

Mitogenic functions of endocrine gland-derived vascular endothelial growth factor and *Bombina variegata* 8 on steroidogenic adrenocortical cells

Michelle Keramidas^{1,2,3}, Caroline Faudot^{1,2,3}, Agnès Cibiel^{1,2,3}, Jean-Jacques Feige^{1,2,3} and Michaël Thomas^{1,2,3}

¹Institut National de la Santé et de la Recherche Médicale, Unité 878, Grenoble, France

²Commissariat à l'Énergie Atomique, Institut de Recherches en Technologies et Sciences pour le Vivant, 38054 Grenoble, France

³Université Joseph Fourier, 38054 Grenoble, France

(Correspondence should be addressed to M Thomas who is now at INSERM Unité 878, iRTSV, CEA-G, 17 rue des Martyrs, 38054 Grenoble, Cedex 09, France; Email: michael.thomas@cea.fr)

Abstract

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) and its homolog *Bombina variegata* (Bv8), also termed prokineticin-1 and -2 (PK1 and PK2) respectively, are newly identified peptides with specific mitogenic activity on endocrine gland-derived endothelial cells. In the present study, we analyzed the sites of expression of EG-VEGF/PK1, Bv8/PK2, and their receptors (PKR1 and PKR2) in the adrenal cortex and checked for new biological functions of these factors on the endocrine cell compartment. RT-PCR and immunostaining analyses revealed that glomerulosa and fasciculata cells express both factors and both receptors.

EG-VEGF/PK1 had no effect on the steroidogenic activity of both bovine glomerulosa and fasciculata cells but appeared to be mitogenic for both cell types. Binding of EG-VEGF/PK1 to fasciculata cells stimulated the phosphorylation of ERK1/2. Pretreatment with pertussis toxin suppressed this effect, indicating that it was Gi mediated. EG-VEGF/PK1 also increased the phosphorylation of Akt in endocrine cells of the adrenal cortex. EG-VEGF/PK1 and Bv8/PK2 thus represent new regulatory peptides acting as autocrine mitogens for endocrine cells.

Journal of Endocrinology (2008) **196**, 473–482

Introduction

Like most other endocrine glands, the adrenal cortex is a highly vascularized organ in which a dense network of quiescent fenestrated capillaries is maintained throughout adult life (Thomas *et al.* 2003). Interestingly, the level of expression of angiogenic factors, such as vascular endothelial growth factor-A (VEGF-A), is high in these organs despite the absence of active angiogenesis, suggesting that these factors play an important role in the homeostasis and the hormonal control of the microvasculature (Shweiki *et al.* 1993). Our team has previously established that VEGF expression is under the hormonal control of adrenocorticotropin (ACTH) both in primary cultures of bovine steroidogenic adrenocortical cells *in vitro* and in mouse adrenal gland *in vivo* (Gaillard *et al.* 2000, Thomas *et al.* 2004, Cherradi *et al.* 2006). However, besides VEGF, a number of other angiogenic factors have been described, which may also be expressed in the adrenal cortex and participate in the regulation of its vascularization.

Recently, a novel family of secreted proteins comprising about 80 amino acids and possessing the N-terminal amino

acid sequence AVITGAC has been characterized and named the AVIT protein family (Kaser *et al.* 2003). Among these members, endocrine gland-derived-VEGF (EG-VEGF) and *Bombina variegata* 8 (Bv8) represent a novel class of angiogenic factors with expression profiles restricted to endocrine glands and a few other tissues, and with selective mitogenic, motogenic, and prosurvival activities for endocrine gland-derived endothelial cells (LeCouter & Ferrara 2003, Ferrara *et al.* 2004). In humans, EG-VEGF, whose amino acid sequence is totally different from those of VEGF and the other known angiogenic factors, was found to be expressed in the steroidogenic cell compartment of ovary, testis, placenta, and adrenal glands and to a much lower level in non-steroidogenic organs including prostate and spleen (LeCouter *et al.* 2001, Zhang *et al.* 2003). EG-VEGF is identical to prokineticin-1 (PK1) which was initially identified as a stimulant of gastrointestinal smooth muscle contraction (Li *et al.* 2001). EG-VEGF/PK1 shares 44% amino acid identity with prokineticin-2 (PK2), the mammalian homolog of the frog skin peptide Bv8. The expression of Bv8/PK2 is somewhat different from that of EG-VEGF/PK1 with major sites of expression in the central nervous system where it promotes

neuronal survival, in the suprachiasmatic nucleus of the brain where it affects circadian rhythm, and in the seminiferous tubules of the testis (Wechselberger *et al.* 1999, Melchiorri *et al.* 2001, Cheng *et al.* 2002, LeCouter *et al.* 2003a,b). Moreover, the distinct patterns of expression of both factors have been observed between murine and human tissues, which are likely to be explained by divergence of promoter sequences (LeCouter *et al.* 2003a,b, Ferrara *et al.* 2004). Both EG-VEGF/PK1 and Bv8/PK2 appear able to bind two small, highly identical Gi-coupled receptors, named PK receptor-1(PKR1)/GPCR73a and PKR2/GPCR73b, with similar nanomolar affinities (Lin *et al.* 2002a,b, Masuda *et al.* 2002, Soga *et al.* 2002). PKR1 and PKR2 appear to be expressed on distinct cell types within many organs including ovary, endometrium, corpus luteum, and placenta (Kisliouk *et al.* 2003, Hoffmann *et al.* 2006, Ngan *et al.* 2006).

The aim of the current study was to characterize the pattern of expression of EG-VEGF/PK1 and Bv8/PK2 and of their cognate receptors in the bovine adrenal cortex. In addition, to gain more information on the biological action of these factors in adrenal cortex physiology, we investigated their effects on proliferation and steroidogenic functions of primary cultures of bovine glomerulosa and fasciculata cells.

