Endocrine, *vol. 16, no. 3, 207–215, December 2001* 0969–711X/01/16:207–215/$12.25

Tissue Kallikrein and Bradykinin B2 Receptor in Human Uterus in Luteal Phase and in Early and Late Gestation

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This study was addressed to evaluate the temporospatial pattern of key components of the kallikrein-kinin system in human uterus in luteal phase (*n* = 7), early pregnancy (isolated spontaneous abortions, *n* = 11; ectopic pregnancies, *n* = 9), idiopathic preterm deliveries (*n* = 5), and term gestations (*n* = 12). Tissue kallikrein mRNA and protein and the type 2 bradykinin receptor (B2R) protein were expressed in luminal and glandular epithelium and in endothelial cells of stromal and myometrial blood vessels, while tissue kallikrein mRNA and B2R, but not tissue kallikrein protein, were observed in decidual cells and in arteriolar and myometrial muscle. A greater signal intensity for tissue kallikrein mRNA and protein and of B2R protein was observed in the early pregnancy samples. The sites and variations of the tissue kallikrein mRNA and protein and of the B2R protein in the human uterus and in fallopian tubes during the luteal phase and in pregnancy coincide with those described for other vasoactive effectors such as nitric oxide, prostacyclins, growth factors, and renin. The uterine localization of the main enzyme and receptor of the tissue kallikrein-kinin system in key sites for embryo attachment, implantation, placentation, maintenance of placental blood flow, and parturition supports the notion that the kallikrein-kinin system participates in these processes, probably through vasodilation, increased vasopermeability, enhanced matrix degradation, stimulation of cell proliferation, and myometrial contractility.

Key Words: Tissue kallikrein; type 2 bradykinin receptor; human uterus; pregnancy; spontaneous abortions; ectopic pregnancy.

Introduction

Tissue kallikrein is a serine protease that cleaves kininogens and generates kallidin and bradykinin *(1)*. Bradykinin exerts its actions through two known receptors, the bradykinin type 1 receptor (B1R), related to proliferation, and the type 2 bradykinin receptor (B2R), related to vasodilation and edema formation *(2,3)*. In the rat uterus, tissue kallikrein mRNA has been demonstrated, and the corresponding protein (rK1), as well as the B2R, have been immunolocalized in luminal and glandular epithelium, implantation node, deciduomata, and subplacental sinusoids *(4–9)*. In human uterus, tissue kallikrein (hK1) mRNA and protein, as well as B2R, are present in luminal and glandular epithelial cells *(6,10,11)*. In human placenta, kallikrein-like activity *(12)* and the kallikrein mRNA and protein *(13)* are present in trophoblast and fetal endothelium. Interestingly, both the tissue kallikrein mRNA and protein levels are higher in early pregnancy samples *(13)*.

To study the variations in the kallikrein-kinin system in maternal human reproductive tissues during pregnancy, the presence of uterine kallikrein mRNA and protein and the B2R were studied by *in situ* hybridization (ISH) and immunohistochemistry in uteri obtained in luteal phase and in idiopathic pregnancy loss as well as in preterm and term pregnancies. To evaluate the response to the embryo and to the hormonal milieu of pregnancy, we used the clinical model of ectopic pregnancy.

Results

Luteal Phase of Menstrual Cycle

Tissue kallikrein mRNA was expressed in uterus in the luminal and glandular epithelium, the endothelium, and the stromal cells of all samples; a few samples exhibited signal in the myometrial cells. In the fallopian tubes, tissue kallikrein mRNA (Fig. 1A) was expressed in the epithelial cells and vascular endothelium, and occasionally in the smooth muscle of blood vessels and of the fallopian wall.

The tissue kallikrein protein was expressed in uterus in luminal and glandular epithelium and in endothelial cells.

Received July 9, 2001; Revised November 25, 2001; Accepted December 6, 2001.

