Luteinizing hormone increases human endometrial cancer cells invasiveness through activation of protein kinase A.

[CANCER RESEARCH 63, 4281–4286, July 15, 2003]

Luteinizing Hormone Increases Human Endometrial Cancer Cells Invasiveness through Activation of Protein Kinase A

Sara Dabizzi, Ivo Noci, Patrizia Borri, Elena Borrani, Massimo Giachi, Manuela Balzi, Gian Luigi Taddei, Mauro Marchionni, Gian Franco Scarselli, and Annarosa Arcangeli[2]

Departments of Gynaecology, Perinatology, and Human Reproduction [S. D., I. N., P. B., E. B., M. G., M. M., G. S.], Experimental Pathology and Oncology [A. A.], Clinical Physiopathology [M. B.], and Human Pathology and Oncology [G. L. T.], University of Firenze, 50134 Firenze, Italy

ABSTRACT

Endometrial cancer (EC) is a hormone-dependent cancer that currently represents the most frequent malignancy of the female reproductive tract. The involvement of steroid hormones in its etiology and progression has been reported. The possibility that even gonadotropins (GT) could play a role in the genesis and establishment of EC is supported by the fact that specific receptors for the GT luteinizing hormone/human chorionic GT (LH/hCG) have been detected in a high percentage of ECs, and their expression is apparently related to the cancer grading. However, the precise mechanisms by which GTs might exert their effect on EC is still obscure. The aim of this study was to determine the effects of LH/hCG on the invasion potential of EC cell lines and primary human EC cells. Human recombinant (hr) LH (and hCG) induced a significant increase in cell invasiveness through Matrigel-coated porous membranes in an EC human cell line Hec1A, which expresses the LH/hCG receptor. This effect turned out to depend on hrLH binding to its specific receptors and to the subsequent activation of protein kinase A (PKA). Moreover the hrLH-induced increase in Hec1A invasiveness relied upon a PKA-dependent functional activation of β_1 integrin receptors, as well as the subsequent induction of matrix metalloproteinase-2 secretion in its active form. The same mechanisms were also found to be operative in primary EC cells. In fact, a significant percentage of primary ECs expressed the LH/hCG receptor, and hrLH addition to primary EC cells, which expressed the specific receptors produced an increase in cell invasiveness only in those tumor cells possessing the specific receptors. This effect was also dependent on PKA activity. We conclude that LH/hCG can regulate EC cells invasiveness, and this result provides a rationale for the use of inhibitors of LH secretion such as GnRH analogues in the treatment of EC.

INTRODUCTION

EC[3] is currently the most frequent malignancy of the female reproductive tract, with an incidence of 22 of 100,000 and a mortality rate of 3.2 of 100,000 in the Western countries (1). The etiology of EC is well understood: prolonged exposure to Es without P can modulate the mitogenic effect of E, thus contributing to the malignant transformation of endometrial surface epithelium (2, 3). The level of E and P is well regulated by the appropriate secretion of GTs, FSH, and LH/hCG. The median age for EC is 61 years, *e.g.,* in the postmenopausal period (4), when GTs levels are high; although EC incidence does not increase dramatically after menopause (5), some cancers arising in this reproductive period are often more aggressive and apparently unlinked to E secretion (6). It is possible that these E-independent ECs (which make up only a proportion of total EC morbidity) might be sensitive to elevated levels of LH/hCG that occur during postmenopausal period and that these GTs might be involved in the natural history of at least some types of ECs. In this light, it has been recently reported that LH/hCG regulate cell proliferation in EC cell lines (7).

On the other hand, the correlation between GTs and several other aspects of tumor progression is still poorly known. The process of tumor progression includes the acquisition of malignant characteristics such as the ability of invading surrounding tissues and the bloodstream. This invasion eventually is critical to the establishment of metastases, which is the major hindrance to cancer therapy. Although most mechanistic studies of tumor invasion in EC have focused on the role of E (8–10), it has recently emerged that GTs promote neoangiogenesis both inside tumors (11) and in the chicken chorioallantoic membrane (12).

This opens the possibility that GTs could directly regulate tumor progression of EC by binding to specific receptors. In fact specific LH/hCG-Rs have been detected in human EC, and their expression is apparently related to the cancer grading (13). In particular, two isoforms of this receptor arising from alternative splicing of the corresponding gene are documented in EC samples (13), as well as in neoplastic ovarian tissues (14).

The LH/hCG-R is a transmembrane glycoprotein receptor, which is a member of the G protein-coupled receptor family (15). The ligand-receptor binding induces the activation of signaling pathways centered on the cAMP/PKA system as well as on PLC/inositide tris-phosphate pathway (15, 16). It has been reported (15) that the prevalence of one or the other signaling pathways depends on the cell type involved and the amount of receptor expressed on the plasma membrane (17). In endometrial cells of porcine origin, it has been recently reported (18) that LH induces the activation of both cAMP/PKA and PLC/inositide tris-phosphate pathways.

On the basis of these findings, we started a study aimed at determining the effects of LH/hCG system in tumor progression of EC by analyzing the effects of such hormones on the invasion potential of both EC cell lines and primary human EC cells. We report here that hrLH (as well as hCG) induced a significant increase in cell invasiveness through Matrigel-coated porous membranes in the human EC cell line Hec1A, which expresses the LH/hCG-R. This effect turned out to depend on the hrLH binding to its specific receptor and on the following activation of the cAMP/PKA signaling pathway. Moreover, the hrLH-induced increase in Hec1A invasiveness was dependent upon the functional activation of β_1 integrin receptors and the subsequent induction of MMP-2 secretion. Interestingly, these mechanisms were found to be operative in primary EC cells transferred *in vitro* because hrLH addition produced an increase in cells invasiveness in those primary EC tumors that expressed the LH/hCG-R. Here again, this effect was dependent on PKA activity.

On the whole, data presented in this article show that LH/hCG can regulate EC cell invasiveness, and this provides a rationale for the use of inhibitors of LH secretion such as GnRH analogues in the treatment of EC (19).

Received 1/31/03; accepted 4/25/03.

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

[1] This work was supported by Takeda Italia (Roma) and by grants from the Associazione Italiana contro le Leucemie (Firenze) and the Ministero dell'Università e Ricerca Scientifica e Tecnologica (Murst, Cofin 2001) (to A. A.).