Materials and Methods

Tissue collection and primary cell cultures

Adrenal glands from 2-year-old steers were collected in ice-cold PBS at the local slaughterhouse and processed in the laboratory within 60-min postmortem. One adrenal gland was cut into pieces and fragments were either fixed overnight at 4 °C in 4% paraformaldehyde and embedded in paraffin or frozen in tissue-freezing medium (Jung, Nussloch, Germany). Primary glomerulosa and fasciculata/reticularis cells were prepared as described elsewhere (Negoescu *et al.* 1994, Python *et al.* 1995). Hormonal stimulations were performed on day 4 after 24-h serum deprivation. Bovine adrenal cortex-derived capillary endothelial cells were provided to us by Dr A Baird (Salk Institute, La Jolla, CA, USA) and kept in culture for several passages in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 2 ng/ml fibroblast growth factor (FGF)-2 (Gospodarowicz *et al.* 1986). The samples of ovary, testis, liver, lung, spleen, skeletal

muscle, and adrenal cortex were also collected at the local slaughterhouse and snap frozen in liquid nitrogen for subsequent RNA analysis.

RNA isolation and semiquantitative RT-PCR analysis

Total RNA was extracted from bovine tissues or cells in 4 M guanidium isothiocyanate buffer using a commercial kit (RNAagents, Promega). Reverse transcription was performed on 1 µg total RNA using 1 µl ImProm-II reverse transcriptase (Promega) according to the manufacturer's procedures. Volumes of RT reactions yielding equal amplification of ribosomal protein L27 (RPL27) mRNA for each bovine sample were first determined in a setup experiment. These same amounts were used in specific PCRs for EG-VEGF/PK1, Bv8/PK2, PKR1, PKR2, PECAM, and RPL27. The oligonucleotide primers were designed based on published bovine or human sequences (Table 1). PCRs were performed in a final volume of 25 µl containing 1×PCR buffer, 0.5–1.5 mM MgCl₂, 200 µM dNTPs, 400 nM each primer, and 0.2 U Taq polymerase (Q.Biogene, Montreal, Canada). The PCR conditions were as follows: step 1, 94 °C for 1 min; step 2, 28–35 cycles for 1 min at hybridization temperature indicated in Table 1; and step 3, 72 °C for 1 min. To ensure semiquantitative results in the RT-PCR assays, the number of PCR cycles for each set of primers was selected to be in the linear range of amplification (data not shown). PCR products were visualized after electrophoresis on 1.5% agarose gels by ethidium bromide staining and quantified on a FluorImager (Molecular Dynamics, Palo Alto, CA, USA) using the ImageQuant software (Molecular Dynamics).

Immunohistochemistry

Rabbit polyclonal antibodies against human Bv8/PK2 (Hoffmann *et al.* 2006), PKR1, and PKR2 (Hoffmann *et al.* 2007) were prepared for our laboratory by Covalab (Lyon, France). Rabbit antiserum to human EG-VEGF/PK1 was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). For Bv8/PK2 immunodetection, 4 µm thick sections of paraffin-embedded tissues were deparaffinized, rehydrated, and microwaved at 800 W for 5 min in 10 mM citrate buffer, pH 6.0. The slides were then incubated for 1 h with rabbit anti-human Bv8/PK2 antiserum (dilution 1:50) at room

Table 1 Primers used for semiquantitative RT-PCR

Gene	Species	Forward primer	Reverse primer	Annealing temperature (°C)
EG-VEGF	h	aggtccccttctcaggaacg	tccaggctgtctcagaaaag	56
Bv8	b	gagcatacggattgacac	aaacgaagtccgtgaacagg	54
PKR1	b	ggatgagaatgccaccaatag	tgaagtggccgataccacagac	54
PKR2	b	gcagcccagaatggaatgc	aagtgccaccccgataacaatc	55
PECAM	b	ttgccattcccacagcaagatgtc	ttgttcagttcagaagcggatcgg	59
RPL27	b	gaacattgatgatgacacctc	ggggatattccacagagtacc	55

temperature. For EG-VEGF/PK1, PKR1, and PKR2 immunohistochemistry, frozen sections (8 μm) from fresh adrenal were fixed either in 4% paraformaldehyde for 10 min for EG-VEGF/PK1 and PKR1 or in acetone for 10 min for PKR2. The slides were then incubated for 1 h either with anti-EG-VEGF/PK1 (dilution 1:1500), anti-PKR1 (dilution 1:50), or anti-PKR2 (dilution 1:100) antibodies. The sections were washed twice for 5 min in Tris-buffered saline (TBS) containing 0.1% Tween 20, and then sequentially incubated with a biotinylated goat anti-rabbit secondary antibody for 1 h and with an avidin-biotin peroxidase complex (Dako A/S, Glostrup, Denmark) for 45 min at room temperature. The peroxidase activity was revealed with diaminobenzidine and the sections were briefly counterstained with hematoxylin. The negative controls were performed either by omitting the primary antibody in the case of EG-VEGF/PK1, PKR1, and PKR2 staining or by preabsorbing Bv8/PK2 antibody with blocking peptide for 2 h at room temperature (data not shown).

Immunocytochemistry

Primary bovine glomerulosa and fasciculata cells were seeded at a density of 10^5 cells/chamber on chamber tissue culture-treated glass slides (Becton-Dickinson, Franklin Lakes, NJ, USA) with four chambers per slide in 500 μl Dulbecco's Eagle's Medium/Ham's F12 1:1 supplemented with 10% fetal bovine serum and 2.5% horse serum. Two days after seeding, the medium was removed and the cells were rinsed in TBS, fixed for 10 min at room temperature in 4% paraformaldehyde, and finally rinsed thrice in TBS/0.1% Tween 20. The primary antibodies were diluted in 0.5% BSA in TBS: 1:500 for EG-VEGF/PK1, 1:50 for Bv8/PK2, 1:50 for PKR1, and 1:100 for PKR2 and added to the cells overnight at 4 °C. The following steps were conducted as described for the immunohistochemistry staining. Control sections were treated with the antibody that had been preabsorbed for 2 h at room temperature with the appropriate blocking peptide at a molar concentration 20 times higher than that of the corresponding primary antibody.

Corticosteroid secretion

Aldosterone was measured from the conditioned medium of bovine glomerulosa cells using a commercial EIA kit from Cayman Chemical (Ann Arbor, MI, USA). Detection of cortisol in the conditioned culture medium of bovine fasciculata cells was performed using a sensitive RIA. Cortisol antiserum was obtained from Endocrine Sciences (Calabasas, CA, USA) and [^3H]cortisol (80 Ci/mmol) from NEN (Zaventem, Belgium).