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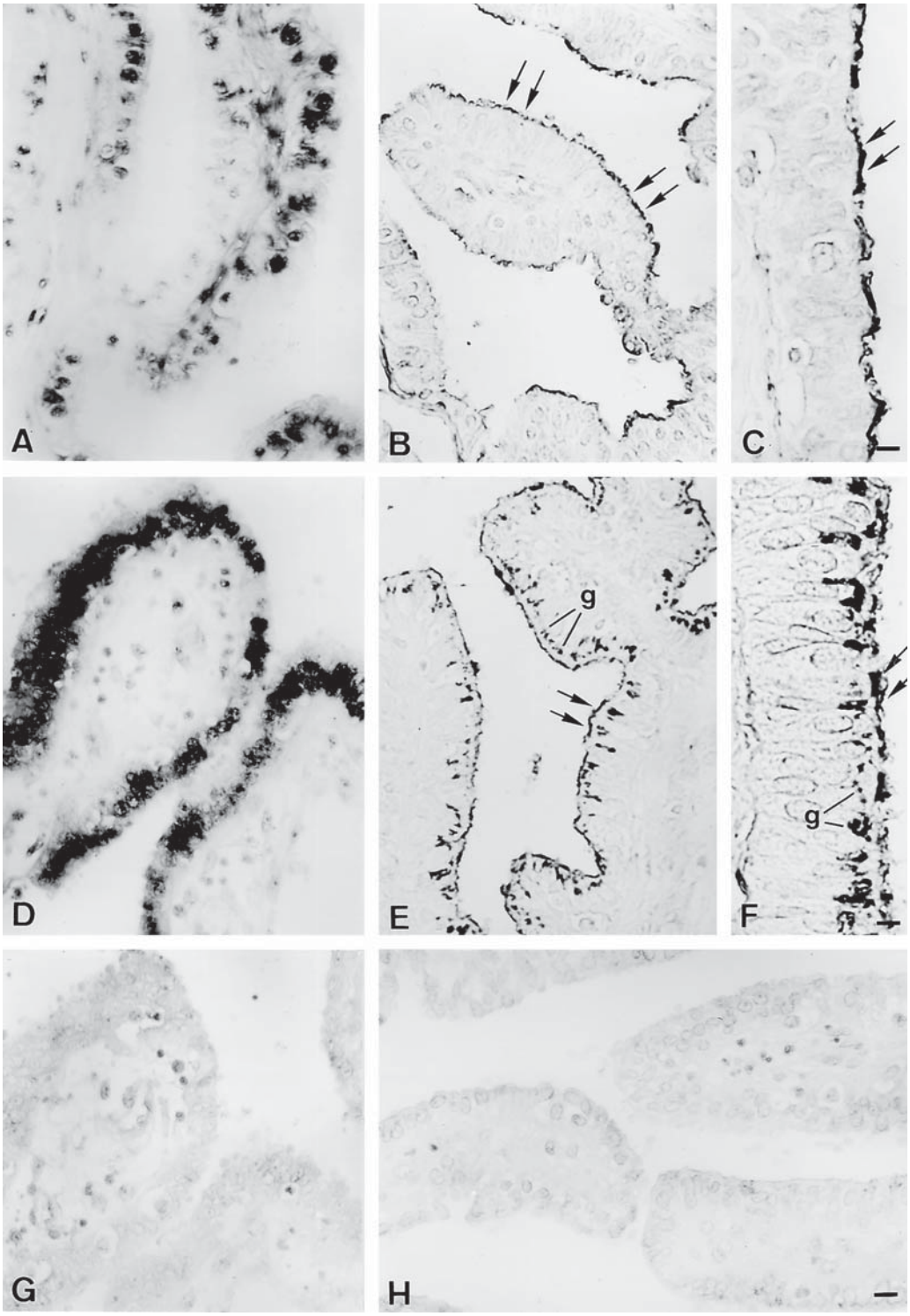

Fig. 1. Expression of tissue kallikrein mRNA (**A,D**) and immunoreactivity to tissue kallikrein protein (**B,C,E,F**) in fallopian tube epithelium in luteal phase (A,B,C), and in implantation site of a 4-wk ectopic pregnancy (D,E,F). Negative control sections were prepared by incubating the sections with labeled sense riboprobes (**G**) or with an excess of purified human urinary kallikrein (**H**). Arrows and g, apical membrane and intracytoplasmic granules expressing tissue kallikrein, respectively. (A,B,D,E,G,H): ×400 (bar = 13 μm); (C,F): ×1000 (bar = 5 μm).

Absence of staining was observed in endometrial stroma and in the smooth muscle of the blood vessels. In the fallopian tube, the epithelial immunoreactivity for tissue kallikrein was predominantly apical and linear (Fig. 1B,C), while the smooth muscle of the blood vessels and the fallopian wall were devoid of staining.

Early Pregnancy

Tissue kallikrein mRNA in ectopic pregnancy was expressed lightly in the uterine luminal and glandular epithelium, whereas a marked signal was observed in the tubal implantation site, the epithelium (Fig. 1D), the endothelium and smooth muscle of small- and large-bore tubal blood vessels, and the smooth muscle. In samples showing decidualization of the tubal stroma, the decidualized cells expressed tissue kallikrein mRNA. In spontaneous abortions, the decidual cells and the vascular endothelium expressed kallikrein mRNA (Fig. 2A,B). In half of the specimens, vascular smooth muscle showed tissue kallikrein mRNA signal. The cytotrophoblast invading maternal blood vessels also expressed kallikrein mRNA (Fig. 2A). The luminal and glandular epithelial cells of the endometrial stroma were positive for kallikrein mRNA.