[2] To whom requests for reprints should be addressed, at Department of Experimental Pathology and Oncology, Viale Morgagni 50, 50134 Firenze, Italy. Phone: 0039-055-4282326; Fax: 0039-055-4282333; E-mail: annarosa.arcangeli@unifi.it.

[3] The abbreviations used are: EC, endometrial cancer; E, estrogen; P, progesterone; GT, gonadotropin, FSH, follicle-stimulating hormone; LH/hCG, luteinizing hormone/human chorionic GT; LH/hCG-R, receptors for the LH/hCG peptide hormone; PLC, phospholiphase C; cAMP, cyclic AMP; PKA, protein kinase A; RT-PCR, reverse transcription-PCR; hr, human recombinant; MMP, matrix metalloproteinase; FN-R, fibronectin receptor; gapdh, glyceraldehyde-3-phosphate dehydrogenase.

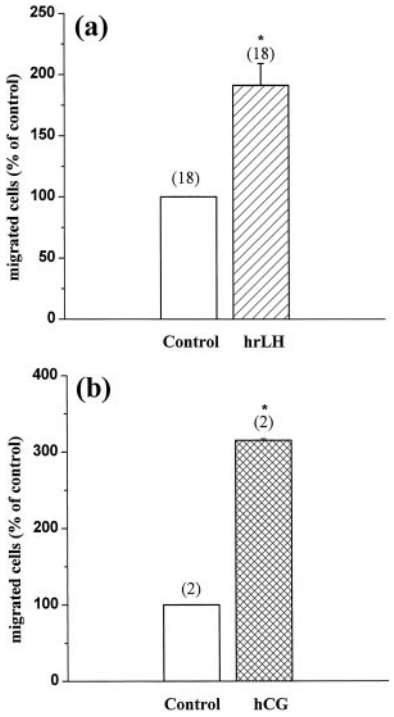

Fig. 1. Effect of hrLH and hCG on Hec1A invasion. Hec1A cells were inoculated into Boyden chamber where a Matrigel-coated porous membrane (8-μm pore diameter) was inserted as described in "Materials and Methods." At time 0, either 0.3UI/ml hrLH (*a*) or 5000 UI/ml hCG (*b*) were added to the medium; migrated cells were determined after 24 h of incubation. Data are reported as the percentage of migrated cells as compared with untreated cells (control) and are the means ± SE of the number of experiments reported in the figure (*in bracket*), each carried out in triplicate. * = significantly different as compared with "control"; $P = 1.26\ 10^{-5}$ (*a*) and $P = 8.65\ 10^{-5}$ (*b*; Student's *t* test).

MATERIALS AND METHODS

Chemicals. Matrigel Basement Membrane Matrix by Becton Dickinson Labware (Becton Dickinson, Franklin Lakes, NJ) was used at a final concentration of 250 μg/ml and following the instructions from the Thin Coating Method. hrLH: Lhadi (Lutropina α) 75 UI (3.4 μg; Serono Laboratories, Geneva, Switzerland) was used at 0.3 UI/ml (6). hCG: Profasi 5000 HP (Serono Laboratories, Geneva, Switzerland) was used at 5000 UI/ml. The inhibitor of PKA H89 (N-5-isoquinolinesulfonamide hydrochloride; Sigma Chemical Company, St. Louis, MO) was used at a final concentration of 50 μM. The inhibitor of PLC U73122 (Sigma Chemical Company) was used at a final concentration of 5 μM. Doses of inhibitors were chosen according to various data reported in literature and to preliminary dose response courses.

Cell Culture. The human endometrial adenocarcinoma cell line Hec1A (American Type Culture Collection, Manassas, VA) was routinely grown in DMEM (Euroclone; Wetherby, West Yorkshire, United Kingdom) supplemented with L-glutamine (DMEM-GLUT) and 10% FCS (Euroclone, Wetherby; complete medium) and incubated at 37°C in 5% CO_2. Primary EC cultures were obtained from surgical specimens and prepared as reported previously (19, 20).

Matrigel Invasion Assay. Hec1A cells, cultured as described above, were harvested by trypsinization and resuspended in DMEM-GLUT containing 250 μg/ml heat-inactivated BSA (DMEM + BSA). Cell suspension (200 μl) containing 600,000 cells was seeded into the upper well of a Boyden chamber (NeuroProbe, Inc., Gaithersburg, MD), separated from the lower compartment (filled with DMEM + BSA) by a porous membrane (8-μm pore diameter) previously overlaid with Matrigel (see above). All of the additions were performed both into the upper and the lower wells. Boyden chambers were incubated at 37°C in humidified atmosphere containing 5% CO_2 in air for 24 h. Migrated cells, which remained layering onto the lower face of the porous membrane, were fixed with absolute methanol at 4°C overnight and stained with Diff-Quick staining solution (Dade-Behring Holding GmbH, Liederbach, Germany). Cells were counted on the whole migration field at ×40 magnification.

RT-PCR. Total RNA was extracted from either the Hec1A cells or fresh tissue EC samples by the guanidinium/isothyocyanate method (21). cDNA was then synthesized using 1 μg of total RNA in a mix reaction with 2.5 units/μl reverse transcriptase murine leukemia virus-RT (Applied Biosystem, Foster City, CA), 1 mM of each deoxynucleotide triphosphate, and 2.5 μM random exhamers in a 20-μl final reaction volume for 30 min at 42°C and 5 min at 99°C. Ten μl of each resulting cDNA template was then used to perform a nested PCR specific for LH/hCG-R, which consists of a first amplification using 1.25 units AmpliTaq polymerase (Applied Biosystem), 200 μM of each deoxynucleotide triphosphate, 2 mM $MgCl_2$, and 0.15 μM of the specific primers. Ten μl of the initial PCR product were used as template for a second PCR in a reaction mix as above except that different primers, internal to the first ones, were used. In both cases, amplification was subjected to 43 cycles each consisting of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and to a 72°C for 5 min cycle for final elongation. The primers used were those described by Lin *et al.* (12). The omission of template in each PCR reaction was performed as negative control.

