogenesis with vascular endothelial growth factor (VEGF) has been validated in peripheral arterial disease. Its use in myocardial ischemia may be delayed as the result of the description in a porcine model of peripheral vasodilation after intraluminal injections of VEGF resulting in a 50% fatality rate by hypotension. We carried out this study to test whether VEGF-induced hypotension (1) is species specific, (2) is mediated by the receptor mediating angiogenesis, (3) is prevented by inhibition of nitric oxide synthase.

Methods: In the rabbit corneal pocket assay we tested whether a previously published anti-idiotypic antibody AIA agonist of the VEGF receptor Flk-1/KDR could elicit angiogenesis. Various doses of recombinant VEGF or AIA were injected into anesthetized normotensive Wistar-Kyoto rats and the mean arterial blood pressure (MABP) was recorded. To test the implication of nitric oxide in VEGF-induced hypotension we treated the animals with a competitive inhibitor of nitric oxide synthase prior to the injection of VEGF.

Results: Both VEGF and AIA induce angiogenesis but only intravenous injections of VEGF induced a rapid, transient and dose-dependent decrease in MABP. The ED50 was 0.5 μg. The interval between two VEGF injections required to lead to a decrease of MABP was 40 minutes. Nitric oxide synthesis inhibitor prevented, in a reversible fashion, the effect of VEGF.

Conclusion: VEGF-induced hypotension is not species specific. It is prevented by nitric oxide inhibition. VEGF-induced angiogenesis and hypotension are not mediated in vivo by the same VEGF receptor.

Keywords: Angiogenesis; Blood pressure; Vascular endothelial growth factor; Flk-1/KDR; Flt-1; Nitric oxide; Wistar-Kyoto rat; Rabbit

1. Introduction

Ischemic coronary disease is a leading cause of morbidity and mortality in the western world. Direct revascularization techniques, i.e. angioplasty or bypass surgery, aim to restore blood supply to the myocardium. In this context they could be associated with, or replaced in critical situations by, therapeutic agents designed to induce the outgrowth of new collateral vessels. During the past decade the cloning of the genes coding for angiogenic factors such as basic fibroblast growth factor and vascular endothelial growth factor led to obtaining recombinant proteins, which enabled to validate their potential use as therapeutic agents [1–5]. VEGF is 38– to 46-kDa heparin-binding homodimeric glycoprotein, acting as an endothelial cell-mitogen in vivo and vitro [6]. VEGF exerts its effects on endothelial cells through the activation of two cognate tyrosine kinase receptors, flt-1 and flk-1 [7,8]. Diverse strategies have been tested in peripheral vascular diseases. For instance intramuscular injections of basic fibroblast growth factor (bFGF) [1], intravascular injections of vascular endothelial growth factor (VEGF), using either the protein [2–4] and/or a plasmid DNA coding for VEGF [5] result in an increase of blood flow, angiographic score and vascular density. Such an armamentarium has validated the concept of therapeutic angiogenesis and paved the way for VEGF-based therapeutic application [9].

In acute ischemia VEGF plays an important role in the angiogenesis associated with myocardial infarction [10]. In
a model of chronic ischemia, injections of VEGF improved regional blood flow [11–13] making this growth factor of almost immediate clinical relevance. However in the latter experiment 50% of the animals succumbed to severe hypotension. Although VEGF can stimulate angiogenesis and vascular permeability in animal models, it is not yet known whether such biological actions are mediated by different VEGF receptor activations. The present study was designed to determine whether this effect was species specific or could be prevented by inhibitors of nitric oxide and whether angiogenesis and hypotension were mediated by the same VEGF-receptor.

2. Methods

2.1. Materials and in vitro assays

Human recombinant VEGF (165 aminoacid isoform) was expressed in a baculovirus expression system [14]. Anti-VEGF antibodies were raised in rabbits by monthly injections of 10 µg of VEGF165 in lymph nodes as previously described [15]. IgG were purified using protein A sepharose chromatography and further injected in co-isogenic rabbits, 4–7 Months later blood samples were collected and IgG were purified using protein A sepharose chromatography and used for in vivo experiments. The specificity of anti-idiotypic antibodies (AIA) for Flk-1/KDR was assessed by radioreceptor-assay using COS cells expressing either Flt-1 or Flk-1/KDR as already described [16]. One IgG preparation was further purified using anti-VEGF IgG affinity chromatography, enabling the purification of flk-1 specific agonists that represent 2% of the total IgG, and were used for in vitro experiments. Proliferation assays were performed on fetal bovine aortic endothelial cells (FBAE) seeded at 5000 per 12 multiwell plates in DMEM supplemented with 5% newborn calf serum, 2 mM glutamine and antibiotics. Modulators (50 pM; 2 ng/ml VEGF or 8 ng/ml AIA) were inoculated every other day and the cells trypsinized and counted after 4 days in a Coulter counter.

