Differential expression of VEGF receptors in adrenal atrophy induced by dexamethasone: a protective role of ACTH

CHRISTINE MALLET,1 OLIVIER FÉRAUD,2 GAELI OUENGUE-MBÉLÉ,3 ISABELLE GAILLARD,1 NICOLAS SAPPAY,1 DANIEL VITTET,2 AND ISABELLE VILGRAIN2

1Equipe Mixte Institut National de la Santé et de la Recherche Médicale (EMI) 01-05 Angio, 2EMI 02-19 Laboratoire de Développement et Vieillissement de l’Endothélium, and 3EMI 01-04 Transduction du Signal, Commissariat à l’Energie Atomique, 38054 Grenoble, France

Submitted 1 October 2001; accepted in final form 5 August 2002

Mallet, Christine, Olivier Féraud, Gaeli Ouengue-Mbélé, Isabelle Gaillard, Nicolas Sappay, Daniel Vittet, and Isabelle Vilgrain. Differential expression of VEGF receptors in adrenal atrophy induced by dexamethasone: a protective role of ACTH. Am J Physiol Endocrinol Metab 284: E156–E167, 2003; 10.1152/ajpendo.00450.2001.—Although ACTH is important to adrenal growth and steroidogenesis, its role in vascular development and function has not been established in vivo. In the present study, we demonstrate the expression of mRNA for all four VEGF isoforms (mVEGF120, 144, 164, 188) and for Flk-1/KDR and Flt-1 receptors in the mouse adrenal in vivo. Suppression of the pituitary-adrenocortical axis by dexamethasone (0.5 mg·kg−1·day−1 during 6 days) induced a decrease in corticosterone levels, adrenal weights by 50% (P < 0.001), VEGF188 mRNA, and Flk-1/KDR mRNA, whereas Flt-1 remained consistent during steroid treatment. A daily injection of ACTH-(1–39) restored the transcript for Flk-1/KDR and both VEGF188 and plasma corticosterone to control levels. To gain further insights into the effects of ACTH, cultured endothelial cells (ECs) were treated with forskolin, which increases cAMP, the second messenger in ACTH action. We demonstrated that Flk-1/KDR protein expression was markedly increased by forskolin within 24–48 h of treatment in a dose-dependent manner (0.1–10 μM). The biological effect of ACTH on ECs was then tested by use of coincubations of ACTH on ECs. Within 5–7 days of culture, ECs organized into multicellular structures that resemble networks of microvasculature, which characterize angiogenesis in vitro.

vascular endothelial growth factor receptors; adrenocorticotropin; angiogenesis; mouse adrenal; steroidogenesis; hypothalamic-pituitary disconnection; vascular permeability

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

THE ADRENAL CORTEX is physiologically important as the site of formation and secretion of several steroid hormones and less-active steroids. The secreted hormones gain access to the bloodstream through an intense and complex adrenal vasculature that is essential for organ growth and maintenance. The vessels of the adrenal cortex are characterized by richly fenestrated endothelia (2, 40). These fenestrations are the pores through which releasing factors within the hemal milieu can access the cells. In adult mammals, adrenal growth and steroidogenesis are regulated by adrenocorticotropic (ACTH) (27), which acts through interaction with a specific melanocortin receptor (MC2R) specifically expressed in the adrenal cortex (6, 32). This guanine nucleotide-binding protein-coupled receptor elicits the activation of adenylate cyclase and the production of cAMP, which activate the protein kinase A-signaling pathway (39, 53) and the normal functioning of adrenocortical cells. Glucocorticoid hormones are thought to exert negative feedback inhibition on hypotalamic-pituitary-adrenocortical axis activity at the pituitary, hypotalamic, and extrahypothalamic levels. This ultimately results in decreased synthesis and secretion of both corticotropin-releasing hormone (CRH) and other corticotropic secretagogues and in an inhibition of the production and secretion of ACTH (8, 24). A rapid atrophy of the adrenal cortex and the reduction of its steroid machinery ensue. Morphological studies of the adrenal gland after steroid treatment have shown a decrease in the average volume of the fasciculata cell and in the number of adrenocortical cells in the entire cortex. Furthermore, a prompt and potent inhibition of proliferative activity of adrenocortical cells was described as assessed by the counting of metaphases per adrenal section (36). Despite numerous in vivo studies showing that the adrenal cortex homeostasis is regulated by ACTH, a possible role of ACTH in influencing the physiological activity of endothelial cells has not been investigated until now.

