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Tissue Kallikrein in Human Placenta in Early and Late Gestation

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This study was addressed to identify kallikrein mRNA and protein in early, preterm, and term human placenta and to evaluate their temporospatial pattern. Kallikrein mRNA was expressed in syncytio/cytotrophoblasts and in the endothelial cells of the floating villi, with a greater intensity in early samples (isolated spontaneous abortions and ectopic pregnancies). Cytotrophoblasts at the base of the anchoring villi, maternal decidua and decidual arteries, endothelial cells of chorionic and basal plate blood vessels, and the amniotic epithelium presented a positive signal. Tissue kallikrein was predominantly observed in syncytiotrophoblasts and had a greater immunoreactivity in first-trimester samples. Intraarterial trophoblasts, blood vessels of the floating villi, basal and chorionic plates, and the amniotic epithelium showed positive immunoreactivity. The sites and variations of the tissue kallikrein mRNA and protein in the human placenta, in different stages of pregnancy, support the hypothesis that this enzyme may participate in the establishment and maintenance of placental blood flow through vasodilation, platelet antiaggregation, cell proliferation, and trophoblast invasion.

Key Words: Tissue kallikrein; human placenta; pregnancy.

Introduction

Tissue kallikrein (hk1) is a serine protease that cleaves low molecular weight kininogen and generates kallidin and bradykinin. These bioactive deca- and nonapeptides participate in vasodilation, increased vascular permeability, angiogenesis, and cell proliferation, which are key conditions for embryo implantation. In normal human pregnancy, urinary kallikrein increases between wk 6 and 12 and decreases thereafter, suggesting an enhancement of renal synthesis related to the vasoactive adaptations of early pregnancy *(1)*.

In the rat uterus, kallikrein mRNA has been demonstrated, and the tissue kallikrein protein (rK1) has been immunolocalized in luminal and glandular epithelium, implantation node, deciduomata, and subplacental sinusoids *(2–5)*. In the human uterus, tissue kallikrein is found in the luminal and glandular epithelial cells, and bradykinin receptors are localized in the epithelial cells and myometrium *(6–8)*. In addition to the tissue kallikrein gene expression (*hKLK1*), *hKLK2* and *hKLK3*, which code for hk2 and prostate-specific antigen (PSA), have been described in the human uterus *(9)*. In pregnancy, kallikrein-like activity has been described in myometrium, placenta, and amniotic fluid *(10)*.

With the purpose of advancing in the understanding of the role of the kallikrein-kinin system in human pregnancy, the temporospatial pattern of tissue kallikrein mRNA and its protein expression were evaluated in placentas obtained from the first-, early-, and late third trimesters.

Results

In Situ Hybridization

Tissue kallikrein mRNA was mainly expressed in trophoblast cells (syncytio- and cytotrophoblasts) and in the endothelial cells of the floating villi. The intensity of mRNA expression, as well as the number of positive cells, was greater in the first-trimester samples compared to term (Fig. 1A,C,E, G and Table 1). Trophoblastic villi obtained from spontaneous abortions and ectopic pregnancies showed a similar localization and grade of staining (Fig. 1A,C). No signal was detected in control sections hybridized either with labeled sense riboprobes, or in the absence of riboprobes (Fig. 2A,C,E).

Cytotrophoblasts located at the base of the anchoring villi (Fig. 3), in the maternal decidua (intramural cytotrophoblast), and in decidual arteries (intraarterial cytotrophoblast) (Fig. 4), showed a positive reaction. Endothelial cells of small blood vessels in the chorionic and basal plate and the amniotic epithelium also presented a positive signal (Figs. 4A and 5A).

Immnohistochemistry

Tissue kallikrein was predominantly observed in syncytiotrophoblasts, with a preferential apical localization. In agreement with the findings of *in situ* hybridization, the width, extension, and intensity of the staining was significantly greater in the first-trimester samples compared to term (Fig. 1B,D,F,H and Table 1). Trophoblastic villi obtained