Heparin (140 IU per mg) was from Sigma (L’Isle d’Abeau Chesnes, France) and Nω-monomethyl-L-arginine (L-NMMA) from Nova Biochem (Meudon, France).

2.2. Animal studies

All experiments were conducted in conformity with the guiding principles in the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No 8523, revised 1985).

2.3. Rabbit corneal pocket assay

The angiogenic response to anti-idiotypic antibodies was investigated using the rabbit corneal pocket assay [17]. Male New Zealand rabbits (Elevage du Trotti, Esperce, France) were anesthetized by Ketamin (Ketalar®, intramuscularly). Corneal pockets (2 per cornea) were created 2 mm away from the limbus and implanted with slow-release hydrogel implant containing AIA (30 µg/implant, corresponding to 600 ng or 4 pmol of immunopurified AIA) in one cornea and pre-immune antibodies (30 µg/implant) in the other cornea. In a parallel experiment implants containing VEGF (200 ng or 4 pmol/implant) in one cornea and vehicle alone (50 µg of bovine serum albumin in phosphate buffered saline) in the other cornea were tested in a similar manner. A total of 32 implants were implanted (8 implants per condition studied) in 8 animals.

Pictures of the corneas were taken after 8 days (×2.5, OPMI microscope, Zeiss) and the surface of the neovessels was assessed in a single-blind manner by an independent operator according to a 5 grades scale. (grade 0: no neovessel, grade 1: less than 1 mm long neovessels sprouting from the limbus, grade 2: 1 mm long neovessels, grade 3: 1 to 2 mm long neovessels, grade 4: neovessels extending to the implant).

The results are expressed as the mean and the standard error corresponding to the scores of 8 implants.

2.3.1. Hemodynamic studies

Normotensive male Wistar–Kyoto rats (Mean Arterial Blood Pressure, MABP = 95.3 ± 3.6 mmHg; 200–300 g; 8–12 week of age; IFFA-CREDO, L’Abresle, France) were anaesthetized with sodium thiopental (Inactin; 50 mg/kg intraperitoneally). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket. The carotid artery was cannulated and connected to a TA400 pressure transducer (Gould Electronics, Ballainvilliers, France) for the measurement of MABP. During surgical preparation, a first jugular cannula was used to infuse 100 µl/min of a Ringer–Lactate solution containing 3% w/v gelatin (Plasmon®, Roger Bellon Laboratories) to replace fluid loss. It was thereafter used to infuse 20 µl/min of a mixture of Plasmon® and isotonic dextrose (1–1). A second jugular cannula was used to infuse 10 µl/min of 0.9% NaCl and 75 µg/kg/min of sodium pentobarbital. A third jugular cannula was inserted to allow direct intravenous bolus injection of substances to be studied (total volume of 200 µl). All animals were allowed a 45 minute stabilisation period after completion of the surgical procedure.

MABP expressed in mmHg is reported, unless otherwise stated, as mean and standard error corresponding to four observations.

2.4. Statistical analysis

Rabbit corneal pocket assay: The Mann–Whitney test was used for comparison between different treatments. A p-value of less than 0.05 was considered significant.
Hemodynamic studies: The Wilcoxon test was used for comparisons of data before and after the bolus injections of VEGF or other substances. The Mann–Whitney test was used for comparison between different treatments. A \( p \)-value of less than 0.05 was considered significant.

3. Results

3.1. Anti-idiotypic agonists of Flk-1/KDR induced cell proliferation and angiogenesis

We took advantage of the anti-idiotypic network to obtain VEGF internal images specific for the VEGF binding domains to each VEGF receptor. We found that 15–20% of the rabbits elicited anti-idiotypic antibodies which bound to flk-1 but not to flt-1. IgG purified from one blood sample collected five months after the immunization (AIA) induced a mitogenic response on endothelial cells stronger than that obtained upon VEGF stimulation, whereas pre-immune IgG (PI-Ab) did not (Fig. 1A). When similar amounts —on a molar basis— of VEGF or AIA were inserted in slow releasing implants in the corneal stroma they were equally potent (scores 2.90 and 2.72 respectively) in inducing sprouting of new capillaries from the limbus whereas PI-Ab remained inefficient. Angogenic scores obtained eight days after surgical insertion of the implants are reported in Fig. 1B.