Members of the vascular endothelial growth factor (VEGF) family of proteins have been characterized as critical regulators of the endothelial system (for review, see Refs. 14, 15, 35, and 47). VEGF is a potent inducer of endothelial fenestration (14) and a major...
mediator of normal and abnormal angiogenesis (13, 14). At least five different VEGF isoforms are known in mammals; they derive by alternative splicing of a single gene and differ in their secretion and heparin-binding properties (15). These different isoforms, which respectively have 121, 145, 165, 189, and 206 amino acid residues in human and bovine species, are glycosylated and expressed as dimeric protein (15). With the exception of VEGF121, they are able to bind heparin, and all VEGF isoforms have been reported to be mitogenic toward vascular endothelial cells and to increase blood vessel permeability. The various VEGF forms bind to two high-affinity tyrosine kinase receptors, the kinase insert domain-containing receptor (Flk-1/KDR) and the Fms-like tyrosine kinase (Flt-1), which are expressed in vivo almost exclusively on endothelial cells and their precursor cells (35). Although these two VEGF receptors are structurally highly homologous, their biochemical features are quite distinct, and they can mediate different biological responses. Activation of Flk-1 induces a mitogenic response, whereas the activation of Flt-1 does not induce endothelial cell proliferation either in vitro or in vivo (38).

Targeted gene inactivation studies have revealed that members of the VEGF family of proteins and the VEGF receptors are major regulators of the development of the vasculature (16, 43, 52). In the adult, regulation of VEGF and its receptors has been observed in some endocrine tissues, such as ovary, thyroid, and prostate, in various conditions pointing to a hormonal control of the vasculature (3, 25, 28, 45, 49). Immunohistochemical experiments have reported that the steroidogenic cells in adult bovine adrenal gland express VEGF (17). In addition, VEGF mRNA can be detected in human fetal (44) and in adult bovine (17) adrenocortical cells in vitro culture. Furthermore, the expression of VEGF receptors (VEGFRs)-1 and -2 at the mRNA level has also been detected in adrenocortical capillary endothelial cell in culture (51) but not in steroidogenic cells (data not shown).

On the basis of these data and because ACTH regulates adrenal cortex homeostasis in vivo, we hypothesized that ACTH could influence adrenal vascularization in a coordinated manner by regulating the VEGF/VEGF receptor system. In the present study, we examined the expression of VEGF and its receptors in adult mouse adrenal in vivo, and we investigated the involvement of the pituitary gland in their regulation by using both an animal model of adrenal regression-regeneration and in vitro-cultured adrenocortical capillary endothelial cells.

MATERIALS AND METHODS

Reagents. ACTH-(1–39), benzamidine, dexamethasone, leupeptin, pepstatin A, horseradish peroxidase-conjugated affinity-purified goat anti-rat IgG, rabbit anti-goat, Triton X-100, and Tween 20 were purchased from Sigma (Rueil Malmaison, France). Goat polyclonal antibody against mouse Flt-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dr. S.-I. Nishikawa (Kyoto University, Kyoto, Japan) kindly provided rat monoclonal antibody directed against mouse Flk-1/KDR. Rat monoclonal platelet endothelial cell adhesion molecule (PECAM) antibody was from Pharmingen (BD Pharmingen, Le Pont de Claix, France). Rabbit polyclonal von Willebrand antibody was from Dako (Trappes, France). The metal-enhanced dianobenzidine substrate kit was from Pierce Chemical (Rockford, IL). Biotinylated donkey anti-rat IgG antibodies and Hybrid N+ substrate kit was from Amersham (Les Ulis, France). The RNAgents Total RNA Isolation System was from Promega (Madison, WI). The enhanced chemiluminescence detection reagents were purchased from DuPont NEN (Les Ulis, France). Nitrocellulose was obtained from Schleicher and Schuell (Écouvilly, France). A BCA protein assay reagent kit was from Pierce (Oud Beijerland, the Netherlands). Transformed stable endothelial H5V cell lines from embryonic heart were obtained as previously described (18).

Animals. The study, which was conducted in accordance with both institutional guidelines and those formulated by the European Community for the use of experimental animals, was performed with 2-mo-old Swiss female NMRI mice weighing 20–30 g (Charles River laboratories, Les Oncins, France). For chronic experiments (see protocol to follow), animals were randomly divided into three groups, each group receiving one of the following treatments: 1) a control (CTL) group, intraperitoneally receiving sterile water (equivalent volume 100 μl), 2) a dexamethasone (DEX) group receiving daily dexamethasone (0.5 mg/100 g body wt ip) for 6 days, 3) a DEX-ACTH group, receiving first daily dexamethasone (0.5 mg/100 g body wt ip) for 6 days, and for the next 6 days, a daily intraperitoneal injection of ACTH-(1–39) consisting of 30 IU/kg. At the end of the treatment, mice were anesthetized by intraperitoneal injection of tribromoethanol (2 mg/g body wt), and blood was collected for measurements of corticosterone concentration. Adrenal glands were immediately removed to be weighed, and then tissues were processed for RNA and protein extraction.

Histology and immunochemistry. Fresh adrenal glands were embedded in OCT compound (Miles Scientific, Elkhart, IN) after fixation in 4% paraformaldehyde for 1 h at 4°C and cryoprotection in 20% (wt/vol) sucrose. Frozen sections (10 μm thick) were mounted onto gelatin-coated glass slides, air-dried overnight, and processed for indirect immunofluorescence microscopy as previously described (17).

