Endocrine, *vol. 18, no. 1, 75–78, June 2002* 0969–711X/02/18:75–78/$11.00

Calcitonin Immunostaining in Monkey Uterus During Menstrual Cycle and Early Pregnancy

Hong-Lu Diao,[1] Shi-Jie Li,[1] Hong-Bin Wang,[2] and Zeng-Ming Yang[1]

[1]College of Life Sciences and [2]College of Animal Medicine, Northeast Agricultural University, Harbin, China

Calcitonin has been shown to be a progesterone-regulated potential marker of the receptive endometrium in the rat and human. The present study was undertaken to immunohistochemically investigate the changes in calcitonin in the monkey uterus during the menstrual cycle and periimplantation period. Calcitonin immunostaining was primarily localized in the glandular epithelium on d 16, 20, and 25 of the menstrual cycle. During early pregnancy, calcitonin immunostaining was strongly observed in the glandular epithelium only on d 9 of pregnancy, the day before implantation. Since the high level of calcitonin immunostaining in the glandular epithelium during the luteal phase of the menstrual cycle and periimplantation period matched the high level of maternal progesterone during this period, the expression of calcitonin in monkey endometrium may be under the regulation of maternal progesterone.

Key Words: Calcitonin; monkey; endometrium; menstrual cycle; implantation.

Introduction

Calcitonin, a 32 amino acid peptide hormone, is mainly synthesized and secreted by the parafollicular C-cells of the thyroid gland. Its most well known physiologic role is to regulate calcium levels in bone and kidney *(1)*. A low level of calcitonin is also localized in lung, liver, intestine, pituitary, and hypothalamus *(2,3)*. Calcitonin is highly expressed in the glandular epithelium of rat uterus at the time of implantation and is significantly induced by progesterone in the uteri of ovariectomized (OVX) rats *(4,5)*. Implantation stage-specific expression of calcitonin can be specifically attenuated by administering antisense oligodeoxynucleotides directed against calcitonin mRNAs into the uterine horns on d 2 of gestation, resulting in a dramatic reduction in the number of implanted embryos *(6)*. In human uterus, progesterone-induced expression of calcitonin in the secretory endometrium temporally coincides with the putative window of implantation *(7)*. Therefore, calcitonin might be a progesterone-regulated potential marker for mammalian implantation.

In view of the close relationship and similar menstrual cycle between rhesus monkey and human, the present study was undertaken to immunohistochemically investigate the changes of calcitonin in the monkey uterus during the menstrual cycle and periimplantation period.

Received February 4, 2002; Revised May 2, 2002; Accepted May 2, 2002.

Author to whom all correspondence and reprint requests should be addressed: Zeng-Ming Yang, College of Life Sciences, Northeast Agricultural University, Harbin 150030, China. E-mail: zmyang@mail.neau.edu.cn

Results

Calcitonin Immunostaining During Menstrual Cycle

Calcitonin immunostaining in monkey uterus during the menstrual cycle was determined by immunohistochemistry (see Fig. 1). The level of calcitonin immunostaining in the glandular epithelium depended on the locations of glands in the whole endometrium. In the glandular epithelium near the myometrium, no immunostaining was detected on d 6, 9, and 12 (Fig. 1A,B), while a strong level of calcitonin was seen only on d 16, 20, and 25 (Fig. 1C–E). In the luminal epithelium during the menstrual cycle, only a basal level was seen on d 16 and a strong level on d 20, while no immunostaining was seen on d 6, 9, 12, and 25 (Fig. 1B). There was no detectable immunostaining in the stroma and superficial glandular epithelium. Moreover, no immunostaining was shown after rabbit antihuman calcitonin was replaced by normal rabbit IgG.

Calcitonin Immunostaining During Early Pregnancy

Calcitonin immunostaining in monkey uterus during early pregnancy is shown in Fig. 2. No immunostaining was seen in the uterus from d 1 to 5 of pregnancy (Fig. 2A,B). In the glandular epithelium near the myometrium, a strong immunostaining was detected only on d 9 (Fig. 2D), and a basal level on d 7 and 11 (Fig. 2C,E). A basal level of calcitonin immunostaining was also detected in the superficial glandular epithelium on d 9. No immunostaining was shown in the luminal epithelium and stroma on d 7, 9, and 11.

