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Original Article

Inhibitory effect of recombinant human beta-defensin-2 on angiogenesis and inflammatory factors of rat model of endometriosis

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Abstract: Background: Human β defensin-2 is one of the important members of defensin family in endogenous antibiotic peptides, which plays an important role in immune defense process. This study aimed to investigate the effect of recombinant human beta-defensin-2 (pLXSN-hBD2) on angiogenesis and inflammatory factors of rat model of endometriosis (EMS). Methods: 31 EMS rats were randomly divided into PLXSN-hBD2, PLXSN and PBS groups, which were transfected with 2 μg of PLXSN-hBD2, PLXSN and PBS, respectively. Two weeks later, the volume of ectopic focus, vascular endothelial growth factor (VEGF) in serum, ovarian hormone level and microvessel density (MVD) in tissues as well as the mRNA expression of IL-1β, TNF-α and NF-κB of transfected rats in the three groups were detected. Results: Local multi-point injection of 2 μg of pLXSN-hBD2 resulted in hBD-2 protein expression in the ectopic focus tissue of rat model in the following two weeks. Two weeks after transfection, the volume of ectopic focus, VEGF level in plasma, MVD in focus tissue and the mRNA expression of IL-1β, TNF-α and NF-κB in PLXSN-hBD2 group were obviously smaller or lower than those in both PLXSN and PBS group ($P < 0.01$ and $P < 0.05$). The differences in ovarian hormone level between the three groups were not significant ($P > 0.05$). Conclusion: pLXSN-hBD has an inhibitory effecton angiogenesis and inflammatory factors in ectopic focus of rat model of EMS.

Keywords: HBD2, recombinant plasmid, neovascularization, inflammatory, inhibitory

Introduction

Endometriosis (EMS) is a common benign gynecological disease, which tends to happen in women of childbearing age. The main clinical manifestations of EMS are dysmenorrhea and infertility. Its incidence rate is approximately 10% to 20%, and has an increasing trend. EMS seriously affects physical and mental health, reproductive function and quality of life of women of childbearing age [1]. Currently, the etiology and pathogenesis of EMS are not clear, and the satisfactory treatment methods are insufficient. The recurrence rate is as high as 50% after 12-24 months from treatment with any medicine. In addition, the related side effects, such as low estrogen level, may occur during the treatment [2].

Endogenous antibiotic peptides are an important part of human autoimmune system. Human β defensin-2 (hBD-2) is one of the important members of defensin family in endogenous antibiotic peptides, which is widely expressed in various mucosal cells and epithelial tissues including reproductive organ [3, 4] and plays an important role in immune defense process. It is found in animal experiments that the over-expression of hBD-2 can inhibit the expression of inflammatory factors, such as IL-1β, IL-6 and TNF-α in tumor tissues [5, 6] and thus play a role in resisting immune inflammatory response. Studies have showed that angiogenesis plays an important role in tumorigenesis and metastasis [7]. HBD-2 not only directly inhibits the expression of VEGF, but also enhances the body to indirectly inhibit its expression by stimulating initiative immune response, which results in anti-tumor angiogenesis effect [8-10]. Anti-tumor angiogenesis effect of hBD-2 has become a research hotspot of oncotherapy. Therefore, hBD-2 may

Table 1. Ectopic foci volume EMS rats before and after transfection (mm^3)

Group	4 weeks before transfection	2 weeks after transfection
pLXSN-hBD2	86.94 ± 12.70	39.46 ± 8.02*
pLXSN	82.62 ± 13.99	103.61 ± 15.83*,#
PBS	83.13 ± 16.57	102.62 ± 11.32*,#

*P < 0.01 compared with before transfection; #P < 0.01 compared with pLXSN-hBD2 group.

inhibit EMS by anti-immune inflammatory response and anti-angiogenesis. This study investigated the effect of recombinant hBD-2 on angiogenesis and the expression of inflammatory factors in ectopic focus of rat model of EMS as well as its possible mechanism.