The tissue kallikrein signal in the uterus of ectopic pregnancies was mild in the luminal and glandular epithelium. In the tubal implantation site, it was present both linearly in the apical membrane and as supranuclear granules; immunoreactivity was graded as 2.2 ± 0.7 (21.5–3.0 interquartile range) vs 1.3 ± 0.8 (1.0–2.0) in the luteal phase ($p < 0.04$) (Fig. 1E,F). The endothelium was also immunoreactive, while the smooth muscle cells of large- and small-bore arterial blood vessels and of the tubal wall were negative. In uterine glands with Arias-Stella reaction, immunoreactivity for kallikrein was observed in the apical membrane and in intracytoplasmic granules (Fig. 3E). In spontaneous abortions, the kallikrein protein was expressed in the epithelial cells of lumen and glands, the endothelium, and the cytotrophoblast invading the blood vessels (Fig. 2C,D). The decidual cells were consistently negative for tissue kallikrein (Fig. 2C).

The B2R protein in ectopic pregnancy was expressed in the tubal epithelium, the endothelium of the tubal blood vessels, and the smooth muscle of the arterioles in the implantation site in the majority of samples studied (Fig. 3A,C,D). The trophoblastic villi in the implantation site showed marked staining of the syncytio and cytotrophoblast (Fig. 3F). In the endometrium, the epithelium in glands with Arias-Stella reaction showed labeling for the bradykinin B2R (Fig. 3B). In spontaneous abortions, the luminal and glandular epithelium, decidual cells, vascular endothelium, and intraarterial cytotrophoblast expressed the B2R protein in all specimens (Fig. 2E,F).

Early Third Trimester and Term

Kallikrein mRNA yielded a positive signal in decidual (Fig. 4A) and endothelial cells (Fig. 4C). The mRNA signal was faintly positive in intraarterial trophoblast (Fig. 4A). Tissue kallikrein was expressed mildly in intraarterial trophoblast and endothelium (Fig. 4B,D), while no reactivity was expressed in decidual cells. The B2R immunoreactivity was positive in endothelium, vascular smooth muscle, and myometrium (Fig. 4E,F) and was slightly positive in decidual cells (Fig. 4G).

Discussion

The present study demonstrates tissue kallikrein content, its site of synthesis, and the terminal vasoactive effector, the B2R, in the luminal and glandular epithelium, decidual cells, trophoblast invading spiral arteries, endothelium, and vascular and myometrial smooth muscle—i.e., sites that compose the fetomaternal interface and intervene in the contact and attachment of the embryo, in placental development and perfusion, and in parturition.

In uterine epithelium and endothelium, the expression of tissue kallikrein mRNA and protein, as well as of B2R, coincide. By contrast, decidual cells and smooth muscle (vascular, fallopian, and myometrial) express tissue kallikrein mRNA and the B2R but not the tissue kallikrein protein. Because of the consistent results obtained by hybridization with a riboprobe and an oligonucleotide, to the stringency of the methodology and to the absence of signal in the control sections, the signal has to be considered specific. The lack of protein detection may be due to a lower sensitivity of the immunohistochemical method or may reflect a reduced translation of the corresponding mRNA.

The coincidence, or vicinity, of the sites of synthesis of kallikrein and of the B2R corroborates the autoparacrine mode of action of the kallikrein-kinin system in the pregnant uterus. Thus, the possible actions of kallikrein-kinins have to be searched within the microenvironement of the embryo/fetus and maternal interface. The marked apical reactivity in the luminal epithelium of the ectopic and orthotopic implantation site, as well as the demonstration of kallikrein in uterine flushings in the rat *(8)* and pig *(14)*, suggests that the enzyme is one of the proteinases that participate in the processes of shedding of the extracellular embryo coverings and subsequent attachment to the luminal epithelium *(15,16)*. Tissue kallikrein may degrade extracellular matrix components by collagenase activation *(17,18)* and could in a latter phase participate in trophoblast invasion. Matrix metalloproteinase-2 (MMP-2), and MMP-7 are present in decidua, and stromal and glandular cells *(19)*.