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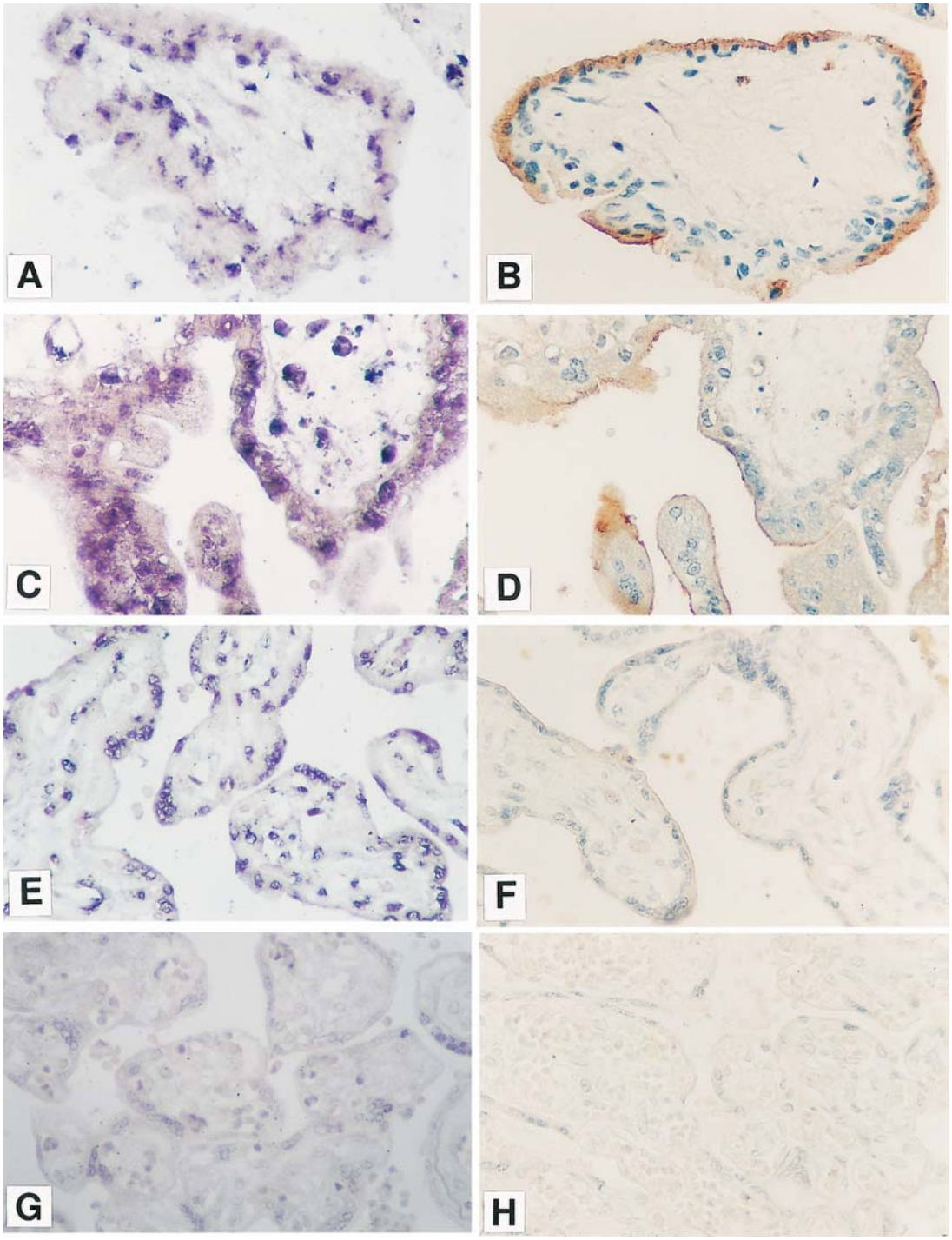

Fig. 1. Expression of kallikrein mRNA and immunoreactive protein in villi of representative samples. Placenta of a spontaneous abortion at 8 wk of gestation shows intense mRNA signal (**A**) and immunostaining in the syncytiotrophoblast (**B**)**;** similar expressions are observed in trophoblast from an ectopic pregnancy of 4 wk of gestation (**C,D**)**.** Placenta from preterm delivery (31 wk of gestation) shows interrupted zones of mRNA signal (**E**) and of cytoplasmic immunoreactivity of the syncytiotrophoblast (**F**)**.** Placenta from a term delivery (38 wk of gestation) shows scant mRNA signaling (**G**) and a thin luminal rim of immunoreactivity (**H**) (×400).

from ectopic pregnancies and spontaneous abortions showed a similar localization and grade of staining (Fig. 1B,D). The correlation coefficient between the expression of the mRNA for tissue kallikrein and the coded protein was 0.68, $p = 0.002$ ($n = 18$). No staining was detected in sequential sections incubated with either nonimmune rabbit serum or antiserum preabsorbed with purified urinary kallikrein (Fig. 2B, D,F).