Integrin Flow Cytometric Analysis. Hec1A cells were seeded onto Matrigel-coated dishes in DMEM + BSA in the absence or in the presence of 0.3 UI/ml hrLH (see above) and incubated for 24 h as above. Cells were harvested in PBS + EDTA (5 mM) both at time 0 and after 24 h of incubation, washed once in PBS, and then incubated with monoclonal antibodies raised against the β_1 integrin subunit (mAb TS2/16; 30 μg/ml), the β_3 subunit (mAb B212; 30 μg/ml), and the α_v subunit (mAb L230; 30 μg/ml) for 15′ at 37°C. All of the antibodies were kindly gifted by Dr. G. Tarone and Dr. P. Defilippi of the University of Torino (Torino, Italy). Afterward, cells were washed and incubated with an antimouse antibody labeled with FITC (Sigma Chemical Company). For the analysis, 20,000 events were collected, and the fluorescence of samples was acquired in logarithmic scale using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 5-W argon-ion laser.

Adhesion Assay. Adhesion assays were performed essentially according to Arcangeli *et al.* (22). Briefly, Hec1A cells were radiolabeled for 48 h of exponential growth in complete medium containing 1 μCi/ml methyl-3H-

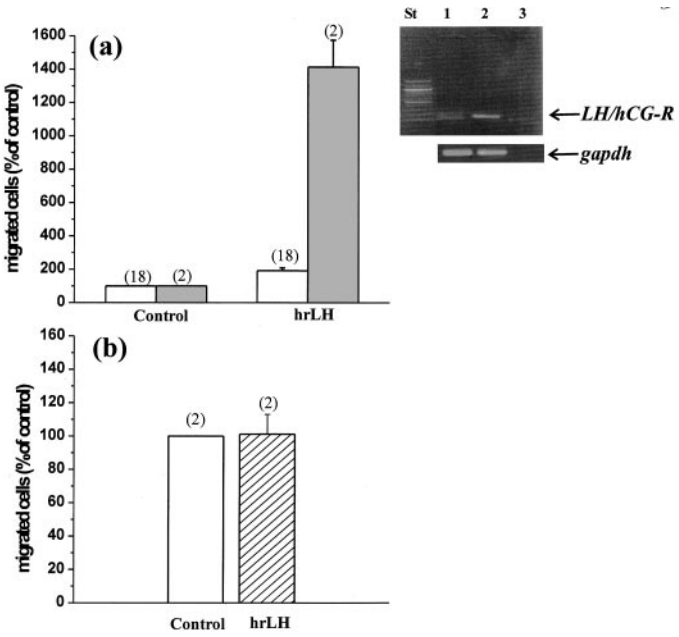

Fig. 2. Effect of hrLH on invasion through Matrigel of Hec1A cells and clone 1s (*a*) and of Hek293 cells (*b*). In *a*, the effect of hrLH on the invasiveness of wild-type Hec1A cells (□) and of the LH-R-overexpressing clone *Hec1A1s* (■) is reported. Conditions were the same as reported in the legend to Fig. 1. *Inset:* RT-PCR analysis of LH/hCG-R expression in Hec1A (*Lane 1*) and Hec1A clone 1s (*Lane 2*). St = molecular weigh standard 100 bp (New England Biolabs, Beverly, MA). *Lane 3:* amplification performed in the absence of any cDNA, to be considered as a qualitative control of the reaction. Amplification of *gapdh* represents the control of RNA integrity. For experimental details, see legend to Fig. 8. In *b* is reported the effect of hrLH on invasiveness of HEK 293. Data are reported as the percentage of migrated cells as compared with untreated cells (control) and are the means ± SE of the number of experiments reported in the figure (*in bracket*), each carried out in triplicate.

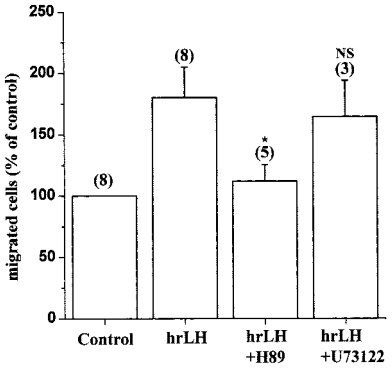

Fig. 3. Effect of the PKA inhibitor H89 and the PKC inhibitor U73122 on the migration of Hec1A cells induced by hrLH. Hec1A cells were inoculated into Boyden chamber as described in the legend to Fig. 1 and treated with or without 0.3 UI/ml hrLH at time 0. Where needed, 10 μM H89 or 5 μM U73122 were added at time 0. In any case, migrated cells were determined after 24 h of incubation. Data are reported as the percentage of migrated cells as compared with untreated cells (control) and are the means ± SE of the number of experiments reported in the figure (*in bracket*), each carried out in triplicate. * = significantly different as compared with "hrLH" (ANOVA; Bonferroni test; $P = 0.044$); NS = not significantly different as compared with "hrLH" (ANOVA; Bonferroni test).

thymidine (specific activity 24 Ci/mmol; Amersham Biosciences Europe GmbH, Freiburg, Germany). After 24 h of labeling, 0.3 UI/ml hrLH were added where needed and cells incubated for an additional 24 h. Cells were then harvested, pelleted, and resuspended in DMEM + BSA; aliquots of cells (1×10^5) were inoculated into each well of 96-well clusters (Corning B.V., Life Sciences, the Netherlands), previously coated with Matrigel (see before). In the appropriate samples, blocking antibodies to FN-R (kindly gifted by Dr. G. Tarone and Dr. P. Defilippi, University of Torino; Ref. 23) at different dilutions were added at time 0. In any case, cells were incubated at 37°C in 5% CO_2 in air; at different times, the medium was aspirated off and adherent cells gently rinsed twice with PBS; the cells were solubilized with 50 μl of 1% SDS in 0.1 N NaOH for 1 h. Radioactive solubilized cells were quantified by scintillation counting and compared on a percentage basis with the radioactivities of the cells inoculated in each well.

Assay of Gelatin-degrading MMPs by Zymography. The release of MMPs in the medium of cell cultures used for the invasion assay was determined as described by Barletta *et al.* (24). Briefly, concentrated supernatants were electrophoresed on 8% polyacrylamide gel added with porcine gelatin (Sigma Chemical Company). After the run, the gel was incubated at 37°C for 24 h in a solution containing 50 mM Tris-HCl, 200 mM NaCl, and 5 mM $CaCl_2$ and then stained with Coomassie blue. Samples obtained from the medium of HT1080 cell cultures, which are known to express MMP-9 and MMP-2 at high levels were used as controls (24).