The B2R in vascular smooth muscle, including large-bore arteries, which in other organs are devoid of immunoreactivity for the B2R *(20)*, underscores the importance of the kallikrein-kinin system in the regulation of blood flow of the pregnant uterus, as both large and small arterial vessels could respond to bradykinin directly by opening potassium channels *(21)* and to its stimulus of nitric oxide (NO) *(22)*, and of prostaglandin I_2 (PGI_2) synthesis *(23)*. Therefore, the sites of NO synthase (NOS) expression may be relevant to the physiologic actions of bradykinin. Endothelial NOS is synthesized in glandular epithelial cells in the menstrual cycle, and in endothelial cells, myometrium,

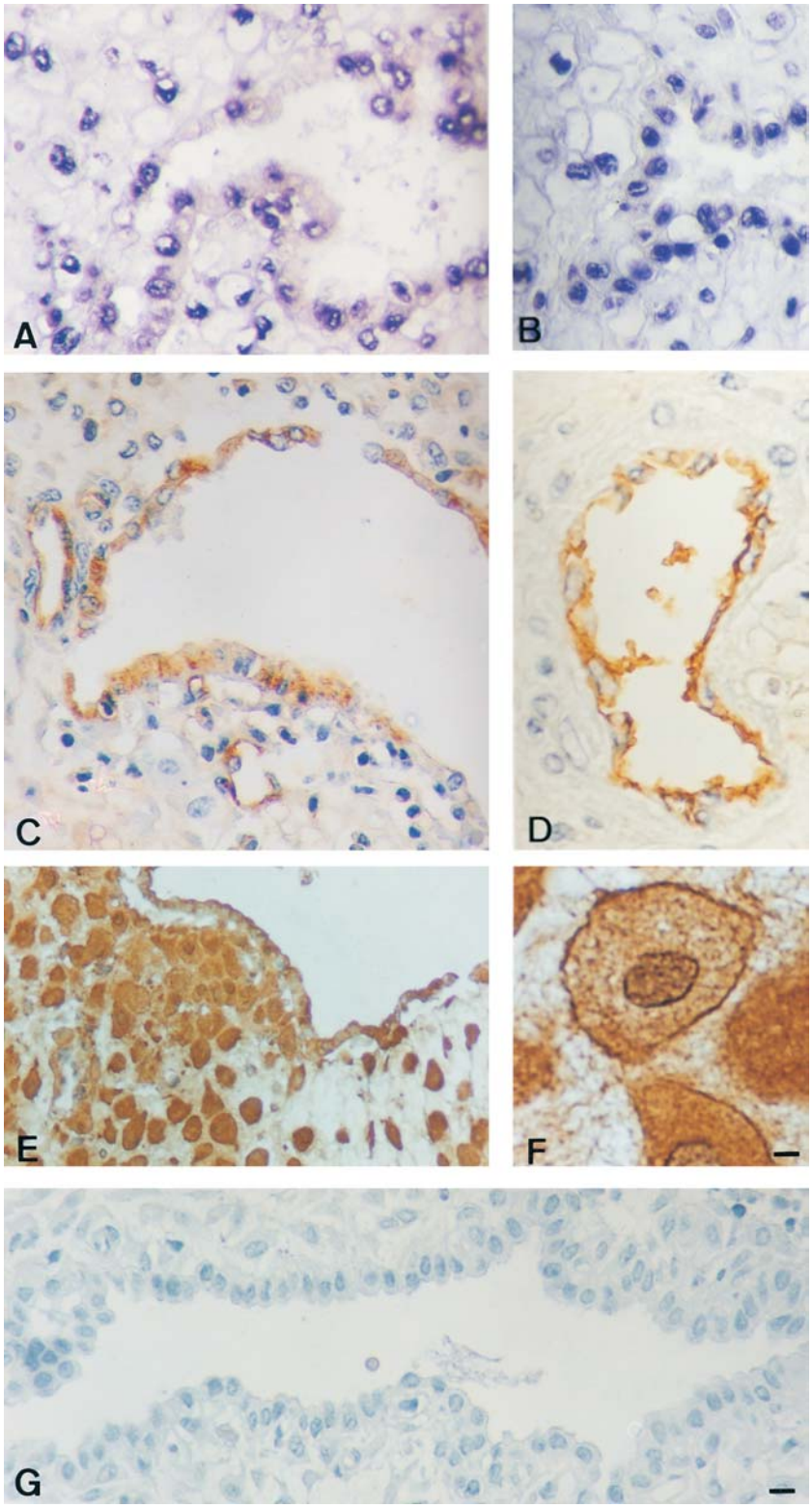

Fig. 2. Endometrial sample obtained from a 9-wk spontaneous abortion depicting expression of tissue kallikrein mRNA in decidual cells and intraarterial cytotrophoblast ([**A**] riboprobe; [**B**] oligonucleotide probe). (**C,D**) The tissue kallikrein protein shows positive reactivity for intraarterial cytotrophoblast and endothelium. (**E,F**) The bradykinin B2R is demonstrated in decidual cells and in intraarterial cytotrophoblast. (**G**) A negative control section incubated without primary antibodies for the B2R is shown. (A,B,C,E,G): ×400 (bar = 13 μm); (D,F): ×1000 (bar = 5 μm).