Table 1
Semiquantification of Tissue Kallikrein mRNA and Protein in Syncytiotrophoblast in Different Stages of Pregnancy (median and 25–75% interquartile range)

	Group I	Group II	Group III
Kallikrein mRNA (grade)	3.0(2.0–3.0)[a] (n = 5)	1.5(1.0–2.0) (n = 5)	1.0 (1.0–1.0) (n = 8)
Kallikrein (grade)	2.5(2.0–3.0)[a] (n = 22)	1.0 (1.0–1.0) (n = 15)	1.0 (1.0–1.0) (n = 22)

[a] $p < 0.05$ for Group I vs Groups II and III, by Tukey's and Dunn's test for *in situ* hybridization and immunohistochemistry, respectively. Group I, first-trimester placentas from spontaneous abortions or ectopic pregnancies; Group II, early third-trimester placentas from idiopathic preterm deliveries; Group III, term placentas. $p < 0.001$ by Kruskall-Wallis analysis of variance for *in situ* hybridization and immunohistochemistry.

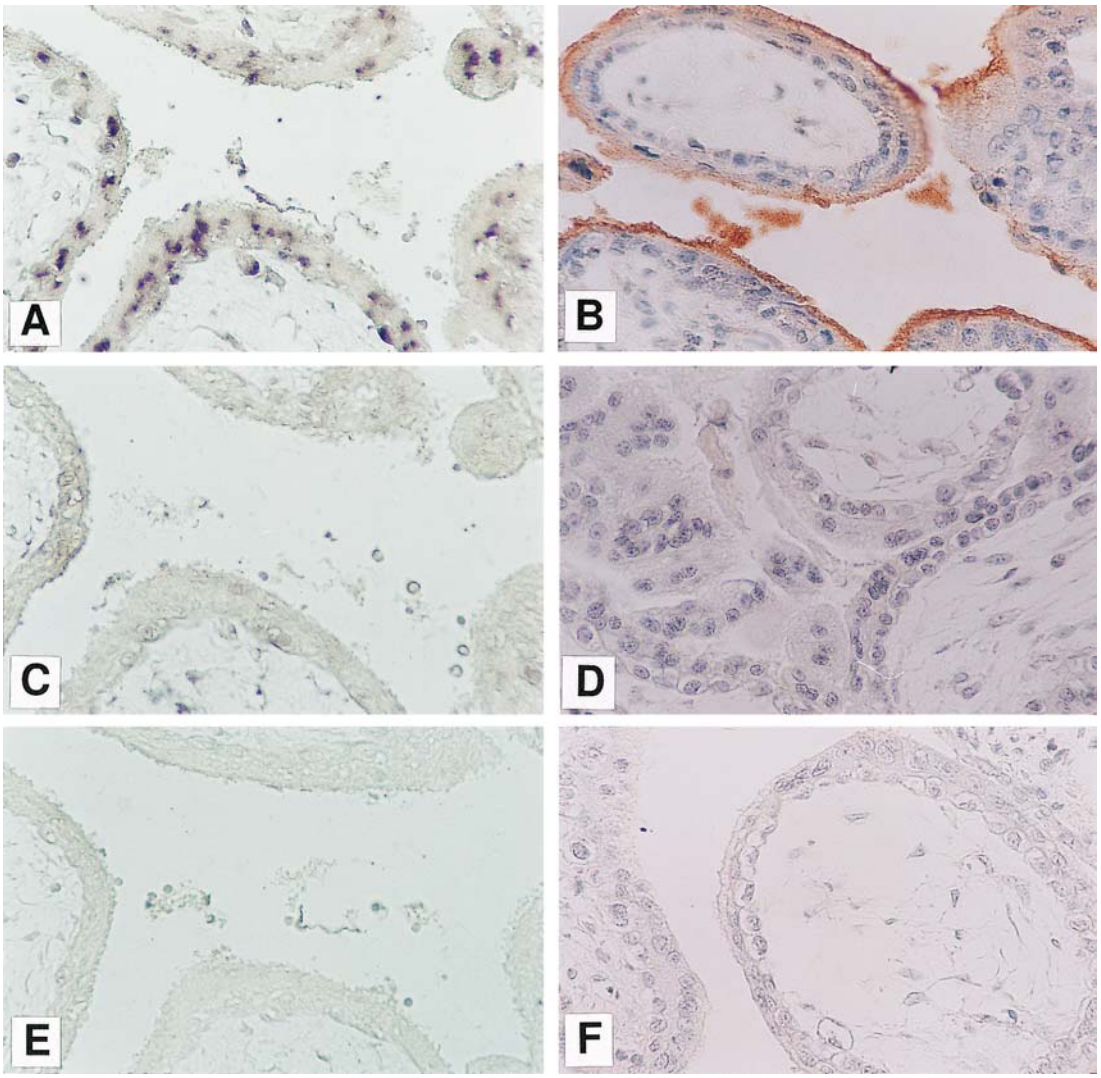

Fig. 2. Expression of kallikrein mRNA and immunoreactive protein in a placenta from an 8-wk gestation spontaneous abortion (**A,B**) is not detected in sections hybridized with labeled sense riboprobes (**C**), in sections incubated in the absence of the first antibody (**D**), in the absence of riboprobes (**E**), or with antiserum preabsorbed with purified urinary kallikrein (**F**) (×400).

Intraarterial trophoblasts in first-trimester samples (Fig. 4B) and in a few third-trimester specimens showed a positive reaction. The blood vessels of the floating villi showed a thin rim of immunoreactivity, slightly more intense in first-trimester samples. The small blood vessels of the basal and chorionic plates exhibited lineal staining. The amniotic epithelium showed either cytoplasmic or apical linear reactivity (Fig. 5B).

No signal was detected in cytotrophoblasts at the base of the anchoring columns (Fig. 3B), the maternal stroma, or the chorion.

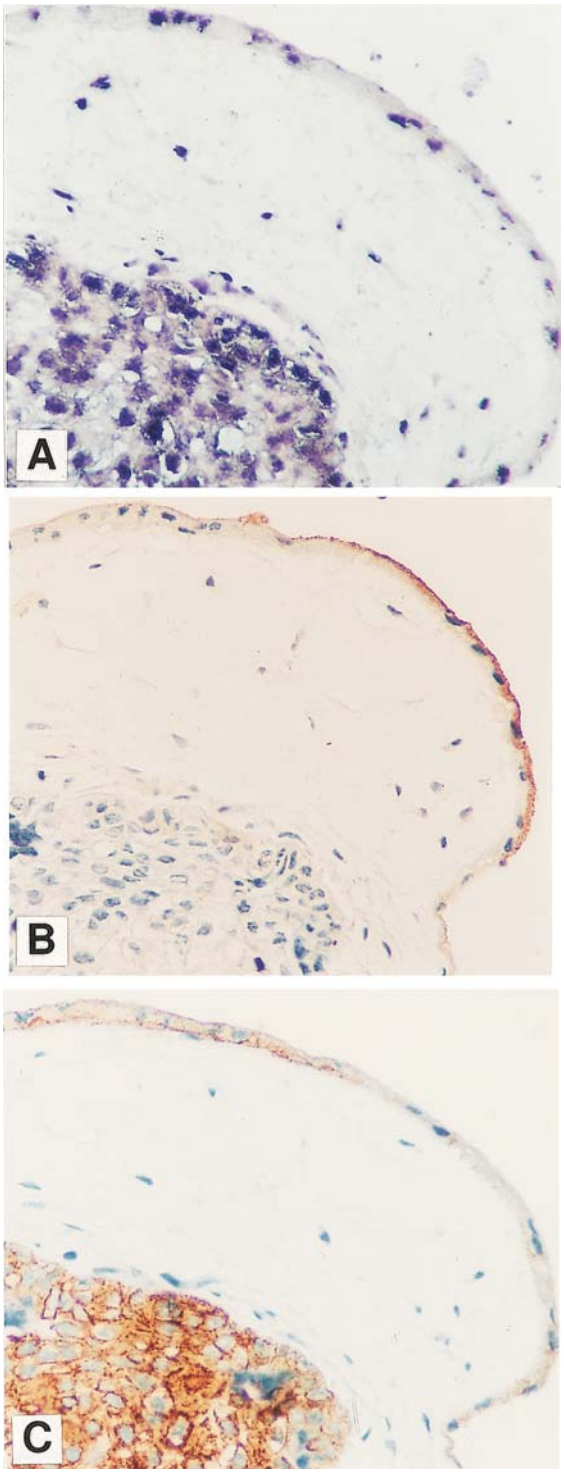

Fig. 3. Anchoring villi of an 8-wk spontaneous abortion expresses kallikrein mRNA in syncytiotrophoblast and basal cell column (**A**) and kallikrein in syncytiotrophoblast (**B**). Trophoblast is characterized by cytokeratin immunoreactivity (**C**) (×400).