RNA extraction and RT-PCR. Total RNAs from adrenals were isolated using the RNAgents Total RNA Isolation System (30). Semiquantitative RT-PCR was performed as previously described (51). The amplification parameters were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min during 25 cycles for hypoxanthine phosphoribosyltransferase (hprt), followed by 5 min at 72°C for final extension. For the other transcripts, hybridization temperature and number of cycles were, respectively, 59°C and 30 cycles for VEGF, 55°C and 30 cycles for Flk-1/KDR, 60°C and 32 cycles for Flt-1, and 57°C and 27 cycles for MC2R. To ensure semiquantitative results, the number of PCR cycles for each set of primers was selected to be in the linear range of amplification. In addition, all cDNA samples were adjusted to yield equivalent amplification of hprt as a reference standard. Reaction products were gel electrophoresed, transferred onto nylon membrane, and hybridized with their respective 32P-labeled 5′-end-labeled internal oligonucleotides probe, according to standard protocols (41). Hybridized filters were visualized and signals quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The primers and probes used are listed in Table 1.

Cell culture. Adrenocortical endothelial (ACE) cells were generously provided by Dr. Jean Plouet (IPBS, Toulouse, France) and were previously characterized (20). Cultures of

---

AdP-Endocrinol Metab • VOL 284 • JANUARY 2003 • www.ajpendo.org
ACE cells were routinely used before passage 15. The endothelial phenotype of the cells was checked by the analysis of VE-cadherin expression (a specific marker of endothelial cells expressed at interendothelial cell junctions). Adrenocortical steroidogenic cells from the fasciculata-reticularis zone were prepared according to the method detailed in Ref. 7. Co-culture in collagen followed the protocol described in Ref. 31. Briefly, adrenocortical cells from the zona fasciculata-reticularis (250,000/well) were suspended in a collagen gel containing ACTH (10 nM) in DMEM 10× and incubated at 37°C. After gel polymerization, another layer of collagen gel was poured, and the gel was incubated again at 37°C for 10 min. ACE cells were then seeded onto the gel, which was flooded with DMEM supplemented with antibiotics and 10% fetal calf serum. The gels were kept under observation for 7–14 days.

**SDS-PAGE and Western blotting.** Proteins were solubilized in 10 mM Tris- HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, 0.5% SDS, and 0.05% (vol/vol) NP-40 and were analyzed by SDS-PAGE (12% acrylamide, 0.1% bis-acrylamide) as described in Ref. 7.

**Hormone determination.** Blood was collected at the end of the treatment in EDTA-treated plastic tubes. Plasma corticosterone was measured by radioimmunoassay on three plasma samples from each mouse of a five-animal group, as described previously (12).

**Data analysis.** Each data point represents the mean ± SD of the measures of three different determinations in the same experiment. Each experiment was reproduced at least three times in identical or similar configuration with similar results.

**RESULTS**

Adrenal cortex capillary vessel-lined columns of steroidogenic cells. A first series of experiments was designed to visualize the distribution of the endothelial capillary network of the adrenal cortex. Immunohistochemical analysis of von Willebrand, PECAM (CD 31), and cytochrome P-450 of side chain cleavage enzyme (P450sc; CYP 11A1) staining in mouse adrenal sections was then performed on paraformaldehyde-fixed sections of whole adrenal. PECAM and von Willebrand factor (vWF) were used as markers of endothelial cells (22). The P450sc, which is a subcomponent of the enzyme complex that catalyzes the first rate-limiting step of steroid synthesis, the conversion of cholesterol to pregnenolone, was used as a marker of steroidogenic cells (37). At the light-microscopic level, the positive immunoreaction for P450sc was found, as expected, in the cells of the zonae glomerulosa, fasciculata, and reticularis (Fig. 1A). Immunostaining experiments were performed either on paraffin sections (vWF staining) or on cryostat sections (PECAM staining). The illustrations provided in Fig. 1 refer to different sections of mouse adrenal gland. As shown in Fig. 1B, the brown positive immunostaining for vWF is detected in loops surrounding steroidogenic cells in both the glomerulosa and the fasciculata zone of the adrenal cortex.

Immunoreactivity of the PECAM molecule showed that endothelial cells appeared as long cells lining cords of steroidogenic cells (Fig. 1C). The staining patterns for vWF and PECAM were not superimposable in Fig. 1, B and C, because slightly different sectioning planes of the sample were used. This immunohistochemistry result shows a highly vascularized tissue (Fig. 1, B and C), where endothelial cells are in close contact with steroidogenic cells and delimit columns of adrenocortical steroidogenic cells for possible paracrine interactions.