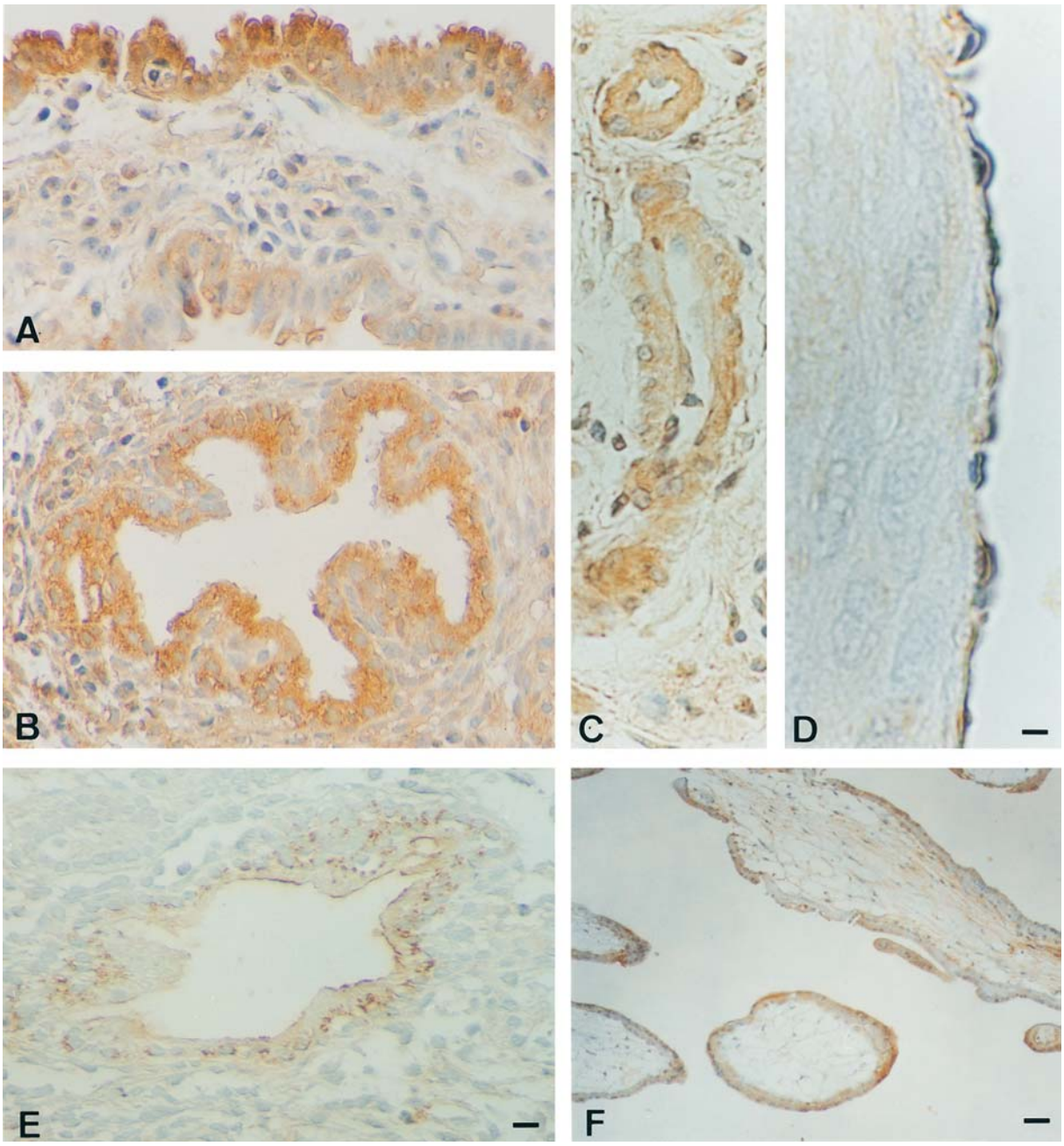

Fig. 3. Immunoreactive B2R (golden brown) in epithelium (**A**), vascular smooth muscle cells, and endothelium of large bore arteries (**C,D**) in tubal implantation site of a 6-wk ectopic pregnancy, in the epithelium of an endometrial gland with Arias-Stella reaction (**B**) and in trophoblastic villi (**F**). (A,B,C,E): ×400 (bar = 13 μm); (D): ×1000 (bar = 5 μm); (F): ×100 (bar = 50 μm).

and decidual cells in early pregnancy *(24)*, localizations that coincide with those of kallikrein and B2R. Bradykinin also increases PGE_2 and PGI_2 in cultured human endometrium and decidua *(25–28)*, and in human umbilical vein endothelial cells stimulates PGI_2 synthesis by the stimulation of phopholipase A_2 and of type 1 and 2 cyclooxygenases *(23)*. Both cyclooxygenases' mRNAs are present in third-trimester decidual homogenates, and decidual endothelial cells are able to synthesize prostacyclin *(29,30)* and could be target enzymes/cells for bradykinin stimulation.