RESULTS AND DISCUSSION

The effect of LH/hCG on cell invasiveness of Hec1A cells line, a human EC cell line expressing the LH/hCG-R (inset in Figs. 2*a* and 8), was tested by analyzing the number of cells migrated through Matrigel-covered porous membranes inserted into Boyden chambers. As shown in Fig. 1*a*, hrLH increased Hec1A cell invasion through Matrigel, almost doubling the number of cells migrated through the membrane after 24 h of incubation. A similar effect was obtained after addition of hCG (Fig. 1*b*). Interestingly, cells seeded on uncoated porous filters displayed a moderate invading activity, which was almost unaffected by hrLH addition (data not shown).

The effect of hrLH on Hec1A cell invasion turned out to be dependent on LH/hCG-R expression on the plasma membrane. In fact, the hrLH-induced increase in cell invasion through Matrigel was 10-fold in a clone of Hec1A (clone 1s; gray bars in Fig. 2*a*), developed in our laboratory, which expresses the LH/hCG-R at high levels (compare *Lane 2* with *Lane 1* in the inset to Fig. 2*a*). On the other hand, hrLH has almost no effect on the invasion of a completely unrelated human epithelial cell line (HEK 293 cells; Fig. 2*b*), which lacks the LH/hCG-R (25, 26). We also confirmed the absence of such receptors in these cells by RT-PCR (data not shown).

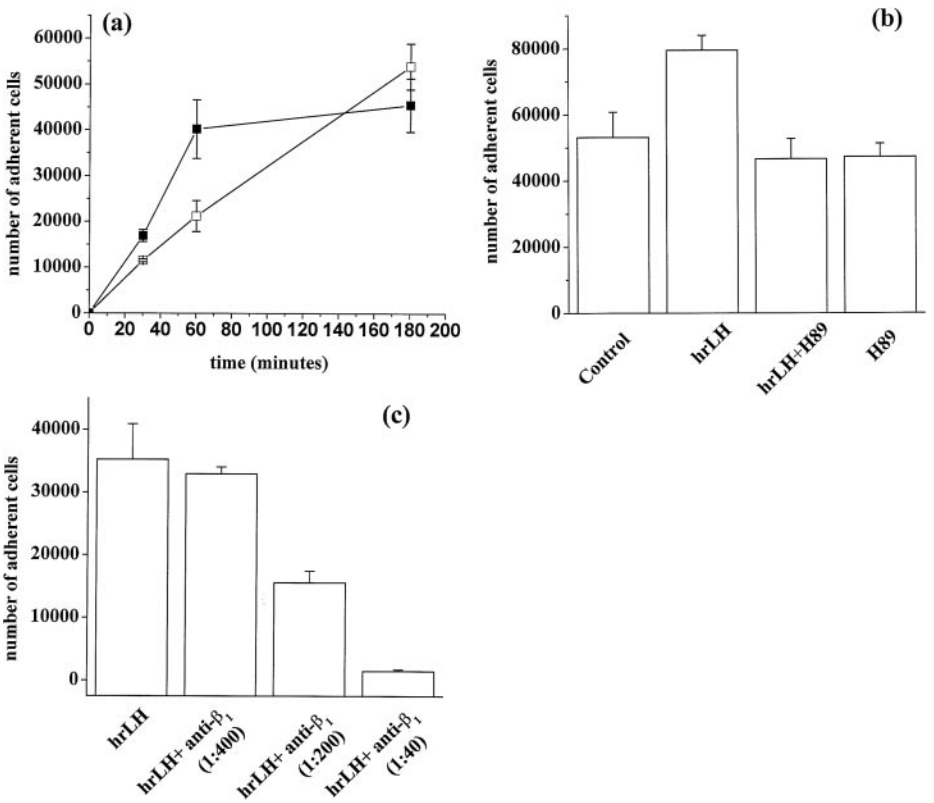

Fig. 4. Effect of hrLH on Hec1A cell adhesion to Matrigel and modulatory effect by H 89 and anti-β_1 antibodies. *a*, effect of hrLH on cell adhesion to Matrigel: cells were radiolabeled for 48 h; appropriate samples were treated during this time for 24 h with hrLH as reported in "Materials and Methods." Afterward, untreated- and hrLH-treated cells were detached from the dish and cell suspensions inoculated into Matrigel-coated wells (time 0) and incubated for different times. Values are reported as adherent cells calculated as detailed in "Materials and Methods" and are the means ± SE of measurements carried out in quadruplicate and refer to a typical experiment; ■ = hrLH-treated cells; □ = control, untreated cells. *b*, effect of H89 on adhesion of Hec1A cells in the presence or in the absence of hrLH. Cells were treated as reported in *a*, except that the appropriate samples were treated for 20 h with 10 μM H89 at the end of the labeling time. At the end of incubation, cells were seeded onto Matrigel-coated wells as above. Values are reported as adherent cells after 30 min of incubation, calculated as detailed in "Materials and Methods" and are the means ± SE of measurements carried out in quadruplicate and refer to a typical experiment. *c*, effect of anti FN-R antibodies on cell adhesion to Matrigel in the presence of hrLH. Experiments were performed as reported in *a*, except that cells were treated with different dilution of anti-FN receptor at the beginning of their incubation onto Matrigel. Antibody dilutions were the following: 1:400; 1:200; and 1:40. Values are reported as adherent cells after 30 min of incubation, calculated as detailed in "Materials and Methods" and are the means ± SE of measurements carried out in quadruplicate and refer to a typical experiment.

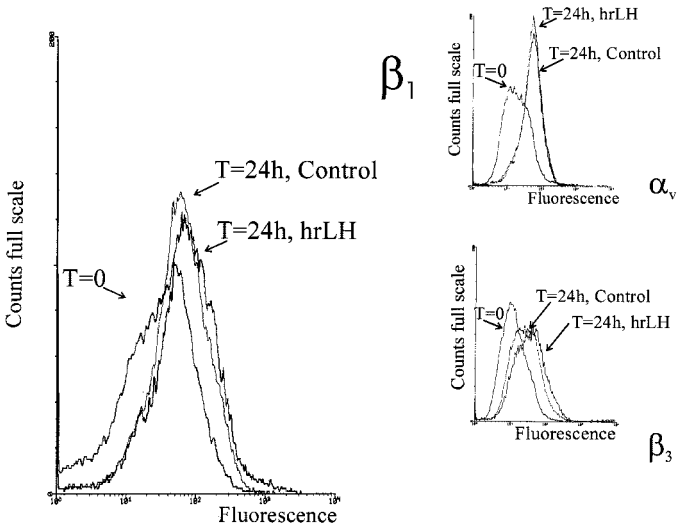

Fig. 5. Flow cytometry analysis of β_1 integrin expression in Hec1A cells. Cells were cultured in DMEM + BSA medium on a Matrigel substrate in the absence (Control) or in the presence (*hrLH*) of 0.3 UI/ml hrLH. At time 0 (T = 0) and after 24 h of incubation (T = 24 h), cells were detached and incubated with anti-β_1 (*a*), anti-β_3, and anti-α_v antibodies (*insets*) as reported in "Materials and Methods," followed by an incubation with a FITC-labeled antimouse antibody. Flow cytometry analysis was performed as reported in "Materials and Methods." *Inset:* flow cytometric analysis of β_3 and α_v integrin expression in Hec1A cells. Experiment was carried out as reported above. Data reported in this figure and inset represent a typical experiment.