**VEGF and its two high-affinity receptors, Flk-1/KDR and Flt-1, are expressed in mouse adrenal in vivo.** To determine the role of VEGF in mouse adrenal, we analyzed VEGF and expression of its receptors, Flk-1/KDR and Flt-1, at the mRNA level by RT-PCR analysis. Oligonucleotide primers were designed to amplify the alternatively spliced VEGF transcript. cDNA templates synthesized from RNA preparations of mouse adrenal were amplified. In the negative control, RNA was substituted by water (no template). As shown in Fig. 2A, the four mRNA spliced variant isoforms VEGF120, VEGF144, VEGF164, and VEGF188 were amplified as the expected 332, 404, 462, 532-bp products (see Table 1). The major isoforms represented were VEGF120 and VEGF164. The expression of VEGF at the protein level was examined by Western blot analysis performed on 80 μg of all protein extracts from mouse adrenal with an antibody directed against VEGF164. Figure 2B showed that one major species of 23 kDa corresponding to VEGF164 was detected in protein extracts from all adrenals.

The expression of mRNA for the VEGF receptors Flk-1/KDR and Flt-1 was investigated within the intact gland by use of mouse VEGF receptor-specific primers (see Table 1). As a positive control, we included

### Table 1. Sequences of oligonucleotide primers and probes used for RT-PCR analyses

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sense 5’ to 3’</th>
<th>Antisense 5’ to 3’</th>
<th>Probe</th>
<th>Size, bp</th>
<th>EMBL No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mc2</strong></td>
<td>5’7GCTGCGCTATTTACGACG89</td>
<td>869GTTGTGTTGAGGAAGGAG850</td>
<td>893TGGATGTTGAGGACAGGAG11</td>
<td>299</td>
<td>S78985</td>
</tr>
<tr>
<td><strong>flk-1</strong></td>
<td>301GCTGCGCTATTTACGACG120</td>
<td>5’7GATGTTGTTGAGGAAGGAG850</td>
<td>869TGGATGTTGAGGACAGGAG11</td>
<td>269</td>
<td>S53103</td>
</tr>
<tr>
<td><strong>flt</strong></td>
<td>274CTCTGCTTTGCTGTTGCG277</td>
<td>3051GATGTTGTTGAGGAAGGAG850</td>
<td>893TGGATGTTGAGGACAGGAG11</td>
<td>316</td>
<td>L07297</td>
</tr>
<tr>
<td><strong>vegf</strong></td>
<td>197CCAATGTTGAGGAAGGAG220</td>
<td>659TCAGCGCTTTGCTGTTG676</td>
<td>227TACGAGGAAGCTACTG238</td>
<td>462</td>
<td>C78970</td>
</tr>
<tr>
<td><strong>hprt</strong></td>
<td>5’7GCTGCGCTATTTACGACG594</td>
<td>824CAGGACTGAAGACACCTG805</td>
<td>699TGGATGTTGAGGACAGGAG11</td>
<td>404</td>
<td>C78970</td>
</tr>
</tbody>
</table>

Sequence locations are given in superscript at both ends of each nucleotide. EMBL no., European Molecular Biology Laboratory GenBank accession no. *Size of amplified cDNA products in base pairs.*
RNA preparation from a hemangioma cell line (H5V), which constitutively expresses VEGF receptor genes (50) (see Fig. 2A, left). Agarose gel analysis by ethidium bromide staining of PCR products showed a single band migrating at a position corresponding to 269 bp for Flk-1/KDR (Fig. 2C, middle) and 316 bp for Flt-1 (Fig. 2D, middle). Western blot analysis was then performed to examine the expression of Flk-1/KDR and Flt-1 at the protein level. With use of the anti-Flk-1/KDR antibody, one species of 220 kDa was detected that corresponds to the Flk-1/KDR protein (Fig. 2C, right). Figure 2B, right, illustrates the Western blot analyses with the anti Flt-1 antibody that detected a 180-kDa species corresponding to the VEGF receptor 1 protein. All together, these results demonstrate a simultaneous expression of Flk-1/KDR and Flt-1 receptors in adult mouse adrenal in vivo that suggests a potential regulation of the adrenal endothelium.