In keeping with the adjacent localization of kallikrein and renin, and the potential of kallikrein to activate prorenin *(31)*, renin has been demonstrated in the first trimester of pregnancy in vascular smooth muscle of unremodeled spiral arteries and in the wall of decidual veins, and at term in decidual cells *(32,33)*. The effects of the tissue kallikrein-kinin system could be modulated by variations in kininase II or angiotensin-converting enzyme, present in spiral artery endothelial cells and perivascular stromal cells in first trimester, declining after wk 10 *(32)*.

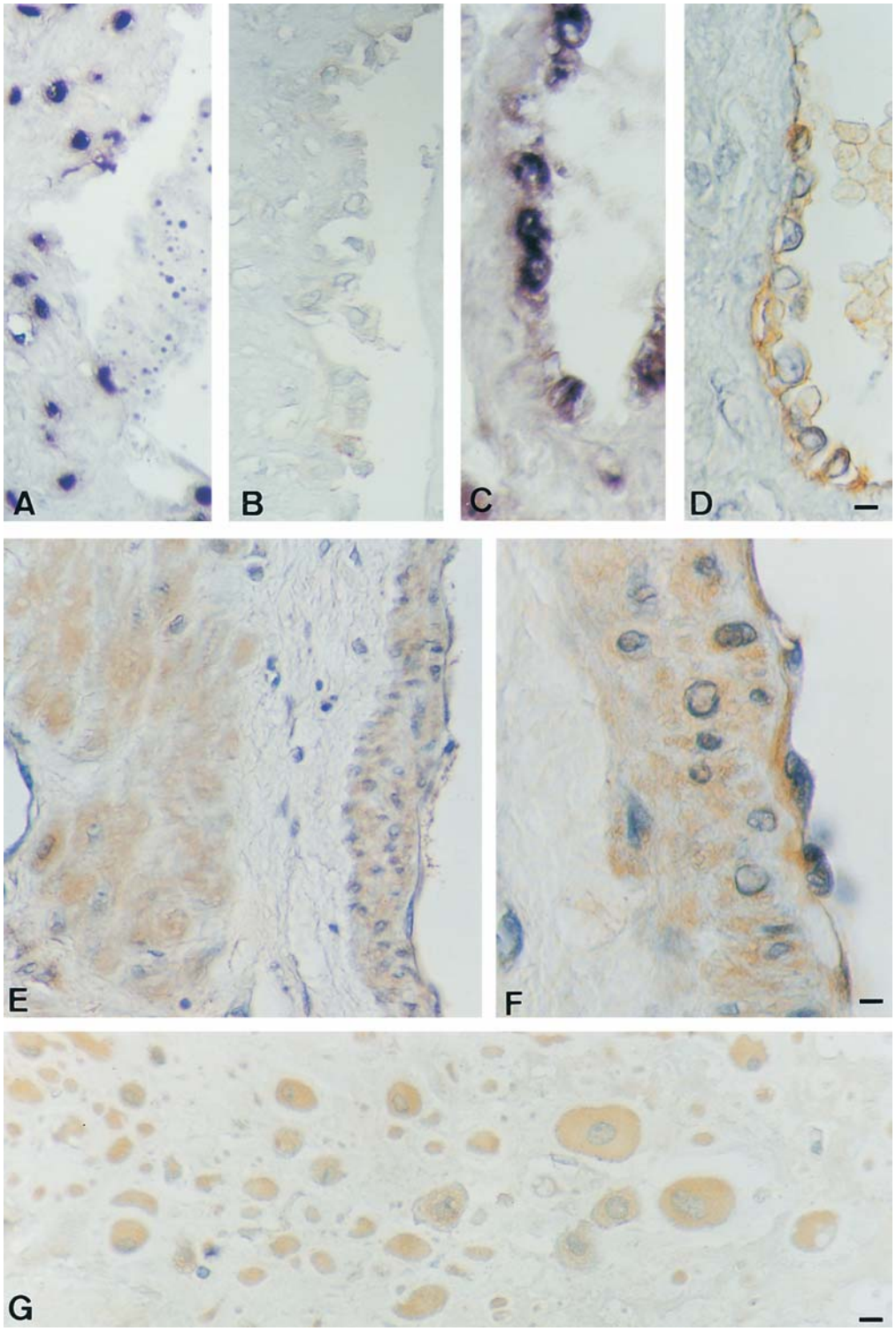

Fig. 4. Decidual section of fetomaternal interface of a term pregnancy showing mRNA for tissue kallikrein in (**A**) intraarterial trophoblast and (**C**) in endothelium. Tissue kallikrein immunoreactivity is depicted in (**B**) intraarterial trophoblast and in (**D**) endothelium. The B2R immunoreactivity is shown in (**E,F**) myometrium and vascular smooth muscle, (**F**) endothelium, and (**G**) decidual cells. (A,B,E,G): ×400. (bar = 13 μm); (C,D,F): ×1000 (bar = 5 13 μm).