The dependence of the proinvasion effect exerted by hrLH on Hec1A cells on the signaling mechanisms induced by binding to its specific receptor was therefore tested. It has been reported (15) that the activation of LH/hCG-R may induce the activation of both the cAMP/PKA and PLC/inositide tris-phosphate signaling pathways. The involvement of both these pathways in the proinvasion effect operated by hrLH on Hec1A cells was therefore analyzed by using specific inhibitors (the PKA inhibitor H89 and the PLC inhibitor U73122). As reported in Fig. 3, although the addition of H89 almost completely inhibited the increase in cell invasiveness through Matrigel induced by hrLH, the PLC inhibitor U73122 had no significant effect. It can be therefore argued that the effect of hrLH on Hec1A cell invasiveness occurs via the signaling pathway initiated by hrLH binding to LH/hCG-R and centered on the activation of PKA.

Because the process of tumor cell invasion is regulated both by the engagement of adhesion receptors with proteins of the basal membrane and of the extracellular matrix (27, 28) and by the secretion of collagenolytic enzymes, named MMPs (29, 30), the involvement of both these mechanisms in the proinvasiveness effect of hrLH was tested. The involvement of integrin receptors was tested by analyzing cell adhesion of Hec1A cells treated or untreated with hrLH and seeded on Matrigel-coated dishes. As shown in Fig. 4, Hec1A cells treated with hrLH adhere more quickly to Matrigel: this effect is already evident after 30 min of incubation, and the vast majority of cells are firmly adherent to the substratum within 60 min of incubation, instead of after 180 min as in the controls (Fig. 4*a*). Moreover, as shown in Fig. 4*b*, the increase in adherent cells operated by hrLH addition after 30 min of incubation onto Matrigel turned out to be completely abolished by cell pretreatment with H89; this suggests that the increase in cell adhesiveness induced by hrLH relies on the activation of the cAMP/PKA signaling pathway. Finally, because the main integrins involved in cell adhesion to Matrigel are those containing the β_1 subunit, the involvement of this integrin subunit in the above reported increase in cell adhesiveness to Matrigel by hrLH was tested. Indeed, Hec1A cell adhesion to Matrigel turned out to be totally dependent on β_1 integrin activity as witnessed by the dose-dependent inhibitory effect of anti-β_1 antibodies (anti-FN-R antibodies; Fig. 4*c*).

To explain these data, the possibility that hrLH induced an up-regulation of β_1 subunit on the plasma membrane was tested. The expression of this subunit on the plasma membrane was analyzed by flow cytometry at time 0 and after 24 h of incubation on Matrigel, both in the absence and in the presence of hrLH. As shown in Fig. 5, although adhesion to Matrigel induces *per se* an up-regulation of β_1, hrLH addition does not alter this expression pattern. Interestingly, an up-regulating effect of Matrigel on integrin expression was evident also for two other integrin subunits, namely β_3 and α_v (Fig. 5, inset). Therefore, the effect of hrLH on integrins is apparently mediated by an increase in integrin

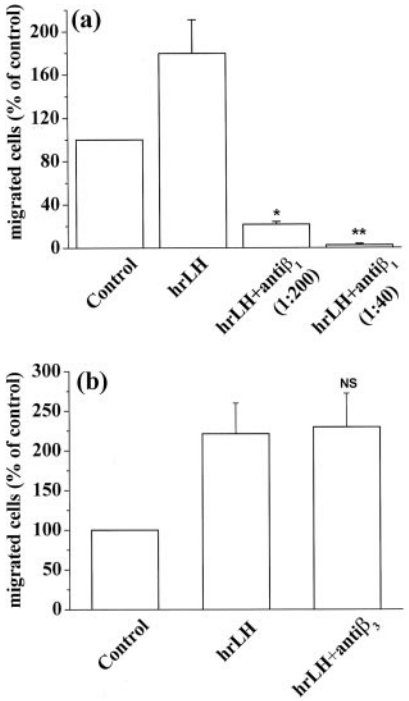

Fig. 6. Effect of anti-β_1 and anti-β_3 antibodies on cell invasion through Matrigel in the presence of hrLH. *a*, effect of anti-β_1 antibodies. Cells treated as reported in the legend to Fig. 1 were treated with different dilution of anti-β_1 (anti-FN receptor) antibodies at time 0. Antibody dilutions were the following: 1/200 and 1/40. Data are reported as the percentage of migrated cells as compared with untreated cells (control), and are the means ± SE of two separate experiments, each carried out in triplicate. * = significantly different as compared with "hrLH" (ANOVA; Bonferroni test; $P = 0.002$); ** = significantly different as compared with "hrLH" (ANOVA; Bonferroni test; $P = 0.001$); *b*, effect of anti-β_3 antibodies. The effect of 9 μg/ml anti-β_3 antibody was tested on Hec1A cells and treated with hrLH as reported in the legend to Fig. 1. Data are reported as the percentage of migrated cells as compared with untreated cells (control) and are the means ± SE of two separate experiments, each carried out in triplicate. NS = not significantly different as compared with "hrLH" (Student's *t* test).