Steroid treatment-induced adrenal atrophy, which can be restored by ACTH. To determine the effect of ACTH on the VEGF/VEGF receptor system expression in vivo during the vascular remodeling of the adrenal, we developed an animal model in which adrenal atrophy was induced by daily injection of dexamethasone for 6 days, and adrenal regeneration was induced by ACTH injections for 7 days after dexamethasone treatment. Three groups of five animals were treated with either vehicle or dexamethasone (0.5 mg/kg) for 6 days or with dexamethasone followed by injection of ACTH-(1–39) (30 IU/kg) for 7 days. We checked hormonal treatment by measuring the adrenal weight (n = 30) and the mouse corticosterone concentration at the end of the treatment (n = 15). As illustrated in Fig. 3A, the mice adrenal weights were reduced by ~50%, from 10.1 ± 0.5 mg for the control group (CTL) to 5.4 ± 0.2 mg after 6 days of dexamethasone treatment (DEX) (P < 0.001). In parallel, the decrease in adrenal weight was associated with a significant decrease in the plasma corticosterone concentration from 435 ± 35 ng/ml at day 1 (n = 10) to 113 ± 15 ng/ml at day 6 (n = 5; P < 0.001; Fig. 3B). This indicates that the atrophic adrenocortical cells were not sufficient to raise the blood level of corticosteroid hormones. This result is in agreement with an inhibitory effect of dexamethasone on the adrenal steroidogenic function in mice. Associated with this decline, the 299-bp products for the ACTH receptor, expressed as a ratio of hprt, were ~60% lower (P < 0.05) in dexamethasone-treated mice (Fig. 3, C–D). A daily administration of ACTH-(1–39) during 7 days fully restored plasma corticosterone level to control levels and partially restored adrenal weight from 5.4 ± 0.62 mg (n = 10) (DEX) to 7.5 ± 0.5 mg (n = 10), whereas the expression of the ACTH receptor was ~65% of the control value (P < 0.05). These results are consistent with in vitro observations demonstrating that ACTH receptor mRNA levels were stimulated by ACTH in cultures of human and bovine adrenocortical cells (32, 48). All together, these data show that the
glucocorticoid treatment of mice inhibits adrenal growth and steroid production, which gives an interesting model of hormonal regulation of the adrenal, because ACTH administration completely restored corticosterone production and stimulated adrenal growth.

Expression of VEGF and its receptors during regression-regeneration of the adrenal gland. To evaluate potential hormone effects on VEGF splice variant expression, we performed RT-PCR on RNA isolated from the CTL, DEX, and DEX-ACTH animals. As shown in Fig. 4, a strong expression of VEGF isoforms was detected in the three groups of mice, and the VEGF188 mRNA was decreased in dexamethasone-treated mice compared with untreated mice and was further restored after 6 days of ACTH injections. Expression of the other VEGF isoforms was maintained during dexamethasone treatment, and the abundance of VEGF120 and VEGF164 remained consistent across the evolution of adrenal in accordance with RT-PCR experiments. Protein analysis was performed by Western blotting by use of an antibody that recognized the isoform 165 as a 23-kDa protein. No commercial antibody was available to detect specifically the 188 isoform. Thus, in our hands, we found a 23-kDa protein present in the same amount in the adrenal extracts of the three groups of mice.

RT-PCR analysis of VEGF receptors showed a single band migrating at a position corresponding to 269 bp for Flk-1/KDR (Fig. 5A) and to 316 bp for Flt-1 (Fig. 5D) for the three groups of mice. The abundance of Flk-1 declined from 6.1 ± 0.8 (arbitrary densitometric units) in the CTL group to 2.75 ± 0.45 (P < 0.01) in the DEX group (Fig. 5A, middle). During the 7-day administration of ACTH after steroid treatment, the expression of Flk-1/KDR returned to the control value (6.5 ± 0.7). These results were confirmed by analysis at the protein level. As shown in Fig. 5C, the 220-kDa form corresponding to Flk-1/KDR protein was highly expressed in the CTL group and became barely detectable after dexamethasone treatment. When adrenal regeneration was induced by ACTH, Flk-1/KDR protein returned to the CTL level (Fig. 5C). By contrast, the Flt-1 expression at the mRNA level was not significantly modified in the three groups of mice as analyzed by agarose gel ethidium bromide staining of the PCR product for Flt-1 (Fig. 5, D-E). At the protein level, the 180-kDa species for Flt-1 remained consistent during steroid and hormone treatment (Fig. 5F). Thus, from these experiments, we conclude that, among the VEGF receptors expressed in mouse adrenal, Flk-1/KDR is under the control of ACTH, and Flt-1 remained at a steady-state.
level. This may reflect a positive regulation of Flk-1/KDR in endothelial cells indirectly exerted by ACTH.

**cAMP stimulates Flk-1/KDR expression in vitro in cultured ACE cells.** A paracrine communication involving growth of blood vessels has been shown to play an important role during follicle maturation in the ovary and in the endometrium (29). Accordingly, our in vivo data imply that, during adrenal atrophy, a paracrine mechanism may be responsible for ACTH-dependent control of endothelial cells. Most effects of ACTH are mediated by a Gs protein-coupled receptor that stimulates cAMP production. Thus, to get further insights into the mechanism of Flk-1 upregulation by ACTH, we examined whether cAMP could reproduce the effects of ACTH on endothelial cells in culture. To that purpose, ACE cells in culture were treated with forskolin, a direct activator of adenylate cyclase, and Flk-1/KDR protein expression was determined by Western blotting analysis. Figure 6A shows Flk-1/KDR expression from cells treated from 0 to 48 h with forskolin (10 μM).