Kallikrein activates growth factors, and in pregnancy could act on endometrial stromal vascular endothelial growth factor (VEGF) in luteal phase, and in VEGF in decidual cells, vascular endothelium, and luminal and endometrial epithelium *(34,35)*, to participate in decidual/placental development.

Finally, in animal models kinins stimulate myometrial contractility, and aprotinin, a kallikrein inhibitor, prolongs parturition *(36)*. The expression of bradykinin receptor in myometrium in late pregnancy supports the participation of kinins in laboor.

In the model of ectopic pregnancy, the marked increase in tissue kallikrein mRNA and protein, and of the B2R in the tubal implantation site vs the milder increase in the epithelial and endothelial cells in the distant endometrium, suggest that the embryo constitutes an important stimulus, superimposed on the hormonal milieu (i.e., estrogen, progesterone, human chorionic gonadotropin). While the bradykinin receptor is more prevalently expressed in decidual cells in early than in late pregnancy, its expression in smooth muscle of myometrium and blood vessels in the third trimester is important. This temporal and cell-specific expression suggests different roles and hormonal modulations. In early pregnancy, the kallikrein-kinin system may act on decidualization and stromal blood flow, while in late pregnancy the vasodilatory action remains unchanged and its contractile action on myometrium becomes important. The increased tissue kallikrein content in the maternal face in early pregnancy parallels that observed in placenta *(13)* and urinary excretion in normal pregnancy *(37)*, suggesting that the regulation of the enzyme levels in epithelium and decidua, placenta, and kidney predominantly responds to common early pregnancy signals. The specific factor(s) that modulate uterine kallikrein and bradykinin receptor synthesis throughout pregnancy deserve further elucidation.

Materials and Methods

The study protocol, approved by the Institutional Review Board, was conducted at the Clinical Hospital of the Pontificia Universidad Católica de Chile. Tissues were obtained from cycling women submitted to hysterectomy because of leiomyomas (n = 7), and from pregnant women who presented with isolated spontaneous abortions (n = 11; gestational age: 9.3 ± 2.7 [SD] wk), ectopic pregnancies (n = 9; gestational age: 6.3 ± 1.6 wk), idiopathic preterm deliveries *(38)* (n = 5; gestational age: 32.9 ± 2.4 wk) and term deliveries (n = 12; gestational age: 38.8 ± 1.0 wk). All samples were obtained from normotensive nondiabetic subjects, with similar ages for the different pregnancy groups (31.2 ± 7.0, 32.0 ± 4.9, 28.0 ± 8.2, and 29.6 ± 6.3 yr), and a higher age range for the control group (42.0 ± 1.6 yr). Light microscopy confirmed that the tissues were free of inflammation or ischemia. Control samples corresponded to the luteal phase by histologic dating and represent sections distant from the leiomyoma. Third-trimester specimens were obtained from normotensive women who gave birth to healthy infants with adequate weight for gestational age (2066 ± 278 and 3519 ± 442 g for preterm and term infants, respectively).

Placentas were fixed with 4% formaldehyde for ISH and immunohistochemistry for tissue kallikrein. Immunohistochemistry for the B2R was performed in a limited number of samples, collected in conditions that permitted fixation in freshly prepared periodate-lysine-paraformaldehyde *(9)*. The tissue blocks were dehydrated in a graded series of ethanol and embedded in Histosec or Paraplast-Plus (Sigma, St. Louis, MO). Sections (5 μm) were mounted on glass slides with polylysine.

In Situ Hybridization

Sense and antisense digoxigenin (DIG)-cRNA probes were obtained by in vitro transcription with T7 or SP6 RNA polymerases (Promega, Madison, MI), respectively, of a 168-bp human tissue kallikrein DNA fragment, which corresponds to the 690–857 bp of hKLK1 *(39,40)* cloned into the pGEM T vector (Promega). The specificity of the riboprobe and the ISH procedure have been previously described *(13)*.