Materials and methods

Synthesis of hBD-2 gene and construction of pLXSN-hBD2 eukaryotic expression vector

hBD-2 gene was synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China). EcoRI and BamHI (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., Shanghai, China) were added to the purified PCR product of hBD-2 gene for double digestion of the target gene. The product was uniformly mixed with pLXSN eukaryotic expression vector plasmids which were double-digested too. Ligation was carried out with T4 ligase. The ligation product was PLXSN-hBD2 recombinant plasmid. Then, it was transferred to E. coli DH5α competent bacteria in late logarithmic growth phase. Recombinant colonies successfully transferred were picked for inoculation. The recombinant plasmids were extracted. At last, EcoRI and BamHI were added to recombinant plasmids extracted for digestion. The product was identified by gel electrophoresis. Meanwhile, target gene was inserted into vector constructed. It was sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Animal grouping and treatment

EMS model of SD rats (SPF grade, 240.6 ± 15.14 g, provided by Experimental Animal Center of Guangdong Province, Guangzhou, China) was established according to the reported method [11]. 31 EMS rats were randomly divided into pLXSN-hBD2 group (11 rats, 22 foci), pLXSN group (10 rats, 20 foci) and PBS group (10 rats, 20 foci). After general observation of the second laparotomy, local multi-point injection was carried out at each ectopic focus site with a 50 μL microsyringe. In pLXSN-hBD2 group, 2 μg of recombinant plasmid lipofectamine-pLXSN-hBD2 complex was injected. In PLXSN group, 50 μL of lipofeotamine-pLXSN complex was injected. In PBS group, 50 μL of PBS was injected. After 2 weeks from transfection, rats were executed by cervical vertebra luxation, followed by determination of related indexes.

Observation indexes

The laparotomy was performed for the third time and the growth situations of ectopic foci in each group were observed with the naked eye; length, width and height of foci were measured with a vernier caliper; with Katsuki's method [12]. The volume of focus was calculated. Ectopic focus tissues on one side of rats were taken, and mRNA expressions of IL-1β, TNF-α and NF-κB were detected by real-time PCR [13]. Ectopic focus tissues on the other side of rats in the three groups (11, 10 and 10 foci respectively) were fixed, embedded and sliced to observe the histomorphology and ultrastructural changes of ectopic foci after transfection under optical microscopyand electron microscopy and detect the changes of microvessel density (MVD) in tissues by immunohistochemistry [14]. The morphological changes of rat ovarian tissues after transfection were observed under optical microscopy. The levels of estradiol (E_2), progesterone (P), luteinizing hormone (LH) and follicle stimulating hormone (FSH) in rat serum after transfection were detected by radioimmunoassay [15]. The VEGF levels in serum of rats in each group were detected by ELISA [16].

Statistical analysis

SPSS13.0 was used for statistical analysis. For continuous variables, mean ± standard deviation was used for description, when normal distribution was met. Comparison between groups was tested by t test. Pearson correlation was used for correlation analysis. Median (lower quartile, upper quartile) was used for description when normal distribution was not met. Comparison between groups was tested by

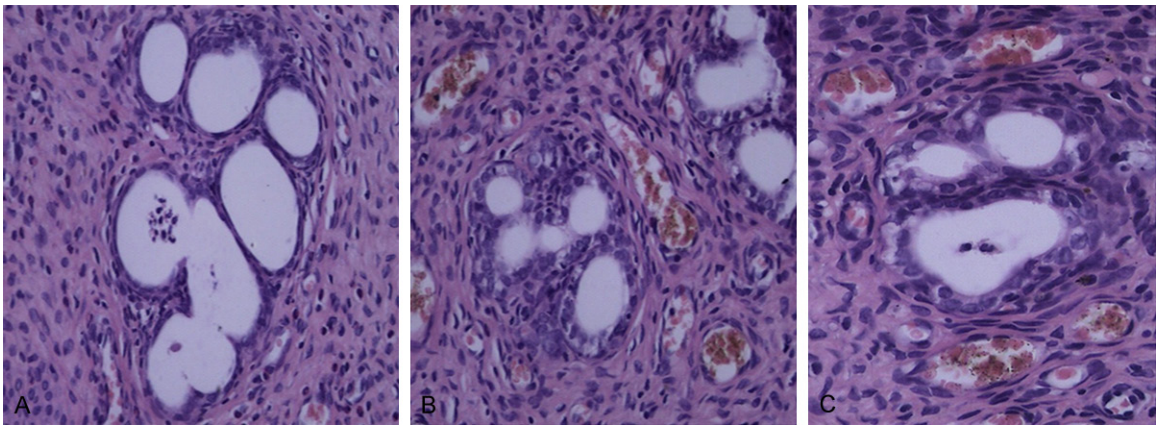

Figure 1. Histological changes 2 weeks after transfection under optical microscope (200 ×). A: pLXSN-hBD2 group; B: pLXSN group; C: PBS group.