Flk-1/KDR was barely detectable in endothelial cells after 3–5 h of forskolin treatment. A trace amount appeared at 24 h, and the 220-kDa protein was readily detectable after 48-h stimulation with forskolin. We performed a dose-response experiment with increasing concentrations of forskolin ranging from 0.1 to 50 μM for 48 h. The increase in Flk-1/KDR expression was detectable from the concentration of 0.1 μM (Fig. 6B) and increased up to 10 μM. These results show that treatment of ACE cells with forskolin could mimic the effect of ACTH; similarly, Flk-1/KDR expression increased in response to treatment with forskolin. In contrast, the expression of Flt-1 was not affected by a forskolin treatment of endothelial cells in culture (data not shown).

**Paracrine communication between steroidogenic cells and adrenocortical capillary endothelial cells.** The growth of new vascular sprouts from existent vasculature is referred to as angiogenesis. The morphogenetic and regulatory processes that mediate and control an-
angiogenesis are difficult to visualize in vivo. Therefore, endothelial cells grown in vitro under conditions that promote their organization into multicellular structures resembling networks of microvasculature develop an adequate model, in terms of morphology, of angiogenesis in vivo (48). To determine whether steroidogenic cells could regulate endothelial cells in the adrenal cortex, we examined whether ACE cells exhibited changes in cellular morphology and orientation, which characterizes angiogenesis in vitro when co-cultured with steroidogenic cells in 3D-collagen. ACE cells maintained on collagen in the presence of 10% fetal bovine serum proliferated to form confluent monolayers, with minimal invasion occurring into the underlying matrix (Fig. 7A). When adrenocortical cells treated with 10 nM ACTH were introduced into the deepest monolayer of the collagen (Fig. 7B), short, cordlike arrays of refractile cells appeared among the flattened cells of the monolayer ~6 days after cultures (Fig. 7C). Within 6 to 10 days, the cellular cords had lengthened considerably and had established connections with one another to form tesselated networks (Fig. 7D). Daily examination of ACE cells alone remained as a confluent monolayer. The effect of ACTH was tested in the co-culture system when ACE cells were grown alone. No formation of capillary-like tubes was observed, which is in agreement with the absence of ACTH receptors in these cells. An effect of ACTH was observed only when the two cell types were co-cultured, separated with one layer of collagen. Because fasciculata cells express ACTH receptors, our results strongly suggest that the observed effect is mediated by ACTH through fasciculata cells.

### DISCUSSION

The establishment and maintenance of the vascular network in the adrenal are essential for organ growth and functioning and involve a complex interaction among multiple cell types. Despite the integral function of the vasculature within the adrenal gland, little has been reported concerning the regulation of capillary survival and permeability in this tissue. It is well established that ACTH has an important role in the regulation of adrenal cortex growth and that VEGF and its receptors exert a major control of the endothelial compartment. ACTH regulation of the VEGF/VEGF receptor system may therefore be relevant when the cortical tissue mass is enhanced in response to functional demand or restored during regeneration. However, in contrast to the ovary, where an active cycle of angiogenesis occurs during the follicular development, neovascularization in the adrenal remains rarely observed. However, ACTH regulation of capillary fenestration in vivo has been demonstrated in the rat adrenal cortex, suggesting a potential role for VEGF in ACTH action (40).

In the present report, we investigated the in vivo status and hormonal regulation of the VEGF/VEGF receptor system in the adrenal gland. To that purpose, we established a protocol to induce adrenal atrophy by glucocorticoid injections, an effect that is reversed by ACTH. It gives an interesting model of an ACTH-dependent control of adrenal growth. Indeed, we show that this treatment decreased adrenal weight and inhibited steroidogenic functions of the adrenal. Our results suggest that ACTH is the major regulator of these effects, since ACTH injections within 7 days restored plasma corticosterone level to control values. These results are in agreement with studies in the hypophysectomized fetal sheep, which showed that adrenal growth could be restored by in utero ACTH replacement therapy (6, 8, 24).

Interestingly, we demonstrate the presence of four mRNA transcripts encoding different VEGF isoforms: mVEGF120, 144, 164, 188, of which the 120- and the 164-amino acid-long isoforms appear to be predominant, which points to a functional role of VEGF in this adult tissue. These data are consistent with the literature, in which VEGF165 and VEGF121 are reported to be the major isoforms secreted by many normal and transformed cells (35). However, the respective physiological functions of these different isoforms remain to be established in the adrenal as well as in other tissues. So far, some differences have been described concerning their mitogenic potential, chemotactic properties, and receptor-binding characteristics (35). We found that dexamethasone and ACTH had a slight effect on VEGF188 splice variant expression in vivo, whereas the other isoforms remained unchanged. Similar findings were also noted in mammary gland development during pregnancy and lactation (41). In rat uterus, upregulation of VEGF188 was also observed after 6-h exposure to estradiol, whereas an earlier upregulatory effect at 2 h was observed for VEGF120 and VEGF164 (9). In
In contrast, in human endometrial stromal cells, estradiol increased VEGF<sub>121</sub> and VEGF<sub>165</sub> expression, whereas the level of VEGF<sub>189</sub> mRNA was unaffected (54).