Discussion

Calcitonin immunostaining was mainly localized in the glandular epithelium near the myometrium, while only a basal level was observed in the superficial glandular epithelium. The level of calcitonin immunostaining strongly depended on the position of the glands in the monkey endometrium. In humans, calcitonin mRNA and protein were also

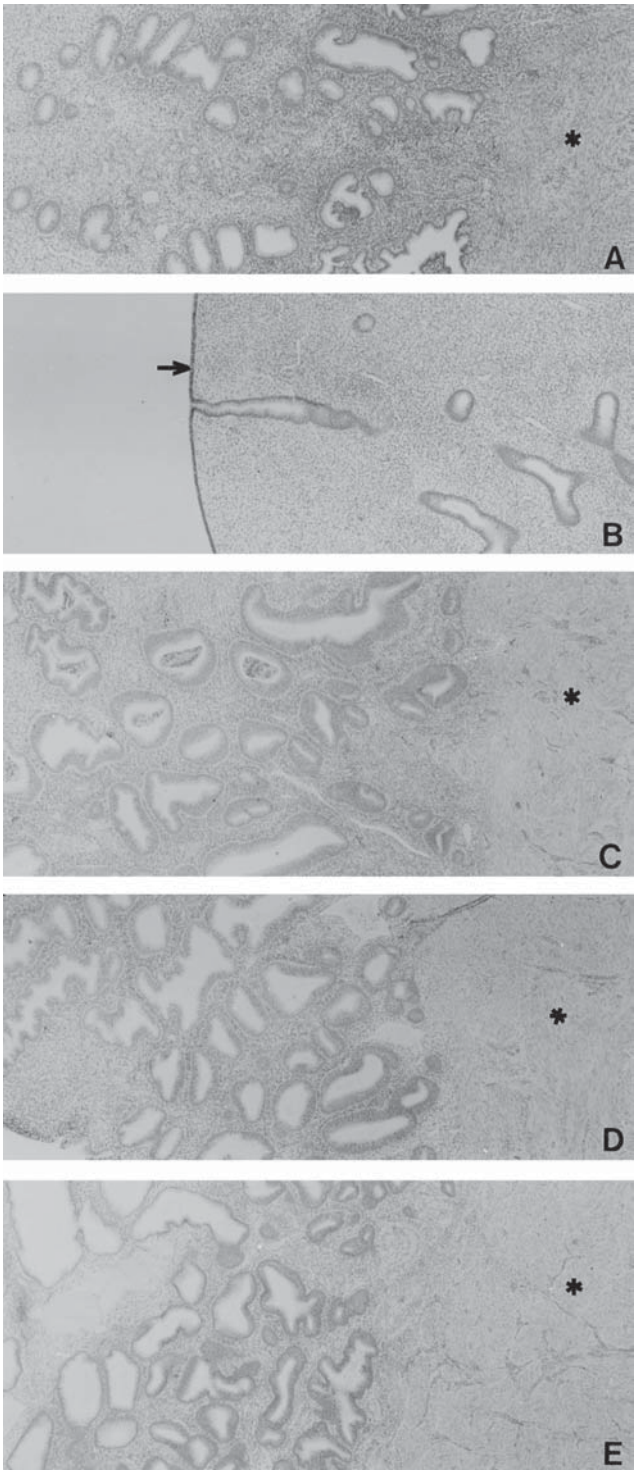

Fig. 1. Calcitonin immunostaining in monkey uterus on d 6 (**A**), 9 (**B**), 16 (**C**), 20 (**D**), and 25 (**E**) during menstrual cycle. *, myometrium; arrow, luminal epithelium. Magnification: ×260.