Cell proliferation assays

Bovine endocrine cells (glomerulosa or fasciculata/reticularis) were serum starved for 24 h in the presence of 0.1% fetal calf serum and subsequently incubated for 24 h in fresh

serum-poor medium containing 0.2 $\mu\text{Ci/ml}$ [^3H]thymidine (86 Ci/mmol, Amersham Biosciences) and the indicated growth factors. The cell layers were then washed thrice with PBS and the radioactivity incorporated into trichloro-acetic acid (TCA)-precipitable material was measured in a liquid scintillation β -counter (Packard). In an independent experiment, the proliferation index of the glomerulosa and fasciculata cells was evaluated by the detection of the proliferation-associated Ki-67 antigen using the monoclonal antibody MIB-1 (Dako A/S). The cells were treated as previously described for the thymidine incorporation and incubated with or without 50 ng/ml EG-VEGF/PK1 (Peprotech, Rocky Hill, NJ, USA) for 24 h. The cells were washed thrice with PBS and fixed with paraformaldehyde 4% for 10 min. The slides were incubated with mouse monoclonal antibody MIB-1 at 1:100 dilution for 1 h at room temperature and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA), as recommended by the manufacturers. The sections were lightly counterstained with hematoxylin. The percentage of Ki-67-positive cells was determined by counting 500–600 adrenal cells per sample.

Cell signaling

Subconfluent bovine fasciculata cells were cultured in serum-supplemented medium for 48 h and subsequently incubated for 24 h in serum-free medium. The quiescent cells (200 000 cells per 9.6 cm^2 well) were then treated for various periods of time with 50 ng/ml EG-VEGF/PK1 or 10 ng/ml FGF-2 (a gift from Dr L Cousens, Chiron Co., Emeryville, CA, USA) in the presence or absence of 200 ng/ml pertussis toxin, an inhibitor of Gi-coupled receptor signaling. At the end of the stimulation, cellular proteins were solubilized in 0.25 ml lysis buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1% NP40, 1% deoxycholate, 0.5% SDS, 0.1% Triton X-100, 10 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin, 100 $\mu\text{g/ml}$ AEBSF (4-(2-aminoethyl) benzene-sulfonyl fluoride hydrochloride), and 1 mM sodium orthovanadate) and separated by SDS-PAGE. After electrophoretic transfer onto a nitrocellulose membrane, the phosphorylated forms of ERK1/2 and Akt were analyzed by western blotting using phosphorylation site-specific antibodies from Promega (dilution 1:5000) and Cell Signaling Technology-Sigma (dilution 1:1000) respectively. The membranes were incubated overnight at 4 °C with primary antibodies. Blots were then incubated for 1 h at room temperature with a peroxidase-coupled anti-rabbit secondary antibody (Pierce Biotechnology, Rockford, IL, USA; dilution 1:10 000). Normalization of the levels of phosphorylated protein was performed by reprobing the blots with an antibody recognizing either total ERK1/2 or Akt.

Statistical analysis

All data are presented as mean \pm S.D. Differences between groups were determined by Student's *t*-test and one-way ANOVA combined with Newman-Keuls multiple

comparison test using the software Graph Pad Prism 4. Results were considered significant at $P < 0.05$.

Results

AVIT proteins and their receptors are differentially expressed in bovine tissues and in endocrine cells of the adrenal cortex

We first checked the status of EG-VEGF/PK1 and Bv8/PK2 expression in bovine tissues as well as that of their common receptors PKR1 and PKR2. As shown in Fig. 1, RT-PCR analysis of the mRNA levels for each of these factors indicated that EG-VEGF/PK1 was poorly expressed in the gonads, slightly expressed in the adrenal cortex, and strongly expressed in lung, spleen, and skeletal muscle. In contrast, Bv8/PK2 was mainly expressed in the spleen and lung but was also detectable in the adrenal cortex. The receptors also showed differential patterns of expression with PKR1, being present in almost every tested tissue, and PKR2, being prominently expressed in the lung and adrenal cortex. As both AVIT proteins and both receptors were expressed in the adrenal cortex, we next analyzed their cellular distribution using primary cultures of isolated cell populations. Using the same RT-PCR technique, we observed that steroidogenic fasciculata and glomerulosa cells express all components of the system whereas adrenocortical capillary endothelial cells express all components but PKR2 (Fig. 2). Amplification of PECAM, a marker of endothelial cells, was performed to exclude any possible endothelial contamination in our steroidogenic cell preparations.

EG-VEGF/PK1, Bv8/PK2, and their receptors are differentially localized in bovine adrenal cortex tissue

We then attempted to immunolocalize EG-VEGF/PK1 and Bv8/PK2 in the sections of bovine adrenal glands using either

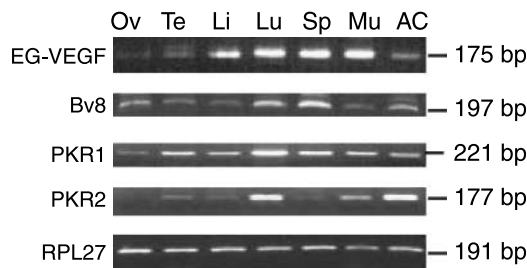


Figure 1 mRNA expression profiles of EG-VEGF/PK1, Bv8/PK2, and their receptors in bovine tissues. The total RNAs were prepared from bovine tissues and the levels of EG-VEGF/PK1, Bv8/PK2, PKR1, and PKR2 mRNA expression were determined by semiquantitative RT-PCR with specific primers as described in Materials and Methods and in Table 1. Amplification of the ribosomal protein L27 (RPL27) mRNA was performed for normalization. PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and photographed. The size of the amplicons is indicated on the right. Tissues included ovary (Ov), testis (Te), liver (Li), lung (Lu), spleen (Sp), skeletal muscle (Mu), and adrenal cortex (AC).