The described effects of dexamethasone on VEGF expression in mouse are contradictory. As an example, in lung no variations in VEGF expression were found (4), whereas several in vitro studies showed that dexamethasone strongly downregulates and/or inhibits induction of VEGF (34, 46). However, in rat glioma cells, it has been shown that dexamethasone induced a differential downregulation of VEGF under normoxic and hypoxic conditions (29). In adrenal in vivo, the effect of dexamethasone was the result of a decreased level of endogenous ACTH, since animals receiving simultaneous administration of ACTH and dexamethasone did not present adrenal atrophy (data not shown).

Interestingly, we have previously shown in primary bovine cell cultures that ACTH induced a rapid increase in VEGF<sub>121</sub> and VEGF<sub>165</sub> splice variant expression. The effect was transient within 2 h of agonist stimulation (17). Similar results were also described in primary cultures of human fetal adrenocortical cells stimulated with ACTH or forskolin (28). Although it is not detected in our in vivo studies, we cannot totally exclude a rapid and transient increase in VEGF expression in response to ACTH in vivo, as described in vitro. Thus, in the adult adrenal, the high expression of the different isoforms of VEGF in vivo could reflect some of the nonangiogenic functions of VEGF, such as the regulation of vascular permeability or the mediation of endothelial cell survival. It is likely that VEGF detected in adrenal cortex (17) acts as a specific mito-

Fig. 5. Effects of dexamethasone and ACTH on VEGF receptor expression. A and D: RT-PCR analysis. Total RNA from whole adrenal of the 3 groups of treated mice (CTL, untreated; DEX, dexamethasone; DEX-ACTH, dexamethasone followed by ACTH) was reverse-transcribed and amplified using exponential nonsaturating conditions with specific oligonucleotides for Flk-1/KDR (A) and Flt-1 (D). Arrowheads point to bands corresponding to VEGF receptors. B and E: relative Flk-1/KDR and Flt-1 mRNA values were quantitated by phosphorimager scanning and normalized to the hprt signals. Data points represent means ± SD of triplicate determinations in a single experiment. VEGF receptor mRNAs are significantly different from control values (**, ***, <0.01). DEX-ACTH values are significantly different from dexamethasone values (P < 0.01) but are not significantly different from control values. C and F: Western blot analysis. An equal amount of protein from adrenal lysates was analyzed by SDS-PAGE and Western blotting with the anti-Flk-1/KDR (C) and the anti-Flt-1 antibody (F). Arrows show the mature form of Flk-1/KDR (220 kDa) and the 180-kDa species corresponding to Flt-1. Molecular mass standards (in kDa) are shown at left. Data points represent means ± SD of triplicate determinations in a single experiment. Values of 3 groups are not significantly different from each other and from control values. This experiment is representative of 2 additional experiments.
gen and chemotactic factor that causes the endothelial cells, which bear the Flk-1/KDR receptor, to proliferate and migrate, leading to capillary alignment along the fasciculata lining. During the time of our studies described in this paper, a new factor specialized for endothelial cells from endocrine glands was described (26). This new growth factor displays biological similarities to VEGF, and further studies are needed to establish whether this new compound can be regulated by tissue-specific hormones. Alternatively, it could rep-

Fig. 6. Forskolin stimulates Flk-1/KDR protein expression in adrenocortical capillary endothelial (ACE) cells. A: ACE cells grown on gelatin-coated plates were treated with 10 μM forskolin for the indicated time. Proteins (100 μg) were analyzed by SDS-PAGE and immunoblotting with the monoclonal anti-Flk-1/KDR antibody. The lower part of the membrane was incubated with an anti-tubulin antibody as an internal standard of the gel loading. Arrows indicate Flk-1/KDR and tubulin proteins. Bands were detected by enhanced chemiluminescence revelation. This experiment is representative of 2 additional experiments. B: ACE cells were treated with increasing concentrations of forskolin (from 0.1 to 50 μM) for 48 h, and protein content was analyzed as described above. A representative result from 1 of 3 independent experiments is shown.

Fig. 7. ACE cells form cellular networks when cultured in a collagen gel containing adrenocortical steroidogenic cells. A: monolayer of endothelial cells alone; B: adrenocortical steroidogenic cells in the deepest part of collagen; C: development of cellular cords in the presence of adrenocortical cells in the presence of ACTH (10 nM). D: cells associated to form a network. Phase contrast for A and B, ×200; for C and D, ×320.
resent the mediator of ACTH action observed in the mouse adrenal.