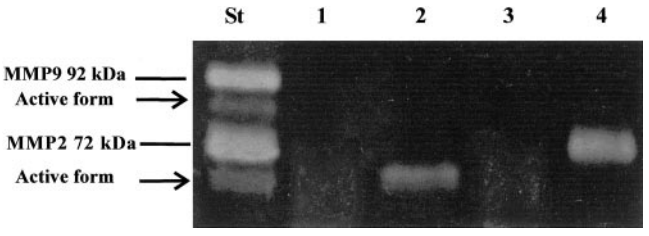

Fig. 7. Effect of hrLH on MMP2 secretion by Hec1A cells seeded onto Matrigel. Cells were seeded in DMEM + BSA onto Matrigel in the absence or in the presence of 0.3 UI/ml hrLH, and then enzymatic activity was detected with zymography. Representative gelatin zymograms of samples from untreated cells (*Lane 1*), cells treated with 0.3 UI/ml hrLH (*Lane 2*), cells treated with 0.3 UI/ml hrLH in the presence of 10 μM PKA-inhibitor H89 (*Lane 3*), and from cells treated with hrLH in the presence of 1:40 anti-β_1 (anti-FN-R) antibodies (*Lane 4*). Purified MMP-2 and MMP-9 from a culture of HT1080 were used as standard reference of both inactive and active forms of the enzymes (23) and were run in parallel (St).

Table 1 *LH/hCG-R expression in primary EC*

RNA extracted from tissue samples of different ECs were retrotranscribed and amplified by a nested PCR using two couple of primers specific for *Lh/hCG-R* (see "Materials and Methods"). Amplification of *gapdh* as a positive internal control as well as for determination of the integrity of RNA was performed for each sample (data not shown).

Histotype	LH-R
AC-SD[a]	−
AC-SD	−
S-AC	+
E-AC	+
E-AC	+
AC-SD	−
E-AC	−
E-AC	−
E-AC	+
E-AC	−
AC-SD	−
E-AC	−
E-AC	−
CC-AC	−
E-AC	+
AC-SD	+
E-AC	−
E-AC	+
E-AC	−
SR-AC	+
AC-SD	+
E-AC	+

[a] AC-SD, adenocarcinoma endometrioid with squamous differentiation; S-AC, secretory adenocarcinoma; E-AC, endometrioid adenocarcinoma; CC-AC, clear cell adenocarcinoma; and SR-AC, serous adenocarcinoma.

avidity for the substratum more than by an increase in the amount of integrin expression on the plasma membrane.

Hence, the possibility that inhibition of β_1 integrins could also interfere with Hec1A cell invasion through Matrigel was analyzed: as shown in Fig. 6, the addition of various amounts of anti β_1 antibodies to hrLH-treated Hec1A cells induced a dose-dependent inhibition of cell invasion through Matrigel (Fig. 6*a*). On the other hand, anti-β_3 antibodies did not affect the hrLH-induced increase in Hec1A cell invasiveness (Fig. 6*b*).

On the whole, data obtained thus far indicate that EC cell invasion through Matrigel occurs via the functional activation of β_1 adhesiveness induced by hrLH.

Therefore, the secretion of MMPs was tested on Hec1A seeded onto Matrigel in the absence or in the presence of hrLH through a zymographic assay. Fig. 7 shows that Hec1A cells are almost unable to secrete MMPs in basal conditions (control), as reported previously (8). The addition of hrLH induces the secretion of active MMP-2 (bottom arrow on the left side of Fig. 7), a MMP widely involved in tumor cell invasion through basement membranes (9, 29, 30). Interestingly, the induction of MMP-2 secretion by hrLH was completely abolished by pretreatment of cells with the PKA inhibitor H89, here again, stressing the relevance of this signaling pathway in the proinvasiveness activity of hrLH on EC cells. Moreover, preincubation with anti-FN-R (anti-β_1) antibodies abolishes the proteolytic activation of the proenzyme MMP-2, thus leading to an accumulation of its inactive form.

On the whole, data gathered thus far in the Hec1A cell model indicate that hrLH (as well as hCG) increases tumor cell invasion through the basal membrane-mimicking substratum Matrigel by activating the LH/hCG-R-dependent cAMP/PKA signaling pathway, which in turn switches on proinvasion mechanisms centered on the activation of β_1 integrin avidity for Matrigel substratum and induction of the secretion of active MMP2.

The possibility that this mechanism could be somehow involved in EC cell invasiveness *in vivo* was then studied. As a first approach, we tested whether the LH/hCG-R was indeed expressed in primary human EC, as reported by Lin *et al.* (13). Results obtained from 22 cases of primary EC indicated that 45% of tumors indeed expressed the receptor (Table 1 and Fig. 8). It is worth noting (Fig. 8, *Lane 4*) that EC samples expressed mainly the splice variant weighing 153 bp as reported by Lin *et al.* (13).

A cell invasion assay was therefore performed as above on selected cases from Table 1 displaying different expression of LH/hCG-R, immediately after surgical removal form the uterus. As shown in Fig. 9*a*, five of seven cases increased their migratory activity after hrLH addition with an average doubling of the number of migrated cells after 24 h of incubation in the presence of the GT. Interestingly, here again, the hrLH-induced effect was apparently related to the expression of LH/hCG-R by tumor cells. In fact, only those primary tumors that expressed the LH/hCG-R increased their invasion through Matrigel in response to hrLH addition (compare samples in Fig. 9 with those in Table 1). Interestingly, the proinvasiveness effect of hrLH turned out to be, here again, almost completely inhibited by the PKA inhibitor H89 (Fig. 9*b*).

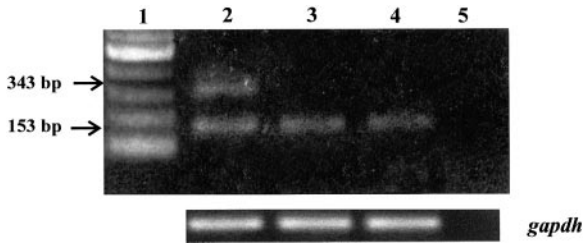

Fig. 8. LH/hCG receptor expression in the human Hec1A cell line and in a representative primary endometrial adenocarcinoma sample. RNA extracted from human testis (as positive control), Hec1A and primary EC samples were retrotranscribed and amplified by a nested PCR using two couple of primers specific for *Lh/hCG-R. Lane 1:* molecular weight standard 100 bp (New England Biolabs); *Lane 2:* testis; *Lane 3:* Hec1A; *Lane 4:* primary EC sample; and *Lane 5:* omissis template as negative control. The panel at the bottom shows the amplification of human (*gapdh*) mRNA as a positive procedural control as well as for determination of the integrity of RNA.