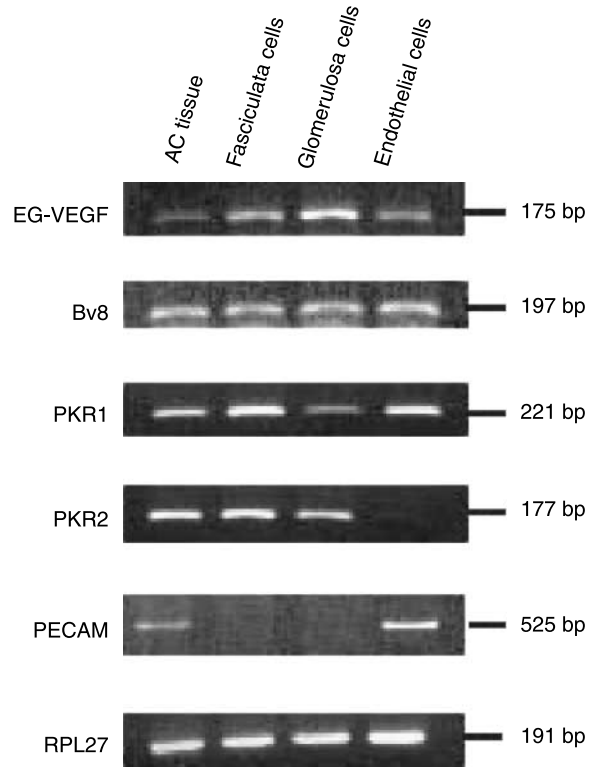


Figure 2 mRNA expression levels of EG-VEGF/PK1, Bv8/PK2, and their receptors in bovine adrenocortical endocrine and endothelial cells. The total RNAs were prepared from bovine adrenal cortex tissue and from steroidogenic cells (from the zona fasciculata/reticularis or the zona glomerulosa) or capillary endothelial cells isolated from this tissue as described in Materials and Methods. The levels of EG-VEGF/PK1, Bv8/PK2, PKR1, PKR2, and PECAM mRNA expression were determined by semiquantitative RT-PCR with specific primers as shown in Table 1. Amplification of the ribosomal protein L27 (RPL27) mRNA was performed for normalization. The size of the amplicons is indicated on the right. AC, adrenal cortex. The results from one representative experiment are shown. Similar results were obtained in three independent cell preparations.

commercially available or home-made antibodies. As shown in Fig. 3A, EG-VEGF/PK1 immunostaining was detectable in all zones of the endocrine compartment of the cortex with no staining in the capsule or the medulla (data not shown). However, the staining was slightly stronger in the zona fasciculata/reticularis than in zona glomerulosa. The distribution of Bv8/PK2 immunostaining was uniformly distributed throughout the zona glomerulosa and the upper part of the zona fasciculata cortex (Fig. 3B). The lower part of the fasciculata/reticularis zone demonstrated an attenuated expression of the Bv8/PK2 protein. No staining was observed in the capsule and the medulla. As concerns PKR1 immunodetection, we noticed a similar expression throughout the cortex (Fig. 3C). Finally, PKR2 staining was observed at a stronger intensity in the zona glomerulosa than in the zona fasciculata/reticularis with the notable exception of a layer of cells between zona glomerulosa and zona fasciculata/reticularis

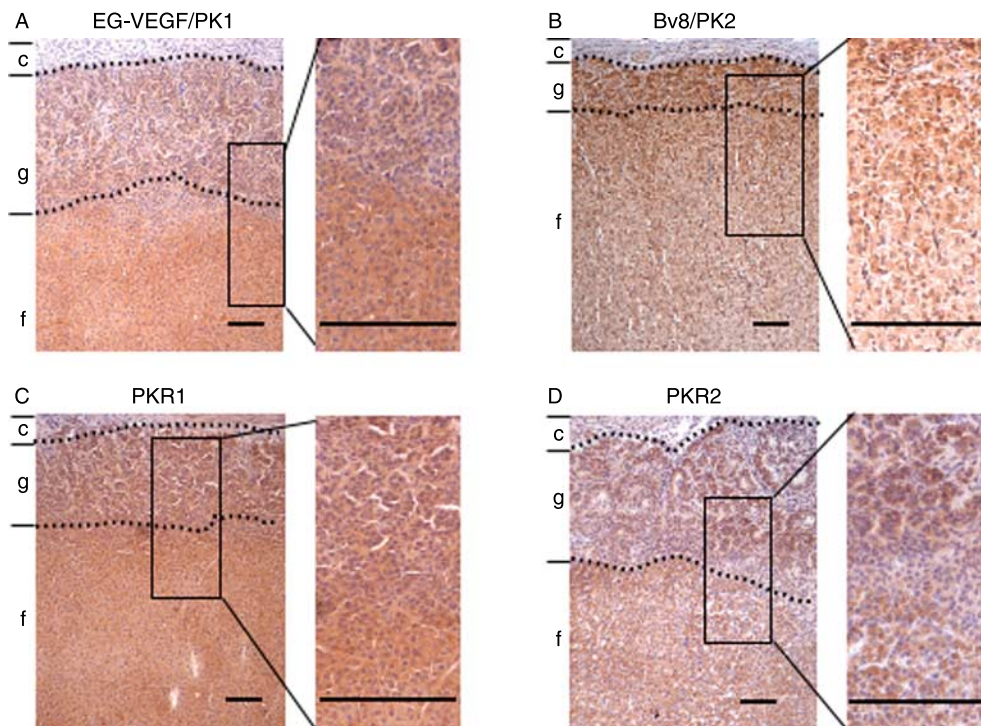


Figure 3 Immunohistochemical localization of EG-VEGF/PK1, Bv8/PK2, and their receptors PKR1 and PKR2 in the bovine adrenal cortex. The sections of a bovine adrenal gland were immunostained for (A) EG-VEGF/PK1, (B) Bv8/PK2, (C) PKR1, and (D) PKR2 as described in Materials and Methods. Immunoreactivity is indicated as cytoplasmic brown staining. The border between the capsule (c), the zona glomerulosa (g), and the zona fasciculata (f) are indicated as dotted lines in (A–D). Scale bars, 250 μ m (A–D) and 10 μ m (selected areas in A–D).

where the immunostaining was much weaker (Fig. 3D). The staining was specific since no signal was detected in the control sections (data not shown). The expression of EG-VEGF/PK1, Bv8/PK2, and their receptors was confirmed on isolated cells from the zona glomerulosa (Fig. 4A) and the zona fasciculata (Fig. 4B). Moreover, preabsorption of each antibody with the appropriate blocking peptide beforehand the incubation period with the glomerulosa cells precluded the detection of a specific signal (Fig. 4A).