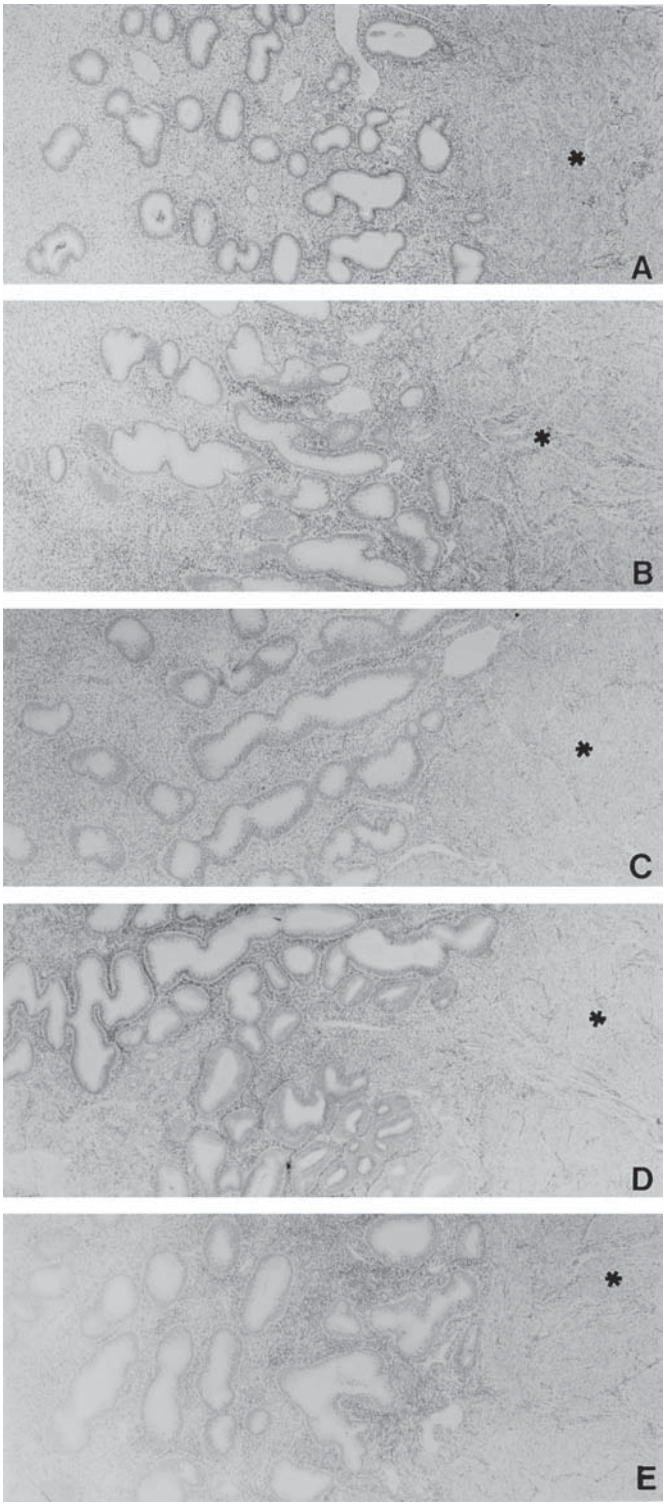

Fig. 2. Calcitonin immunostaining in monkey uterus on d 2 (**A**), 5 (**B**), 7 (**C**), 9 (**D**), and 11 (**E**) during pregnancy. *, myometrium. Magnification: ×300.

found to be strongly localized in the glandular epithelium *(7)*. However, it was not clear whether there was a difference for calcitonin expression in the glands of different position in the whole endometrium since human samples were from curettage. In rat uterus, calcitonin mRNA and protein were located in the whole glandular epithelium *(4,5)*.

During the menstrual cycle of the rhesus monkey, calcitonin immunostaining was strongly localized in the glan-

dular epithelium on d 16, 20, and 25. In humans, calcitonin expression peaked on d 19–21 of the midsecretory phase of the menstrual cycle, whereas none was detected during the preovulatory or late secretory stage *(7)*. The implantation window in humans was supposed to be open between d 19 and 22 of the menstrual cycle *(7)*. In view of the close relationship and similar menstrual cycle between rhesus monkeys and humans, the implantation window in rhesus monkeys should also be open between d 19 and 22 of the menstrual cycle. In the rat, the expression of calcitonin increased by d 2 (postfertilization) of gestation; reached a peak on d 4, the day before implantation; and declined on d 5, the day implantation occurred *(4,5)*. Moreover, calcitonin immunostaining was also highly detected in the glandular epithelium only on d 9 of pregnancy in our study. It was reported that blastocyst implantation in monkey occurred on d 9.5 of pregnancy *(8,9)*. It seems that calcitonin may also be a good marker for embryo implantation.

Nevertheless, leukemia inhibitory factor (LIF) had also been shown to be essential for mouse implantation *(10)*. LIF was also highly expressed in monkey endometrium during the luteal phase of the menstrual cycle and periimplantation period. Pregnancy rate could be significantly reduced with an injection of goat antihuman recombinant LIF IgG into the uterine lumen on d 8 of pregnancy *(11)*. It seems that embryo implantation may be controlled by a multiple redundant pathway.

Progesterone significantly stimulated calcitonin mRNA and protein synthesis in the uteri of OVX rats. The antiprogestin drug RU486 markedly reduced the expression of calcitonin mRNA and protein in rat uterus *(4,5)*. Moreover, treatment of women with an antiprogestin, RU486, drastically reduced calcitonin expression in the endometrium *(7)*. Because RU486 is known to exert its inhibitory effects by impairing the gene regulatory activity of the progesterone receptor *(12)*, the downregulation of calcitonin expression by RU486 treatment indicated that synthesis of calcitonin in the uterus was regulated by progesterone. In our study, the high level of calcitonin immunostaining in the glandular epithelium during the luteal phase of the menstrual cycle and periimplantation period also matched the progesterone pattern during this period since circulating progesterone levels were high during the midluteal phase and periimplantation period in monkey *(13,14)*. The expression of calcitonin in monkey endometrium may also be under the regulation of maternal progesterone.