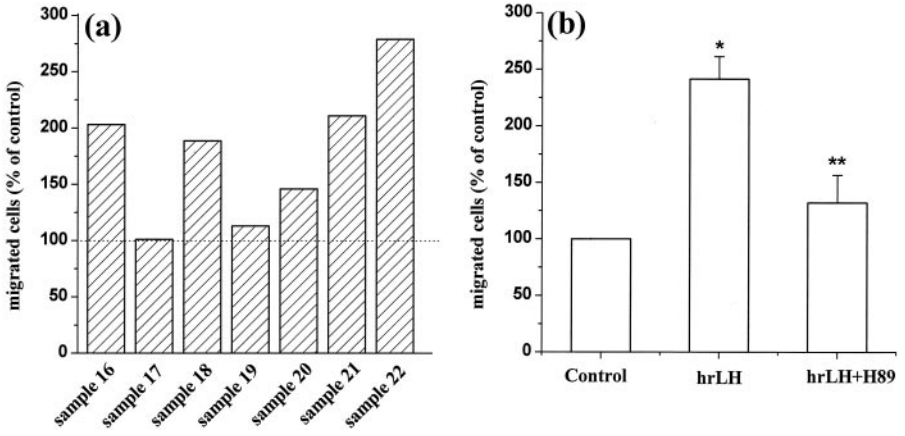

Fig. 9. *a*, effect of hrLH on invasiveness of tumor cells from primary selected EC samples. The invasion assay was performed as reported in the legend to Fig. 1 on endometrial adenocarcinoma cells extracted directly from fresh tissues belonging to 7 of 22 samples reported in Table 1. Sample numbers correspond to those reported in Table 1. *Bars* represent the percentage of migrated cells in the presence of 0.3 UI/ml hrLH as compared with the untreated controls, represented in the figure by the continuous line intercepting the y axis at the value of 100. *b*, effect of the PKA inhibitor H89 on the migration of adenocarcinoma cells induced by hrLH. The invasion experiment was carried out as described in the legend to Fig. 3 on three adenocarcinoma samples, two of which correspond to samples reported in *a* and in Table 1. Data are reported as the percentage of migrated cells as compared with untreated cells (control) and are the means ± SE of three separate experiments, each carried out in triplicate. * = significantly different as compared with "control" (Student's *t* test; $P = 0.002$); ** = significantly different as compared with "hrLH" (Student's *t* test; $P = 0.026$).

On the whole, these results indicate that the activation of LH/hCG-R by the proper ligand induces an increase in cell invasiveness also of primary EC cells, here again, through the activation of PKA signaling.

In this study, we provide evidence that LH/hCG is involved in tumor progression of EC because it induces an invasive phenotype in the moderately differentiated EC cell line Hec1A. The mechanism for this involves LH/hCG binding to its receptor and inducing the activation of PKA, which in turn activates β_1 integrin adhesiveness to Matrigel and therefore the secretion of active MMP-2. In this light, PKA has been indeed recently reported to positively regulate cell spreading and motility in different cellular models (31, 32); moreover, our data show that the PKA-dependent activation of β_1 integrin appears to be also necessary for the induction of the enzymatic activation of MMP-2.

Data demonstrating that this mechanism is also operative in primary EC are presented. No data regarding a direct effect of LH/hCG on EC cell lines and primary EC invasiveness have been published previously to the best of our knowledge.

Data reported in this article not only stress the role of GTs as tumor progression factors but also lend support to the use of GnRH analogues to inhibit pituitary LH secretion in the therapeutic protocols of LH/hCG-R-expressing tumors (19) because this use could block the invasive features of the tumor and thus permit longer survival, often without interfering with other aspects of neoplastic growth such as proliferation and differentiation as reported by Noci *et al.* (19).

ACKNOWLEDGMENTS

We thank Dr. Emanuela Barletta for her help in zymographic experiments and Dr. Serena Pillozzi for contributing to adhesion test. We also thank Dr. Guido Tarone and Dr. Paola Defilippi (University of Torino) for supplying anti-integrin antibodies. We thank Dr. Harry Witchel for the kind revision of the English (University of Bristol) and Dr. Vieri Boddi (University of Firenze) for the statistical analysis.