EG-VEGF/PK1 stimulates adrenocortical cell proliferation but does not affect steroidogenesis

As adrenocortical cells express both AVIT proteins and their receptors, we then evaluated whether this factor might regulate their steroidogenic activity. Primary cultures of bovine glomerulosa and fasciculata cells were stimulated by ACTH or angiotensin II in the presence or absence of 50 ng/ml recombinant EG-VEGF/PK1 and their production of aldosterone or cortisol were measured respectively. As shown in Fig. 5A and B, EG-VEGF/PK1 had no steroidogenic activity by itself and did not alter hormone-induced production of corticosteroids. Bv8/PK2 was not

tested as it was previously established that both AVIT proteins activate their common receptors PKR1 and PKR2 with similar affinities (Lin *et al.* 2002a,b, Masuda *et al.* 2002).

We then checked the effects of EG-VEGF/PK1 on the proliferation of glomerulosa and fasciculata cells. Incubation of sparse serum-deprived primary cultures of bovine glomerulosa cells for 24 h in the presence of 50 ng/ml EG-VEGF/PK1 resulted in a marked increase in cell number ($P < 0.05$; Fig. 6A). EG-VEGF/PK1 was also shown to significantly stimulate [3 H]thymidine incorporation into DNA of fasciculata cells ($P < 0.05$), although less potently than fetal calf serum or FGF-2 (10 ng/ml; $P < 0.01$; Fig. 6A). The dose–response curve shown in Fig. 6B indicated that the EC_{50} of the proliferative effect of EG-VEGF/PK1 was 40 ng/ml (3.3 nM) which is comparable with the EC_{50} for EG-VEGF/PK1 binding to its receptors (2 and 7 nM for PKR1 and PKR2 respectively) determined by Lin *et al.* (2002a,b). The proliferative effect of EG-VEGF/PK1 was confirmed by a third method namely immunohistochemical staining of the proliferation-associated Ki-67 antigen. The Ki-67-labeling index, defined as the number of Ki-67-positive cells per 100 adrenocortical cells, was doubled after fasciculata cells were exposed to 50 ng/ml EG-VEGF/PK1 for 24 h ($9.0 \pm 0.8\%$ of control cells versus $18.2 \pm 1.0\%$

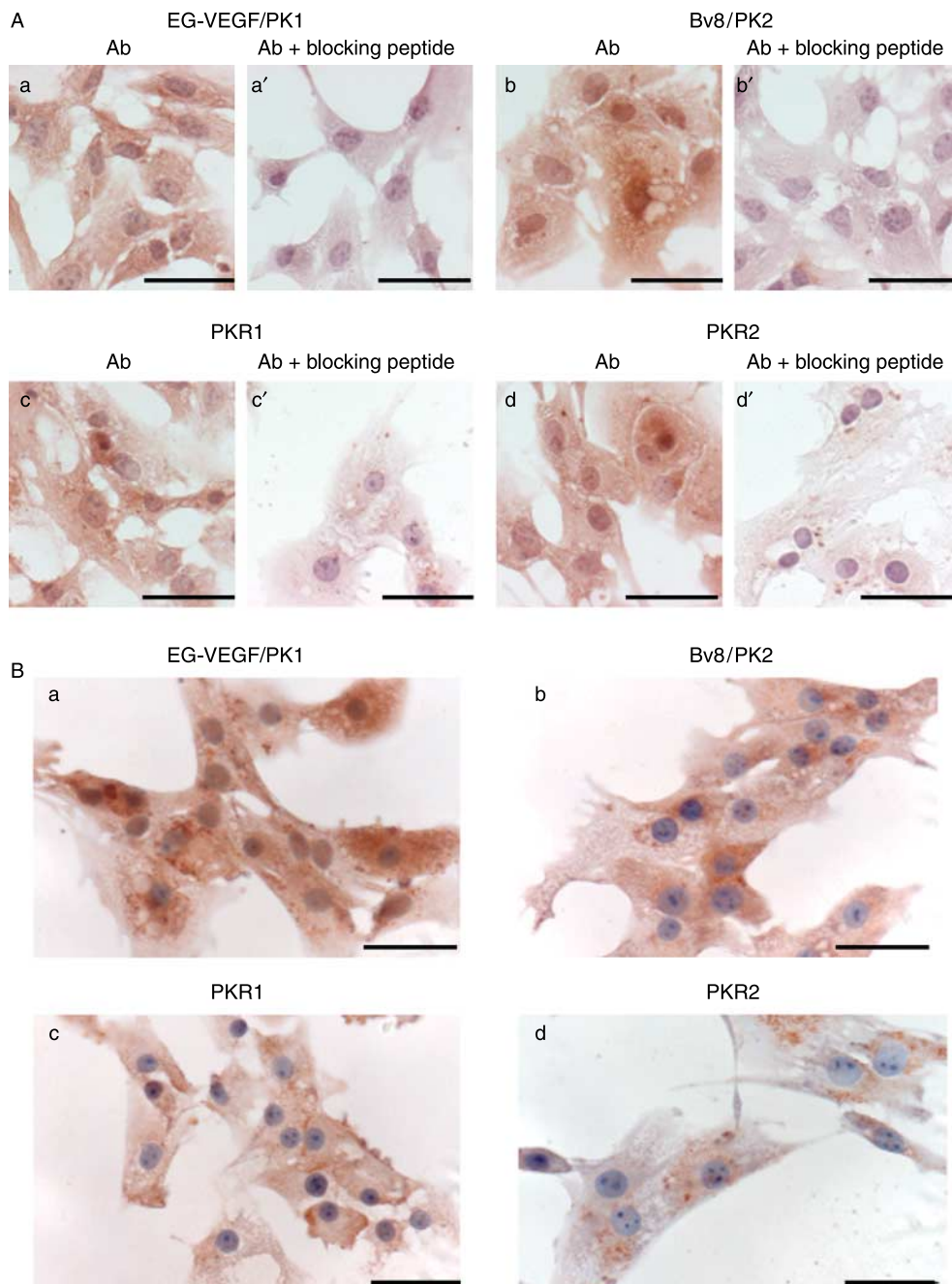


Figure 4 Immunocytochemical localization of EG-VEGF/PK1, Bv8/PK2, and their receptors PKR1 and PKR2 in glomerulosa and fasciculata cells. Isolated cells from (A) the zona glomerulosa and (B) the zona fasciculata were immunostained for EG-VEGF/PK1 (a), Bv8/PK2 (b), PKR1 (c), and PKR2 (d) as described in Materials and Methods. Immunoreactivity is indicated as brown staining. a', b', c', and d' in (A) show undetectable immunoreactivity when the antibody was incubated with the corresponding blocking peptide. Scale bars, 50 μ m.