rank-sum test. Spearman correlation was used for correlation analysis. Comparison of enumeration data was tested by chi-square test. $P < 0.05$ was considered as statistically significant.

Results

Ectopic foci volume of EMS rats before and after transfection

Average volumes of ectopic foci in the three groups before transfection were compared and the differences were not statistically significant ($P > 0.05$). After transfection, average volume of ectopic foci in pLXSN-hBD2 group was obviously smaller than those in pLXSN group and PBS group. The differences were statistically significant ($P < 0.01$). The comparison of average volumes of ectopic foci in two latter groups was not statistically significant ($P > 0.05$). Average volumes of ectopic foci in three groups before transfection were compared with those after transfection. The differences were all statistically significant. Average volume of ectopic foci in pLXSN-hBD2 group after transfection was obviously smaller than that before transfection ($P < 0.01$). However, average volumes of ectopic foci in pLXSN group and PBS group after transfection were obviously larger than those before transfection ($P < 0.05$) (**Table 1**).

Histological changes under optical microscope

In pLXSN-hBD2 group, 2 weeks after transfection, the ectopic endometrial tissue was significantly reduced. The glandular cavity was narrow. The cells were sparse and atrophic, and the peripheral vessels were reduced (**Figure 1A**). In pLXSN group and PBS group, the ectopic endometrial tissues grew well. The glandular epithelial cells were trim, and the mesenchyme grew well (**Figure 1B**, **1C**).

Utrastructure changes of ectopic endometrial cells under electron microscope

Two weeks after transfection, in pLXSN-hBD2 group, there were obvious mitochondrial swelling, karyopyknosis (**Figure 2A**), basic disappearance of villus and cellular atrophy (**Figure 2B**). However, in pLXSN group and PBS group, the cell size was normal. The cellular morphology was regular and the cells were trim (**Figure 2C**, **2D**).

MVD after transfection

Two weeks after transfection, the MVD in ectopic focus tissue in pLXSN-hBD2 group was 10.08 ± 2.72 HPF, which was significantly lower than those in 20.58 ± 3.30 HPF in pLXSN group and 21.02 ± 3.71 HPF in PBS group (all $P < 0.01$). There was no significant difference between pLXSN group and PBS group ($P > 0.05$).

Expressions of IL-1β, TNF-α and NF-κB mRNA in focus tissues after transfection

Two weeks after transfection, the expression levels of IL-1β, TNF-α and NF-κB mRNA in ectopic endometrial tissue in pLXSN-hBD2 group were significantly lower than those in pLXSN group and PBS group ($P < 0.01$ and $P < 0.05$, respectively). There was no significant differ-