REFERENCES

1. Wingo, P. A., Ries, L. A. G., Giovino, G. A., Miller, D. S., Rosenberg, H. M., Shopland, D. R., Thun, M. J., and Edwards, B. K. Annual report to the nation on the status of cancer, 1973–1996. J. Natl. Cancer. Inst. (Bethesda), *91*: 675–690, 1999.
2. Li, S. and Wang, E. (eds.) Endometrial Carcinoma, pp. 1–22. New York: The People's Medical Publishing House Sprinter-Verlag, 1990.
3. Pike, M. C., Peters, R. K., Cozen, W., Pobst-Hensch, N. M., Felix, J. C., Wan, P., and Mack, T. M. Estrogen-progestin replacement therapy and endometrial cancer. J. Natl. Cancer Inst. (Bethesda), *89:* 1110–1116, 1997.
4. DiSaia, P. J., and Creasman W. T. Adenocarcinoma of the uterus. *In:* P. J. DiSaia and W. T. Creasman (eds.), Clinical Gynecologic Oncology (Ed. 5), pp. 134–167, St. Louis: Mosby, 1997.
5. Pike, M. C. Age-related factors in cancers of the breast, ovary and endometrium. J. Chronic Dis., *40* (Suppl. 2): 59S–69S, 1987.
6. Deligdisch, L., and Holinka, C. F. Endometrial carcinoma: two diseases? Cancer Detect. Prev., *10:* 237–246, 1987.
7. Davies, S., Bax, C. M. R., Chatzaki, E., Chard, T., and Iles, R. K. Regulation of endometrial cancer cell growth by luteinizing hormone (LH) and follicle stimulating hormone (FSH). Br. J. Cancer, *83:* 1730–1734, 2000.
8. Mizumoto, H., Saito, T., Ashihara, K., Nishimura, M., Takanara, R., and Kudo, R. Acceleration of invasive activity via matrix metalloproteinases by transfection of the *estrogen receptor* α gene in endometrial carcinoma cells. Int. J. Cancer, *100:* 401–406, 2002.
9. Park, D-W., Ryu, H-S., Choi, D-S., Park, Y-H., Chang, K-H., and Min, C. K. Localization of matrix metalloproteinases on endometrial cancer cell invasion *in vitro*. Gynaecologic. Oncol., *82:* 442–449, 2001.
10. Dai, D., Wolf, D. M., Litman, E. S., White, M. J., and Leslie, K. K. Progesterone inhibits human endometrial cancer cell growth and invasiveness: down-regulation of cellular adhesion molecules through progesterone B receptors. Cancer Res., *62:* 881–886, 2002.
11. Shiffenbauer, Y. S., Abramovitch, R., Meir, G., Nevo, N., Holzinger, M., Itin, A., Keshet, E., and Neeman, M. Loss of ovarian function promotes angiogenesis in human ovarian carcinomas. Proc. Natl. Acad. Sci. USA, *94:* 13203–13208, 1997.
12. Zygmunt, M., Herr, F., Keller-Schoenwetter, S., Kunzi-Rapp, K., Munsted, K., Rao, C. V., Lang, U., and Preissner, K. T. Characterization of human chorionic gonadotropin as a novel angiogenic factor. J. Clin. Endocrinol. Metab., *87:* 5290–5296, 2002.
13. Lin, J., Lei, Z. M., Lojun, S., Rao, C. V., Satyaswaroop, P. G., and Day, T. G. Increased expression of luteinizing hormone /human chorionic gonadotropin receptor gene in human endometrial carcinomas. J. Clin. Endocrinol. Metab., *79:* 1483–1491, 1994.
14. Mori, T., and Rao, C. V. Messanger ribonucleic acid expression of LH/hCG receptor gene in human ovarian carcinomas. Eur. J. Cancer, *33:* 1501–1507, 1997.
15. Dufau, M. L. The luteinizing hormone receptor. Annu. Rev. Physiol., *60:* 461–496, 1998.
16. Salvador, L. M., Maizels, E., Hales, D. B., Miyamoto, E., Yamamoto, H., and Hunzicker-Dunn, M. Acute signaling by the LH receptor is independent of protein kinase C activation. Endocrinology, *143:* 2986–2994, 2002.
17. Hirakawa, T., Galet, C., and Ascoli, M. MA-10 cells transfected with the human lutropin/choriogonadotropin receptor (hLHR): a novel experimental paradigm to study the functional properties of the hLHR. Endocrinology *143:* 1026–1035, 2002.
18. Stepien, A., and Ziecik, A. J. Second messenger systems in the action of LH and oxytocin on porcine endometrial cells *in vitro*. Theriogenology, *57:* 2217–2227, 2002.
19. Noci, I., Borri, P., Bonfirraro, G., Chieffi, O., Arcangeli, A., Cherubini, A., Dabizzi, S., Buccoliero, A. M., Paglierani, M., and Taddei, G. L. Longstanding survival without cancer progression in a patient affected by endometrial carcinoma treated primarily with leuprolide. Br. J. Cancer, *85:* 333–336, 2001.
20. Cherubini, A., Taddei, G. L., Crociani, O., Paglierani, M., Buccoliero, A. M., Fontana, L., Noci, I., Borri, P., Borrani, E., Giachi, M., Becchetti, A., Rosati, B., Wanke, E., Olivotto, M., and Arcangeli, A. HERG potassium channels are more frequently expressed in human endometrial cancer as compared to non-cancerous endometrium. Br. J. Cancer, *83:* 1722–1729, 2000.
21. Maniatis, T., Fritsh, E. F., and Sambrook, J. Molecular Cloning: A Laboratory Manual, Ed. 2. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
22. Arcangeli, A., Becchetti, A., Mannini, A., Mugnai, G., Defilippi, P., Tarone, G., Del Bene, M. R., Barletta, E., Wanke, E., and Olivotto, M. Integrin-mediated neurite outgrowth in neuroblastoma cells depends on the activation of potassium channels. J Cell. Biol., *122:* 1131–1143, 1993.
23. Hofmann, G., Bernabei, P. A., Crociani, O., Cherubini, A., Guasti, L., Pillozzi, S., Lastraioli, E., Polvani, S., Bartolozzi, B., Solazzo, M., Gragnani, L., Defilippi, P., Rosati, B., Wanke, E., Olivotto, M., and Arcangeli, A. HERG K+ channels activation during $\beta(1)$ integrin-mediated adhesion to fibronectin induces an up-regulation of $\alpha(v)\beta(3)$ integrin in the preosteoclastic leukemia cell line FLG 29.1. J. Biol. Chem., *276:* 4923–4931, 2001.
24. Barletta, E., Mugnai, G., and Ruggieri, S. Platelet activating factor inhibits the expression of matrix metalloproteinases and affects invasiveness and differentiation in a system of human neuroblastoma clones. Biol. Chem., *383:* 189–197, 2002.
25. Lakkakorpi, J. T., and Rajanemi, H. J. Regulation of intracellular free Ca2+ by the LH/CG receptor in an established cell line 293 expressing transfected rat receptor. Mol. Cell. Endocrinol., *99:* 39–47, 1994.
26. Lee, P. S., Buchan, A. M., Hsueh, A. J., Yuen, B. H., and Leung, P. C. Intracellular calcium mobilization in response to the activation of human wild-type and chimeric gonadotropin receptors. Endocrinology, *143:* 1732–1740, 2002.
27. Prifti, S., Zourab, Y., Koumouridis, A., Bohlmann, M., Strowitzki, T., and Rabe, T. Role of integrins in invasion of endometrial cancer cell lines. Gynecol. Oncol., *84:* 12–20, 2002.
28. Giannelli, G., Astigiano, S., Intonaci, S., Morini, M., Barbieri, O., Noonan, D. M., and Albini, A. Role of $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins in tumor invasion. Clin. Exp. Metastasis, *19:* 217–223, 2002.
29. Boyd, R. S., and Balkwill, F. R. MMP-2 release and activation in ovarian carcinoma: the role of fibroblast. Br. J. Cancer, *80:* 315–321, 1999.
30. Westerlund, A., Hujanen, P. U., and Turpeenniemi-Hujanen, T. Fibroblasts stimulate human ovarian cancer cell invasion and expression of 72-kDa gelatinase A (MMP-2). Gynecol. Oncol., *67:* 76–82, 1997.
31. O'Connor, K. L., and Mercurio, A. M. Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells. J. Biol. Chem. *276:* 47895–17900, 2001.
32. Whittard, J. D., and Akiyama, S. K. Positive regulation of cell-cell and cell-substrate adhesion by protein kinase A. J. Cell Sci., *114:* 3265–3272, 2001.