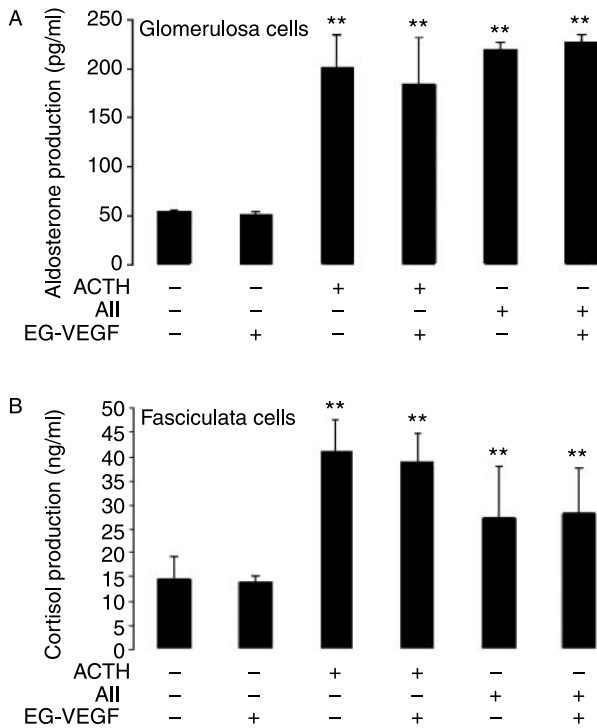


Figure 5 Effect of EG-VEGF/PK1 on basal and hormone-stimulated steroid production by endocrine adrenocortical cells. Primary cultures of bovine endocrine adrenocortical cells from (A) the zona glomerulosa or (B) the zona fasciculata were incubated for 24 h in the presence or absence of 50 ng/ml EG-VEGF/PK1, 10 nM ACTH 1–24, or 10 nM angiotensin II. Aldosterone secretion by glomerulosa cells and cortisol secretion by fasciculata cells were measured by RIA as described in Materials and Methods. Values represent the mean \pm s.d. of triplicate wells; asterisks indicate a statistical difference from the basal condition with no addition: ** $P < 0.01$. This experiment was repeated thrice with similar results.

of treated cells; $P < 0.01$; Fig. 6C). A weaker induction was measured when bovine glomerulosa cells were stimulated under the same conditions ($10.3 \pm 0.3\%$ of control cells versus $13.6 \pm 0.5\%$ of treated cells; $P < 0.05$; Fig. 6C).

EG-VEGF/PK1 stimulates the phosphorylation of p42/44 ERKs and Akt

Previous reports have shown that several downstream signaling pathways are activated in response to EG-VEGF/PK1 in capillary endothelial cells, including the phosphorylation of the two major isoforms of MAPK (ERK-1 and ERK-2) and Akt (Lin *et al.* 2002a,b). We thus determined whether the same signaling cascades were activated in endocrine cells of the adrenal cortex. The treatment of bovine fasciculata cells with 50 ng/ml EG-VEGF induced a rapid and transient activation of the phosphorylation of ERK1/2 (Fig. 7A) and Akt (Fig. 7B), which was detectable 5 min after treatment and vanished after 30 min. As PKR1 and PKR2 have been shown to be Gi

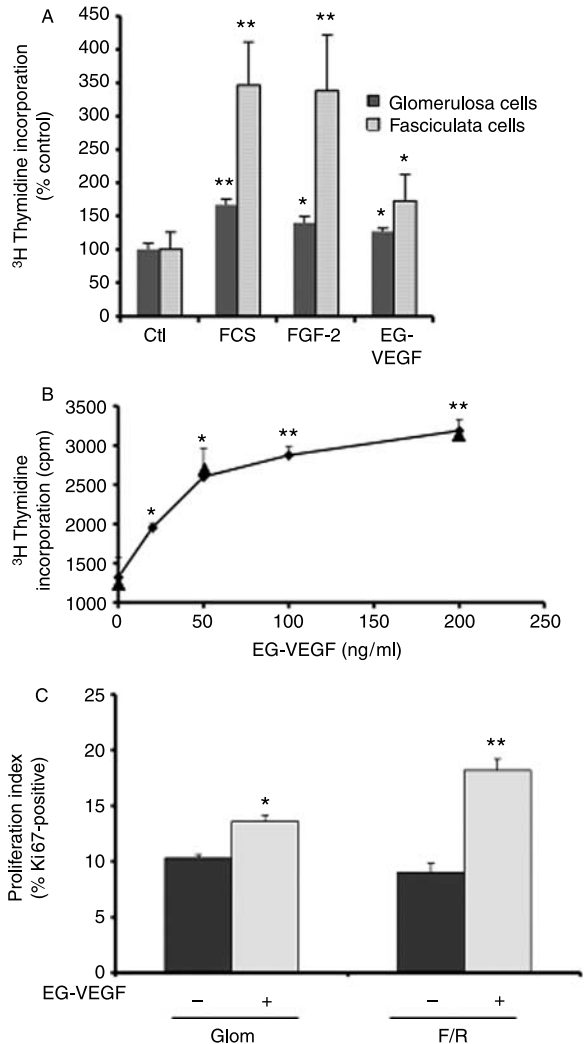


Figure 6 Effect of EG-VEGF/PK1 on adrenocortical endocrine cell proliferation. Serum-starved cells were stimulated either with 10% fetal calf serum (FCS) or 50 ng/ml EG-VEGF/PK1 or 10 ng/ml FGF-2 or without growth factor (Ctl). (A) [³H]thymidine incorporation into growth factor-stimulated subconfluent bovine glomerulosa (black bars) or fasciculata (grey bars) cells was measured as described in Materials and Methods. Bars represent the mean \pm s.d. of triplicate wells. Similar results were obtained in 2 additional experiments. * $P < 0.05$; ** $P < 0.01$ versus control for each cell types. (B) [³H]thymidine incorporation into serum-starved subconfluent bovine fasciculata cells stimulated for 24 h with indicated concentrations of either EG-VEGF/PK1 (diamond) or Bv8/PK2 (triangle) was determined as described in Materials and Methods. Each point represents the mean \pm s.d. of triplicate determinations. * $P < 0.05$; ** $P < 0.01$ versus control without EG-VEGF/PK1. (C) The proliferation index of bovine glomerulosa (Glom) and bovine fasciculata (F/R) cells stimulated with 50 ng/ml EG-VEGF/PK1 was determined as the percentage of Ki-67-immunoreactive cells as described in Materials and Methods. Each bar represents the mean \pm s.d. of triplicate determinations. * $P < 0.05$; ** $P < 0.01$ versus control without EG-VEGF/PK1. Similar results were obtained in two additional experiments.