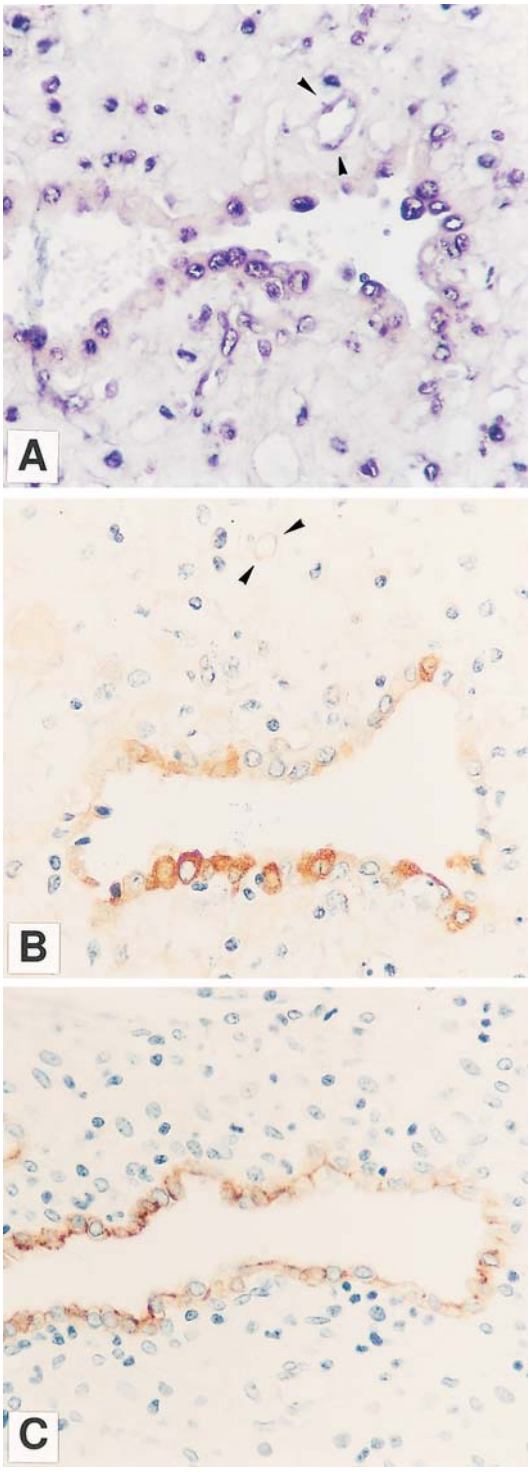

Fig. 4. Decidua from a 9-wk gestation spontaneous abortion shows positive kallikrein mRNA (**A**) and immunoreactive kallikrein (**B**), in the extravillous trophoblast invading maternal blood vessels identified by anticytokeratin antibody (**C**). Maternal blood vessels show positive mRNA and kallikrein (arrowheads) (×400).

Discussion

To our knowledge, this study provides the first evidence that the tissue kallikrein coding gene *(hKLK1)* and protein are found in different human placental cell types, show a greater expression in first trimester, and decrease in the later stages of pregnancy.

A kallikrein-like enzyme activity had been described in human placenta *(10)*, which, according to our results, may