In addition, ISH was performed with a DIG-oligonucleotide probe using a 35-base oligonucleotide complementary to 752–786 bp of hKLK1, 3'-end-labeled with DIG-dUTP/dATP (Roche) *(41)*. Hybridization with DIG-oligonucleotide was performed in dewaxed, hydrated, and permeabilized sections. Prehybridization was done in 4X saline sodium citrate (SSC), 1X Denhardt's solution for 30 min at 60ºC. Thereafter, incubation was done in 50% formamide, 600 m*M* NaCl, 60 m*M* Tris-HCl (pH 7.4), 4 m*M* EDTA, 1 m*M* dithiothreitol, and 5 pmol/mL of probe for 16 h at 35ºC, washed at 45ºC with 2X SSC twice for 10 min, 1X SSC for 10 min.

The presence of DIG-probes was detected with anti-DIG antibodies conjugated with alkaline phosphatase, using nitroblue tetrazolium and bromochloroindolyl phosphate as enzyme substrates, in the presence of levamisole, for 18 h at room temperature.

Control sections were hybridized with labeled sense riboprobes, or with hybridization solution in the absence of riboprobes. The specificity of the DIG-oligonucleotide signal was tested with a competitive reaction with 100-fold unlabeled oligonucleotide, DIG-random oligonucleotide, absence of probe, and with known negative (liver) and known positive (parotid gland) tissues.

Immunohistochemistry for Tissue Kallikrein

The immunostaining technique corresponds to that previously described *(13)*. Dewaxed and rehydrated sections, treated with 10% hydrogen peroxide to block endogenous peroxidases, were incubated sequentially at 22ºC in a humidified chamber with goat polyclonal antiserum against purified human urinary kallikrein (1:2000) (Protogen AG, Switzerland) for 18 h, rabbit anti–goat IgG (1:1000) (Dako, Carpinteria, CA) for 30 min, swine antirabbit IgG (1:80) (Dako) for 30 min, and peroxidase-antiperoxidase complex of rabbit origin (1:100) (Dako) for 30 min. Peroxidase activity was demonstrated with 0.1% (w/v) 3,3'-diaminobenzidine and 0.05% (v/v) hydrogen peroxide for 15 min at room temperature.

The specificity of the staining was determined by incubating sequential sections in the absence of the first antibody, or with antiserum preabsorbed with purified urinary kallikrein (50 μg/mL).

The positivity and intensity of the staining of immunoreactive tissue kallikrein in tubular epithelial cells was evaluated by two independent observers. Grading ranged between 0 and 3, according to the following intensity score: 0 = absence of staining, 1 = faint or scant, 2 = moderate, 3 = intense; the kappa coefficient of agreement *(42)* between gradings of each observer was 0.69, $p = 0.001$.

The crossreactivity of the tissue kallikrein antiserum in dot-blot immunoassay (1:2000) was negative toward 2 and 4 µg of hK3 or prostate-specific antigen (Calbiochem). The different cell types were characterized by the capacity to express vimentin (stromal cell marker; antivimentin 1:500; Sigma), cytokeratin (epithelial and trophoblast cell marker; mAbAntiPAN cytokeratin 1:100; Sigma), and CD34 (endothelial cell marker; QBEnd/10 1:50; Bio Genex, San Ramon, CA). All studied sections were counterstained with hematoxylin (Sigma).

Immunohistochemistry for B2R

The immunostaining technique corresponds to that previously described *(9,20)*. Sections fixed in periodate-lysine-paraformaldehyde were dewaxed, rehydrated, and treated with 1% hydrogen peroxide to block endogenous pseudoperoxidase activity. The sections were incubated sequentially at 22ºC in a humidified chamber with a mixture of 8 polyclonal rabbit antisera (1:1000) raised against the rat kinin B2R for 18 h, swine antirabbit IgG (1:80) for 30 min, and peroxidase/antiperoxidase complex of rabbit origin (1:100) for 30 min. Peroxidase activity was demonstrated with 0.1% 3,3'-diaminobenzidine and 0.03% hydrogen peroxide for 15 min at room temperature.

Immunohistochemical controls were prepared by omission and/or replacement of antipeptide antibodies by preimmune rabbit sera and by incubating tissue sections with the antipeptide antibodies in the presence of an excess (50–100 mg/mL) of the same peptides used for immunization.

Statistical Analyses

Gradings are expressed as medians and interquartile range. Statistical tests to analyze differences in the semiquantitative evaluation of epithelial immunoreactivity for kallikrein were performed using SigmaStat 2.0 (Sigma). Statistical significance, fixed at $p < 0.05$, was tested by equal variance test.

Acknowledgments

We are indebted to Dr. Ignacio Duarte for dating the endometrial samples; to Dr. Helmar Rosenberg for providing kidney and liver samples; to Katya Gysling, PhD, for kindly providing the DIG-random oligonucleotide and for valuable technical advice; to Eliana Lira for excellent technical assistance; and to Alejandro Reitter for the preparation of the microphotographs. This study was supported by grants FONDECYT #1980958-1980964 and by a research and development grant from Gynopharm.

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