Materials and Methods

Animals

Rhesus monkeys (*Macaca mulatta*) at Fujian Province Non-human Primate Research Center in China were caged individually in a controlled environment with a 12-h light: 12-h dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Animals were evaluated daily by visual examination of the perineum for menses, with the onset of menses defined as d 1 of the menstrual cycle. Mature female monkeys with regular menstrual cycles of approx 28 d were chosen for this study. The uterine samples of menstrual cycle were taken on d 3 for the mense; on d 6, 9, and 12 for the proliferative phase; and on d 16, 20, and 25 for the luteal phase, respectively.

Male rhesus monkeys of proven fertility from previous breeding were used for mating. Female monkeys on d 11 of the menstrual cycle were caged with a male monkey. Vaginal smears were done the next morning to check for sperm in the vagina. The day when the sperm checking was positive was designated as d 1 of pregnancy. The uterine samples of early pregnancy were taken on d 1, 3, 5, 7, 9, and 11 of pregnancy, respectively. All of the monkeys were sacrificed by an overdose injection of ketamine. At least three monkeys were used in each group. The uteri were removed and treated as described next.

Immunohistochemistry

The dorsal portion of monkey uterus was immediately cut into small pieces, fixed in Bouin's solution for 24 h, dehydrated with graded ethanol, and embedded in paraffin. Paraffin sections were cut, deparaffinized, and hydrated gradually into phosphate-buffered saline (PBS). Antigen retrieval was performed by incubating the sections in 0.1 m*M* EDTA (pH 8.5) at 89ºC in a water bath for 15 min. Nonspecific binding was blocked with 10% horse serum in PBS at 37ºC for 1 h. The sections were incubated with rabbit antihuman calcitonin IgG (1:100) (Zymed, South San Francisco, CA) at 25ºC for 90 min. Normal rabbit IgG was used to replace the primary antibody for a negative control. The sections were then incubated with biotinylated secondary antibody followed by an avidin–alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit; Vector). Vector Red was visualized as red. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma, St. Louis, MO). The sections were counterstained with hematoxylin and mounted. The degree of staining was assessed subjectively by blinded examination of the slides by two investigators.

Acknowledgment

This work was supported by National Natural Science Foundation of China grants 39825120, 39730250, and 30170110.

References

1. Austin, L. A. and Heath, H., (1981). *N. Engl. J. Med.* **304,** 269–278.
2. Becker, K. L., Snider, R., Moore, C. F., Monaghan, K. G., and Silva, O. L. (1979). *Acta Endocrinol.* **92,** 746–751.
3. Fischer, J. A., Tobler, P. H., Kaufmann, M., Born, W., Henke, H., Cooper, P. E., Sagar, S. M., and Martin, J. B. (1981). *Proc. Natl. Acad. Sci. USA* **78,** 7801–7805.

4. Ding, Y. Q., Zhu, L. J., Bagchi, M. K., and Bagchi, I. C. (1994). *Endocrinology* **135,** 2265–2274.
5. Zhu, L. J., Cullinan-Bove, K., Polihronis, M., Bagchi, M. K., and Bagchi, I. C. (1998a). *Endocrinology* **139,** 3923–3934.
6. Zhu, L. J., Bagchi, M. K., and Bagchi, I. C. (1998b). *Endocrinology* **139,** 330–339.
7. Kumar, S., Zhu, L. J., Polihronis, M., Cameron, S. T., Baird, D. T., Schatz, F., Dua, A., Ying, Y. K., Bagchi, M. K., and Bagchi, I. C. (1998). *J. Clin. Endocrinol. Metab.* **83,** 4443–4450.
8. Enders, A. C., Hendrickx, A. G., and Schlafke, S. (1983). *Am. J. Anat.* **167,** 275–298.
9. Hearn, J. P., Webley, G. E., and Gidley-Baird, A. A. (1991). *J. Reprod. Fertil.* **92,** 497–509.
10. Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S. J. (1992). *Nature* **359,** 76–79.
11. Yue, Z. P., Yang, Z. M., Wei, P., Li, S. J., Wang, H. B., Tan, J. H., and Harper, M. J. K. (2000). *Biol. Reprod.* **63,** 508–512.
12. Baulieu, E. E. (1991). *Trends Endocrinol. Metab.* **2,** 233–239.
13. Ghosh, D., De, P., and Sengupta, J. (1994). *Hum. Reprod.* **9,** 629–637.
14. Zegers-Hoshchild, F. and Altieri, E. (1995). *J. Assist. Reprod. Genet.* **12,** 224–228.