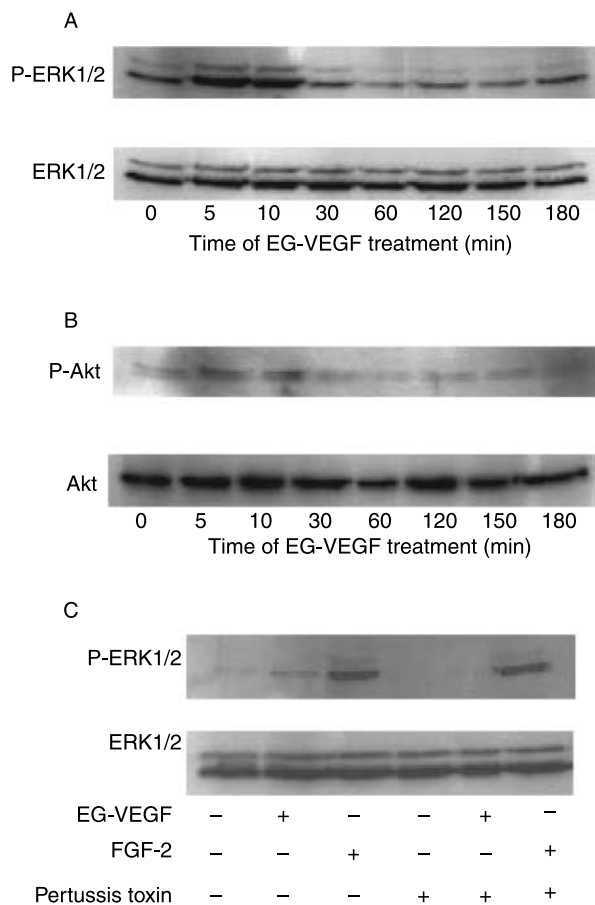


Figure 7 Effect of EG-VEGF/PK1 on ERK1/2 and Akt protein phosphorylation. Subconfluent bovine fasciculata cells were serum-starved and stimulated with 50 ng/ml EG-VEGF/PK1 for indicated time points. (A) Time-dependent activation of ERK1/2 phosphorylation. Cell lysates were analyzed by western blotting with phosphosite-specific antibody as described in Materials and Methods. Equal protein loading in the different lanes was assessed by western blotting of total ERK1/2. (B) Time-dependent activation of Akt phosphorylation. Cell lysates were subjected to western blot using phosphosite-specific antibody as described in Materials and Methods. Equal protein loading in the different lanes was assessed by western blotting of total Akt. (C) Pertussis toxin specifically inhibits ERK1/2 phosphorylation stimulated by EG-VEGF/PK1. Subconfluent bovine fasciculata cells were stimulated by 50 ng/ml EG-VEGF/PK1 or 10 ng/ml FGF-2 for 10 min in the absence or presence of 200 ng/ml pertussis toxin. Phospho-ERK1/2 and total ERK1/2 were then quantitated by western blotting as described in Materials and Methods.

protein-coupled receptors, we then checked whether the induction of the phosphorylation of ERK1/2 was Gi dependent. Pertussis toxin was used to specifically catalyze the addition of ADP-ribose and thus to inhibit Gi and check the phosphorylation of ERK1/2 in response to EG-VEGF/PK1 and FGF-2 (as a control growth factor acting through a tyrosine kinase receptor). As shown in Fig. 7C, pertussis toxin pretreatment abolished the response to EG-VEGF/PK1 but

did not alter the phosphorylation of ERK1/2 induced by FGF-2. Taken together, these experiments clearly indicated that the signaling pathways induced by EG-VEGF in adrenocortical endocrine cells were identical to those induced in adrenocortical endothelial cells (Lin *et al.* 2002a,b).

Discussion

The results presented here demonstrate that EG-VEGF/PK1 and its homolog Bv8/PK2 are autocrine growth factors for steroidogenic cells of the bovine adrenal cortex. This novel statement is supported by the following observations: i) both glomerulosa and fasciculata cells express EG-VEGF/PK1 and Bv8/PK2; ii) both cell types express the PKRs, PKR1 and PKR2; and iii) addition of EG-VEGF/PK1 to purified glomerulosa or fasciculata cells stimulates their proliferation.

The pattern of EG-VEGF/PK1 tissue distribution appears to be quite distinct among mammalian species, due to divergence in the promoter sequences (LeCouter *et al.* 2003a,b). In humans, EG-VEGF/PK1 is predominantly expressed in the endocrine tissues (ovary, testis, adrenal gland, and placenta) and the gastrointestinal tissues (LeCouter *et al.* 2001, Li *et al.* 2001); whereas in mouse, its expression is restricted to the kidney and the liver (LeCouter *et al.* 2003a,b). In contrast, the human and mouse Bv8/PK2 gene promoter sequences are highly conserved and the predominant site of Bv8/PK2 expression appears to be the testis in both species (Wechselberger *et al.* 1999, LeCouter *et al.* 2003a,b), whereas Bv8/PK2 is also expressed in mouse brain subregions (Cheng *et al.* 2002, Negri *et al.* 2006). The pattern of EG-VEGF/PK1 and Bv8/PK2 expression among the bovine tissues that we looked at in the present study surprisingly appears closer to the distribution in the mouse than in the human tissues. EG-VEGF/PK1 is more abundantly expressed in liver than in endocrine tissues and additional sites of high expression include lung, spleen, and skeletal muscle. However, detectable levels of EG-VEGF/PK1 mRNA transcripts are found in the adrenal cortex and the testis. Bovine Bv8/PK2 mRNA levels are highest in the lung and spleen but again detectable in the adrenal cortex. Although the sequence of the bovine EG-VEGF/PK1 promoter has not been published yet, it may be predicted from this distribution pattern that it might also diverge from the human promoter sequence.