Corresponding to the expression of VEGF, the VEGF receptors Flt-1 and Flk-1/KDR were found to be expressed at high levels in the adult adrenal as detected by Western blot analysis and semi-quantitative RT-PCR. These tyrosine kinase receptors are expressed predominantly on endothelial cells in several endocrine tissues, such as thyroid, ovary, pancreas, placenta, and uterus (5). Our finding, showing the coexpression of VEGF and its receptors in adrenal, raises the possibility of autocrine and/or paracrine stimulation. We show that steroid treatment of mice downregulated VEGF-R2 (Flk-1/KDR) and that its expression was restored to control level by ACTH injections during 6 days. Such in vivo data indicate an overlapping with the timing of ACTH-induced proliferation of the adrenocortical zone observed in previous histomorphological studies (21). We also demonstrate that Flt-1 expression remained constant after steroid or ACTH treatment. Similar results have been described in the pituitary gland, where immunohistological studies showed a wide expression of Flt-1 localized to endothelial cells that was independent of hypotalamic input (23). Although considerable experimental evidence links Flk-1/KDR activation to endothelial cell mitogenesis, there is still significant uncertainty concerning the role of individual VEGF receptors for other biological effects such as vascular permeability. Recent work, using VEGF mutants that bind to either Flk-1/KDR or Flt-1 with high selectivity, demonstrates that KDR activation alone is sufficient for the activation of signal transducers involved in mitogenesis and cell migration. Selective KDR engagement also induces in vivo angiogenesis and vascular permeability (19). In adrenal gland, our data showing the hormonal regulation of Flk-1/KDR expression suggest that it may participate in the regeneration of the capillary network under ACTH challenge and regulate vascular permeability (16), which is necessary for access of secreted hormones to the circulation, and may participate also to stabilize the adrenal endothelium. Because the VEGF receptors promote endothelial cell survival, proliferation, and other events necessary for angiogenesis (14), the non-coordinate regulation of Flk-1/KDR and Flt-1 by ACTH suggests that mouse adrenal glands are protected against inappropriate or prolonged loss of VEGF receptors by a homeostatic mechanism important to endothelial cell function.

The mechanism responsible for the elevated expression of Flk-1/KDR after ACTH treatment is currently unknown. Because ACTH modulates cAMP-dependent gene expression by binding to its high-affinity receptors on the adrenocortical cell surface and activating downstream the cAMP-dependent pathway, it can be suggested that Flk-1/KDR regulation by ACTH is mediated through a cAMP-dependent pathway in a paracrine-dependent manner. This hypothesis is supported by our in vitro studies showing that forskolin, a direct activator of adenylate cyclase, increased Flk-1/KDR expression in ACE cells. This result suggests that cAMP is the potential mediator of Flk-1/KDR induction in response to ACTH in whole adrenal in vivo. The mechanism of such a paracrine regulation remains to be explored. However, our immunohistochemical analysis of PECAM and von Willebrand-positive cells showed that interactions between steroidogenic cells and endothelial cells in adrenal are largely dictated by proximity. Inside the adrenal cortex, the cells are linked by gap junctions (33) whose expression in the zona fasciculata is restored by ACTH treatment in hypophysectomized mice (10). Thus gap junction-mediated communication between hormone-responsive and nonresponsive cells is one mechanism for a potential paracrine communication. Further studies are needed to determine whether the blockade of gap junctions by pharmacological agents, such as 18 α-glycyrrhetinic acid (33), could impair ACTH induction of FLK-1/KDR in dexamethasone-treated mice.

A paracrine regulation of endothelial cells by steroidogenic cells was confirmed by our in vitro experiment in 3D collagen, showing that endothelial cells can organize precisely into vascular-like networks in the presence of ACTH-stimulated steroidogenic cells. During angiogenesis in vivo, endothelial sprouts were reported to form channels or vacuoles that extended the lumens of parent vessels into vascular branches (48). Although the molecular events in the observed effects remained to be elucidated, we conclude that the potential exists for steroidogenic cells to induce angiogenesis in vitro.

In summary, our data bring the evidence for an in vivo regulation of VEGF and its receptors by ACTH in the mouse adrenal. These data then allow consideration of ACTH as a major regulator of the adrenal cortex by acting on both the steroidogenic and the endothelial compartments.

We thank Dr. Geneviève Defaye for generously providing the anti-P450scc and anti-corticosterone antibodies, and Vanessa Giraud for skillful technical assistance.

This work was supported by INSERM, Commissariat à l’Energie Atomique, Direction des Sciences du Vivant/ Département Réponse et Dynamique Cellulaire, Association pour la Recherche contre le Cancer (Grant no. 5588), Fondation pour la Recherche Médicale, and Ligue Nationale contre le Cancer.

REFERENCES


11. DeLisser HM, Newman PJ, and Albelda SM. E166 VEGF RECEPTORS IN DEXAMETHASONE-INDUCED ADRENAL ATROPHY

12. Ferrara N.