3.2. VEGF induced MABP decrease in a dose and time-dependent fashion

Intravenous bolus injection of VEGF caused a rapid, transient and dose-dependent decrease in Mean Arterial Blood Pressure (MABP). The MABP fell within seconds of the injection, reached its minimum at 3 ± 0.5 min and, in all instances, returned to baseline value within 20 min. The vehicle control (phosphate buffered saline) did not affect blood pressure. The decrease in MABP was dose-dependent (Fig. 2). Injection of 0.5 µg, 3 µg (n = 8) and 10 µg of VEGF resulted in a maximal decrease in MABP of 18.0 ± 4.8 mmHg, 30.5 ± 3.9 mmHg and 33.2 ± 3.7 mmHg respectively. The \( ED_{50} \) for VEGF was found to be 0.5 µg. A saturating uniform dose of 3 µg of VEGF was used for further tests.

The specificity of VEGF was ascertained by the use of VEGF neutralizing antibodies (V-Ab). No change in MABP
was evidenced when 200 μg of V-Ab were incubated with VEGF prior to injection, whereas the addition of 200 μg of PI-Ab failed to prevent the decrease in MABP (Fig. 3). However, none of these antibodies modified MABP when injected alone. Co-administration of heparin (1000 IU) did not influence the pressure response to VEGF.

The VEGF injections were repeated after recovery of the MABP baseline value (Fig. 4). Compared to baseline MABP no significant decrease could be detected when a 20 minutes interval was allowed between injections, whereas a significant but lower decrease was observed when the interval was extended to 40 minutes (16.5 ± 3.6 mmHg, p < 0.05).

3.3. AIA did not induce hypotension

Similar angiogenic scores were obtained in the rabbit corneal pocket assay, with 5 pmol of VEGF or AIA. We thus compared the haemodynamic effects of 60 nmol of VEGF to similar amounts of AIA.

AIA could not induce a decrease of MABP (n = 8, not shown). Furthermore when injected 10 minutes prior to the injection of 3 μg of VEGF they could not prevent a VEGF-induced MABP decrease similar to that observed in untreated animals (Fig. 4).

3.4. An inhibitor of NO synthesis prevented the VEGF induced MABP decrease

To see whether NO was involved in VEGF-induced hypotension, we investigated the effects of L-NMMA, an inhibitor of NO synthesis [18]. L-NMMA was infused (25 μg/kg/min) for 30 minutes before VEGF injection (Fig. 5). As expected an increase in MABP was observed during L-NMMA infusion; the subsequent injection of VEGF failed to change the MABP significantly (−3.5 ± 2.7 mm Hg, n.s., n = 3). VEGF injections were renewed 70 minutes after the end of L-NMMA infusion and induced a significant decrease in MABP (−29.2 ± 5.3 mm Hg, p < 0.05, n = 3).
4. Discussion

Our results indicate that VEGF activities on angiogenesis and arterial hypotension are mediated by distinct receptors.

Therapeutic angiogenesis has been evaluated as a means of restoring blood supply to the myocardium by providing new vessels for blood flow. VEGF is angiogenic in the chicken chorioallantoïd membrane [6] and in the rabbit corneal pocket assays. It has been shown to increase the blood flow in coronaries when injected for four weeks in the vicinity of a gradual coronary occlusion [11]. Furthermore the angiogenic effect of intracoronary infusions of VEGF on collateral arteries to ischemic myocardium was confirmed in a canine model [12]. In a porcine model intra-coronary injections of VEGF was shown to improve blood flow, but to produce a significant reduction (−40%) in mean arterial pressure. 50% Of the VEGF-treated animals succumbed to hypotension following VEGF injection [13]. However, a recent report demonstrates that short-term treatment with VEGF, in contrast to FGF2 [19,20], failed to improve the collateral blood flow in a canine model.