Analysis of EG-VEGF/PK1 and Bv8/PK2 expression in primary cultures of bovine adrenal cortex-derived endocrine (glomerulosa or fasciculata) or endothelial cells indicated that all cell types express both factors at similar levels. However, the pattern of receptor expression was distinct as endocrine cells expressed both PKR1 and PKR2 whereas capillary endothelial cells expressed only PKR1. In human endometrium, it has also been shown that the endothelial cells coexpressed both factors and both receptors, PKR1 being predominant (Battersby *et al.* 2004). Several studies have reported that PKR1 and PKR2 present similar affinities for both factors and trigger the same signaling cascades in

response to either EG-VEGF/PK1 or Bv8/PK2 binding (Lin *et al.* 2002a, Soga *et al.* 2002). In bovine adrenal cortex-derived capillary endothelial cells, EG-VEGF/PK1 and Bv8/PK2 induce the phosphorylation of Akt, e-NOS, and p42/p44 MAPK (Lin *et al.* 2002a,b, Masuda *et al.* 2002). Distinct patterns of receptor expression may thus not result in distinct biological responses in these two cell types. However, it should be mentioned that little is known at present concerning the additional signaling cascades that may be specifically activated by one or the other of these two G protein-coupled receptors. Interestingly, a proteolytic cleavage fragment of Bv8/PK2 β that only binds to PKR1 was recently characterized, suggesting that specific activation of either one of these two receptors might exist under physiological conditions (Chen *et al.* 2005).

What is the function of EG-VEGF/PK1 in the adrenal gland? The two major hormones that stimulate corticosteroidogenesis transmit their signal through G protein-coupled receptors. Angiotensin II which is the major inducer of aldosterone production in zona glomerulosa acts through the Gq-coupled AT1 receptor, whereas ACTH which is the major inducer of cortisol production acts through the Gs-coupled MC2 receptor. In isolated cells, the specificity of hormone action is looser as both hormones can activate aldosterone synthesis in glomerulosa cells and cortisol synthesis in fasciculata cells. In addition, some locally produced growth factors such as insulin-like growth factor-I (IGF-I) or transforming growth factor- β have been shown to finely tune the steroidogenic response to hormones (Feige *et al.* 1998). Here, we report that EG-VEGF/PK1 is totally unable to stimulate corticosteroidogenesis in any of these two cell types. Although PKR1 and PKR2 are Gi coupled, EG-VEGF/PK1 did not inhibit the steroidogenic response to ACTH or angiotensin II. However, since EG-VEGF/PK1 has been previously reported to be mitogenic for adrenal cortex-derived capillary endothelial cells and to activate the p42/p44 MAPK pathway, we examined whether it could also do so in endocrine cells from the adrenal cortex. Our present data clearly indicate that EG-VEGF/PK1 is mitogenic for both glomerulosa and fasciculata cells although less efficiently than FGF-2, the best known growth factor for these cells (Feige & Baird 1991). However, in contrast to FGF-2 and IGF-1 which trigger their mitogenic signal through tyrosine kinase receptors, EG-VEGF/PK1 is the first reported factor that stimulates adrenocortical cell proliferation through a Gi-coupled receptor via p42/p44 MAPK. Recombinant Bv8/PK2 demonstrates the same mitogenic potency as EG-VEGF/PK1 for the fasciculata cells, which is in accordance with both factors binding to the same PKR1 and PKR2 receptors. On the other hand, the Akt phosphorylation in EG-VEGF/PK1-treated fasciculata cells indicates that EG-VEGF/PK1 may influence cell survival through the inactivation/repression of several targets, such as Bad, Caspase 9, and AFX forkhead, all involved in pro-apoptotic signaling pathways.

Taken together with the previous reports by Ferrara *et al.* (2004) demonstrating that EG-VEGF/PK1 is a selective mitogen for endocrine tissue-derived endothelial cells

(LeCouter & Ferrara 2002), EG-VEGF/PK1 and Bv8/PK2 appear as integrative regulators of adrenocortical tissue growth, acting equally and simultaneously on the endocrine and the endothelial cell compartments. It is already known that these factors present a diversity of biological activities far beyond their tissue-restricted angiogenic activity. They were initially characterized as inducers of gastrointestinal tract contraction and named PKs thereafter (Li *et al.* 2001). In addition, they stimulate hematopoiesis through stimulation of growth, survival, and differentiation of the granulocytic and monocytic lineages (Li *et al.* 2001, LeCouter *et al.* 2004). Generation of knockout mice lacking Bv8/PK2 (Ng *et al.* 2005) or PKR2 (Matsumoto *et al.* 2006) has recently demonstrated that Bv8/PK2 is essential for proper olfactory bulb neurogenesis, and PKR2 is absolutely required for adequate development of the olfactory bulb, the hypothalamic GnRH neurons, and the reproductive system. In the ovary, luteal and follicular steroidogenic cells were shown to express PKR1 but the effect of EG-VEGF/PK1 or Bv8/PK2 on their proliferation was not studied (Kisliouk *et al.* 2005). As knowledge progresses, it thus becomes more and more evident that EG-VEGF/PK1 and Bv8/PK2 are pleiotropic cytokines acting not only on endothelial cells but also on a number of differentiated cell types (steroidogenic cells, neurons, trophoblasts, and hematopoietic progenitors).

In conclusion, we have provided new data indicating that EG-VEGF/PK1 and Bv8/PK2 play important mitogenic roles in the adult adrenal cortex. These factors appear to act not only as paracrine growth factors on the endothelial cells lining the blood sinusoids (LeCouter & Ferrara 2002) but also as autocrine growth factors on the endocrine steroidogenic cells.

Acknowledgements

This work was supported by the Institut National de la Santé et de la Recherche Médicale (U878), the Commissariat à l'Energie Atomique (Life Sciences Division; Institut de Recherches en Technologies et Sciences pour le Vivant), the Fondation de France (Research Grant 2004012837 to M T), and the Groupement des Entreprises Françaises pour la Lutte contre le Cancer (Dauphiné Savoie). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 4 December 2007

Accepted 13 December 2007

Made available online as an Accepted Preprint
13 December 2007