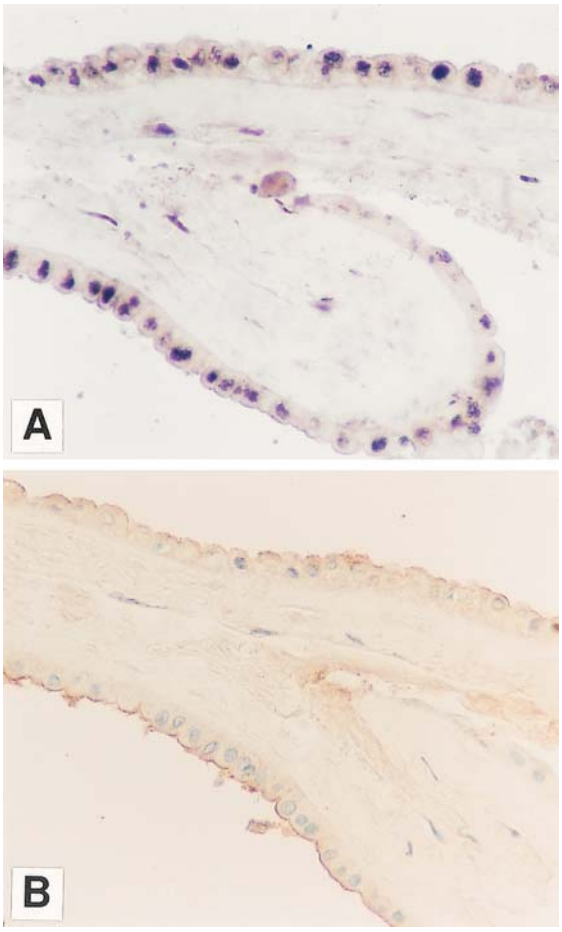

Fig. 5. The amniotic epithelium of a placenta of 31-wk gestation presents kallikrein mRNA (**A**) and kallikrein immunoreactivity (**B**) (×400).

correspond to tissue kallikrein. On the other hand, a more recent study could not detect tissue kallikrein or its mRNA by immunohistochemistry, Western blot, or reverse transcriptase-polymerase chain reaction (PCR) in term placenta *(11)*. The different results are probably because of the varying sensitivity of the antibodies employed in each study and the low kallikrein content of the late gestational samples. Both the specificity of the probes and the lack of cross-reactivity of the antiserum with PSA suggest that this kallikrein-like protein, synthesized and secreted by term syncytiotrophoblast *(12)*, does not constitute an interfering factor in our study.

While the expression of tissue kallikrein and its mRNA coincide in syncytiotrophoblast, intraarterial trophoblast, villous endothelium, chorionic and basal plate, and amniotic epithelium, *in situ* hybridization shows additional signals in the cytotrophoblasts located in villi, in the base of the anchoring columns, and in the chorion. The lack of protein expression in these cell types may be attributed to either a lower sensitivity of the immunohistochemistry or to the modulation of the translation/transcription process, which may provide a reserve population of kallikrein-producing cells in a tissue with high vasodilator requirements. Interestingly, trophoblasts that invade maternal blood vessels express both the coding gene and the protein, while those that invade the decidua or are included in islets in the chorionic plate only express the coding gene. Kallikrein, in intraarterial trophoblast (as in fetal and maternal endothelium), may constitute an additional marker of the switching to an endothelial phenotype, which is crucial for placental development *(13)*.

Tissue kallikrein acts on low molecular weight kininogen to generate kinins. The effect of bradykinin, or the presence of its receptors, has been demonstrated in endothelial cells, in uterine epithelium, myometrium and decidua, and first-trimester cytotrophoblasts, indicating that these cells constitute physiologic targets for kinins *(8,14–17)*. Apical kallikrein in floating villi could be secreted into the intervillous space, where the enzyme may generate bradykinin from maternal plasma kininogen and exert an autocrine effect on the trophoblastic surface. Via an endocrine pathway it could reach the luminal side of the maternal endothelial veins, and through enhanced venous permeability reach the adjacent uterine arteries, as postulated for vascular endothelial growth factor (VEGF) in rat pregnancy *(18)*. In first-trimester trophoblast, the stimulation of bradykinin B2 receptors induces nitric oxide liberation *(17)*. Endothelial nitric oxide synthase (eNOS) in human cytotrophoblast, syncytiotrophoblast, and fetal endothelium *(19,20)* and inducible NOS (iNOS) in syncytiotrophoblast, villous stromal cells, and fetal endothelium *(19,21)* could respond to locally generated kinins.

Bradykinin stimulates prostaglandin E and prostaglandin I_2 in in vitro human decidua *(15)*. The synthesis of both prostaglandins was greater in young placentas *(22)*, as observed for kallikrein in our study. Moreover, the synthesis of the vasodilator prostaglandins in villous vessels and core *(23,24)* could be modulated by kallikrein in endothelial cells and syncytiotrophoblast.