With the aim of stimulating angiogenesis without inducing hypotension it would be important to know whether they are mediated by the same VEGF-receptor. We used a specific flk-1/KDR agonist prepared following the anti-idiotypic antibody strategy and demonstrated that it elicited neovessel sprouting in the rabbit corneal pocket assay and proliferation of bovine endothelial cell proliferation in vitro. AIA induced a stronger mitogenic response than VEGF, confirming our previous results on bovine brain capillary endothelial cells [16] and human stromal cells cultured from neonatal hemangiomas [21]. These results indicate that Flk-1/KDR homodimerization is sufficient to promote both phenomena. The observation that intravenous injections of AIA, in contrast to VEGF, failed to induce MABP decrease does not account for species-restriction since we previously demonstrated that it promoted also the proliferation of human cells [21] and tumoral angiogenesis in mouse [16]. Co-administration of heparin (1000 IU) did not influence the pressure response to VEGF, in agreement with the absence of influence in angiogenesis of adding heparin to VEGF injections in a rabbit ischemic hindlimb model [2]. Yang et al. [22] reported that VEGF (1−250 µg/kg) decreased MABP in rats and Hariawala et al. [13] reported that a dose of 500 µg induced a #40% decrease in mean arterial pressure in pigs of 25−30 kg of weight (17−20 µg/kg) [12]. In our study such a decrease was achieved with 3−10 µg in rats of 300−400 g (10−30 µg/kg), a dose lower than that required in the models designed to detect endothelial cell proliferation, namely the balloon injury model (#50−250 µg/kg, Ref. [23]) and the femoral artery ligation model (250 µg/kg, 300−3000 µg/kg [24]). The hypotensive activity of VEGF appears to be similar among species and thus could be expected in man.

The observation that iterative injections of VEGF required a 40 minute delay to provide a significant effect implies that there is a haemodynamic adaptation to VEGF-induced hypotension that persists 40 minutes. On the other hand the VEGF receptors that mediate the blood pressure effect might be internalized after occupancy. This is supported by in vitro experiments from our laboratory where preincubation of VEGF with bovine aortic endothelial cells abolishes the availability of 90% of VEGF binding sites within 30 minutes. Removal of it enables a full reexpression of the receptors at the cell surface within 6 hours (unpublished results). To investigate the hypothesis that heterodimerization of Flt-1 and Flk-1/KDR by VEGF was required for the MABP decrease we sought to occupy Flk-1/KDR receptors by injecting similar doses of AIA and VEGF (calculated from dose-response curves of their mitogenic effects on endothelial cells). AIA had no effect and subsequent injection of VEGF induced a decrease in MABP similar to that observed in ‘naïve’ animals, thus indicating that heterodimerization of flt-1 and Flk-1/KDR receptors is not responsible for the VEGF-induced hypotension.

The vasodilatory activity of VEGF was not entirely unexpected. Indeed, in an isolated canine coronary artery Ku et al. [25] demonstrated a slow, dose dependent relaxation, also observed by Lazarous et al. [19]. Such a vasodilatory effect could be prevented by mechanical disruption of the endothelium or by pretreatment with L-NMMA. In an attempt to implicate EDRF/NO in VEGF-induced hypotension Hariawala et al. injected L-NNA, a NO-synthase inhibitor, 6 minutes after injection of VEGF when the mean arterial pressure stabilized at its lowest level [13]. This resulted in a rapid restoration of mean arterial pressure. A definitive conclusion as to the implication of EDRF/NO could not be drawn from this experiment since we have shown that the decrease in MABP is spontaneously reversible and reaches its minimum within 3−4 minutes, a time interval comparable to the one reported by Hariawala et al. before the injection of L-NNA and the reversion of hypotension. To avoid such an artefact we pretreated the animals with L-NMMA, a competitive and labile inhibitor of NO-synthase [18] before the injection of VEGF. Such a treatment inhibited the hypotensive activity of VEGF. Furthermore 2 hours later, when L-NMMA was no longer efficient, a second injection of VEGF induced a significant decrease in MABP. Clearly our demonstration of reversible inhibition by L-NMMA of the VEGF-induced decrease in MABP confirms in vivo the EDRF/NO mediation highlighted by others [22,25].

Taken together, our results show that the activation of Flk-1/KDR is sufficient to induce angiogenesis but is not involved in the potentially fatal VEGF-induced hypotension. This demonstration might have implications in coronary therapeutic angiogenesis and attention should be devoted to means of selectively activating Flk-1/KDR. Fortunately such selective ligands were recently obtained by
eliciting antiidiotypic antibodies [16,21] or by site-directed mutagenesis [26].

Acknowledgements

We thank N. Ortéga for purification and characterization of AIA, and Dr. J.F. Amnal for fruitful discussion. This work was supported by fellowships from the Fondation pour la Recherche Médicale (BM and FJ) and by grants from the Association pour la Recherche sur le Cancer and the Ligue Française contre le Cancer.

References