Kinins have been linked to actions other than their classic vasodilator effect. The activation of the B1 kinin receptor in cultured, first-trimester cytotrophoblast promotes cell proliferation *(17)* and supports an effect of the kallikrein-kinin system in placental development. Bradykinin, by increasing the synthesis of plasma tissue-type plasminogen activator *(25)* in cyto- and syncytiotrophoblast *(26)*, could favor a thrombolytic balance. Kinins generated by kallikrein in amniotic epithelium may be related to the synthesis of prostaglandin and cytokines and to water and electrolyte transport *(27)*.

Kallikrein, as a proteolytic enzyme, could participate in the processing of VEGF, localized in early pregnancy in cyto- and syncytiotrophoblast *(28–31)*, as well as in trophoblast invasion, through its capacity of degrading extracellular matrix components by collagenase activation *(32–34)*.

The temporospatial profile of tissue kallikrein is remarkably similar to that of placental renin *(35)* and supports coor-

dinated functions for the kallikrein-kinin and the renin-angiotensin systems, as in the kidney (e.g., renin activation, potentiation of angiotensin 1–7, and electrolyte transport) *(36)*.

The increased kallikrein content in early pregnancy stages parallels the rise in urinary kallikrein observed in normal pregnant women *(1)*, suggesting that this vasoactive enzyme in the placenta and the kidney responds to common early pregnancy signals. Since kallikrein is subject to tissue-specific regulation, the factors that modulate trophoblastic kallikrein synthesis throughout pregnancy deserve to be elucidated by further studies, which must include in vitro and clinical models.

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. Placentas were obtained from women who had given informed consent. Group I consisted of placentas obtained from the first trimester who presented with isolated spontaneous abortions ($n = 12$, gestational age = 9.5 ± 2.2 [SD] wk) or ectopic pregnancies ($n = 11$, gestational age = 7.4 ± 1.9 wk). Group II consisted of placentas obtained from idiopathic preterm deliveries *(37)* ($n = 14$, gestational age = 34.6 ± 2.5 wk). Group III included placentas from term deliveries ($n = 22$, gestational age = 38.7 ± 0.9 wk). All samples were obtained from normotensive nondiabetic subjects, with similar ages for the different groups (29.2 ± 4.8 to 32.6 ± 7.0 yr) and were free from histologic signs of inflammation or ischemia. Samples from spontaneous abortions were compared to those derived from ectopic pregnancies, a condition that has proven to be similar to normal intrauterine pregnancy for other bioactive factors *(38–41)*; results obtained in both groups were similar. To avoid variations in a vasoactive system that may be up- or downregulated in preeclampsia or fetal growth restriction, third-trimester specimens were obtained from normotensive women who gave birth to healthy infants with adequate weight for gestational age (2478 ± 737 and 3505 ± 453 g for preterm and term infants, respectively).

Placentas were fixed with 4% formaldehyde. 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: Preparation of Riboprobes

A 168-bp, human tissue kallikrein DNA fragment, which corresponds to the 690–857 bp of *KLK1 (42,43)*, was generated from human genomic DNA by PCR with the upstream and down stream primers (5'-GATGTGTGATGGTGT GCTCC-3') and (5'-TTTTACT GGGGGTAGGG CAC-3'), respectively, and was cloned into the pGEM T vector (Promega, Madison, MI). The construct was confirmed by DNA sequencing and restriction enzyme mapping, which were consistent with the previously reported sequence of human tissue kallikrein *(42)*. The riboprobe homology to *KLK1*, *KLK2*, and *KLK3* was verified by the National Center for Biotechnology Information basic local alignment tool (BLAST) 2 sequences Program BLASTN 2.0.9 (Bethesda, MD). A 100% homology with hKLK1 was confirmed whereas the homologies to hKLK2 and hKLK3 were 19 and 0%, respectively. Thus, owing to the specificity of the probe and the stringent hybridizing and washing conditions used, the signals obtained in this study are attributed to hKLK1. Sense and antisense digoxigenin-uridine 5'-triphosphate (DIG-UTP) cRNA probes were obtained by in vitro transcription using DIG-UTP (Roche, Boehringer Mannheim GmbH; Mannheim, Germany) and T7 or SP6 RNA polymerases (Promega), respectively.

In situ hybridization was performed on 4% formaldehyde-fixed, Histosec-embedded placental specimens from 23 of the study patients (first trimester, $n = 8$; early third trimester, $n = 5$; and term, $n = 10$). Sections were dewaxed and rehydrated through a series of solutions with a decreasing concentration of ethanol, washed in phosphate-buffered saline (PBS) containing 5 m*M* $MgCl_2$ (PBS/$MgCl_2$), and incubated with 10 μg/mL of proteinase K (Sigma), in a buffer containing the following: 10 m*M* EDTA; 100 m*M* NaCl; and 500 m*M* Tris-HCl (pH 7.5) for 10 min at room temperature. Proteinase treatment was stopped by washing with PBS/$MgCl_2$, followed by 100 m*M* glycine and 200 m*M* Tris-HCl (pH 7.4), prehybridized, and hybridized as described elsewhere *(44,45)*. Following hybridization, the tissue slices were washed twice with 4X SSC/50% formamide for 10 min, 2X SSC/50% formamide for 20 min, and 0.2X SSC/50% formamide for 30 min at 52°C.

The presence of DIG-cRNA 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. Finally, the slides were mounted with Kayser's glycerol-gelatin (Merck, Darmstadt, Germany).

Control sections were hybridized with labeled sense riboprobes or with a hybridization solution in the absence of riboprobes.

The positivity and intensity of the kallikrein mRNA signal of *in situ* hybridization in syncytiotrophoblast was evaluated by two independent observers, blinded to the stage of each preparation; however, ectopic pregnancies could be easily recognized. Grading ranged from 0 to 3, according to the following intensity score: 0 = absence of staining, 1 = segmental perinuclear staining, 2 = continuous perinuclear staining, and 3 = intense continuous perinuclear staining. The kappa coefficient of agreement *(46)* between gradings of each observer was 0.92, $p = 0.000$.

Immunohistochemistry

The immunostaining technique was performed in tissues obtained in all study patients and corresponds, with slight

modifications, to that previously described *(5)*. Briefly, tissue sections were dewaxed with xylene, rehydrated through a graded series of ethanol, and treated with 10% hydrogen peroxide to block endogenous peroxidase activity. After being rinsed three times in 50 m*M* Tris-HCl buffer (pH 7.8) for 5 min the sections were incubated sequentially with the following:

1. Goat polyclonal antiserum against purified human urinary kallikrein (Protogen AG, Switzerland), 1:2000 for 18 h.
2. Rabbit antigoat IgG (Dako, Carpinteria, CA), 1:1000 for 30 min.
3. Swine antirabbit IgG (Dako), 1:80 for 30 min.
4. Peroxidase-antiperoxidase (PAP) complex of rabbit origin (Dako), 1:100 for 30 min.

All incubations were done at 22°C in a humidified chamber. Between incubations the sections were washed three times in PBS-50 m*M* Tris-HCl buffer (pH 7.8, 5 min each). The antisera and PAP complex were diluted in buffer containing 1% (w/v) immunoglobulin-free bovine serum albumin. 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 incubation of sequential sections in the absence of the first antibody, or with antiserum preabsorbed with 50 μg/mL of purified urinary kallikrein.

The positivity and intensity of the staining of immunoreactive kallikrein in syncytiotrophoblast was evaluated by two observers blinded to the group of each preparation. Grading ranged between 0 and 3, according to the following intensity score: 0 = absence of staining, 1 = thin luminal rim of interrupted staining, 2 = thick rim of continuous staining, and 3 = intense continuous staining of the microvillous apical surface or of the whole cytoplasm. The kappa coefficient of agreement between gradings of each observer was 0.69, $p = 0.000$.

The crossreactivity of the kallikrein antiserum (1:2000) was negative towards PSA, as assessed by dot-blot immunoassay using 2 and 4 μg of the purified protein (Calbiochem). The different cell types were characterized on serial sections, using immunocytochemistry to show the expression of vimentin (stromal cell marker; 1:500 antivimentin [Sigma]), cytokeratin (epithelial and trophoblast cell marker; 1:100 mAbAntiPAN cytokeratin [Sigma]), and CD34 (endothelial cell marker; 1:50 QBEnd/10 [Bio Genex, San Ramon, CA]). All of the studied sections were counterstained with hematoxylin.

Statistical Analyses

Statistical tests were performed using SigmaStat 2.0 (Sigma). Results of the semiquantitative grading of the immunohistochemistry are expressed as medians and interquartile range. Statistical significance, fixed at $p < 0.05$, was tested by Kruskal-Wallis one-way analysis of variance on ranks, and post hoc Tukey's and Dunn's tests for the intensity of the immuno- and *in situ* hybridization staining, respectively. Correlation coefficient was analyzed by Spearman's rank-order test.

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