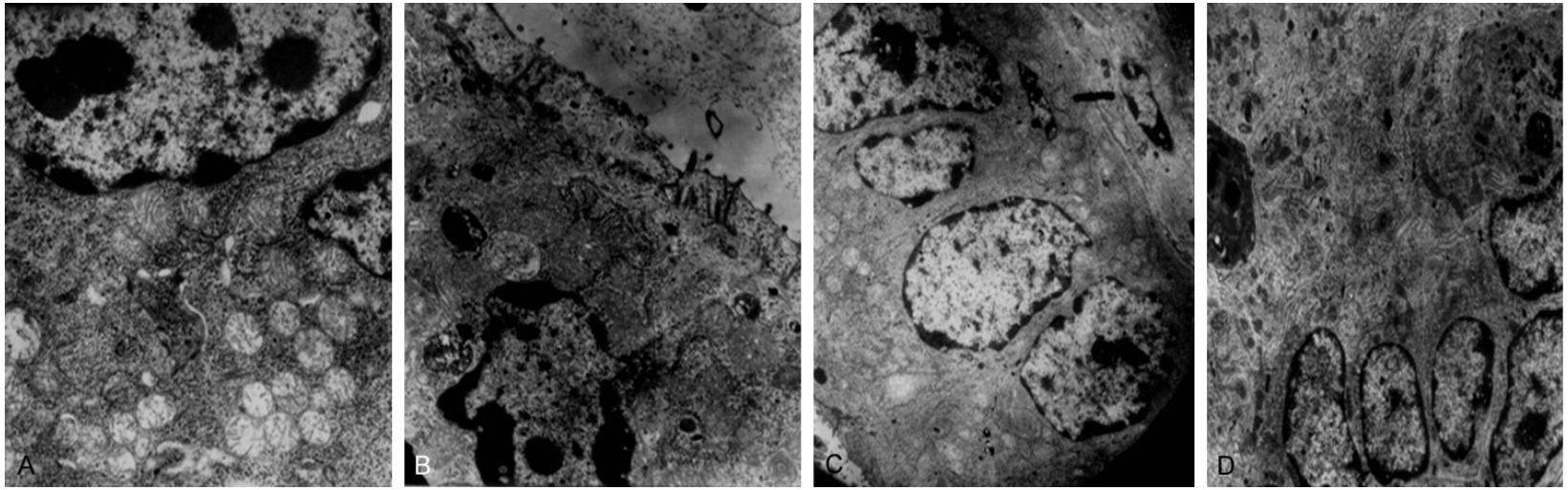

Figure 2. Utrastructure changes of ectopic endometrial cells 2 weeks after transfection under electron microscope. A: pLXSN-hBD2 group (10000 ×); B: pLXSN-hBD2 group (8000 ×); C: pLXSN group (5000 ×); D: PBS group (3500 ×).

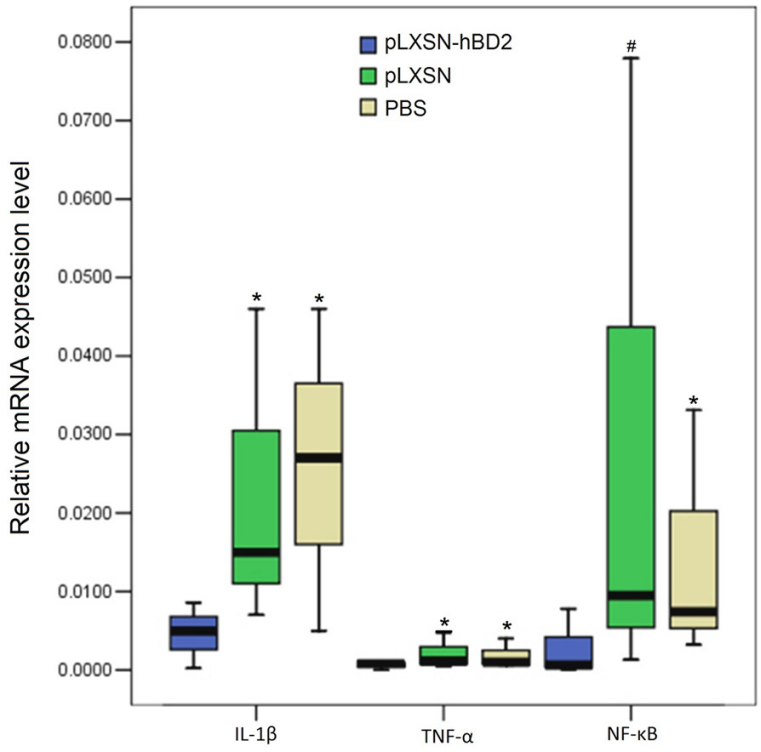

Figure 3. Expressions of IL-1β, TNF-α and NF-κB mRNA in ectopic focus after transfection. *P < 0.05 and #P < 0.01 compared with pLXSN-hBD2 group.

Table 2. VEGF levels in serum before and after transfection (pg/ml)

Group	4 weeks before transfection	2 weeks after transfection
pLXSN-hBD2	61.05 ± 6.10	48.70 ± 6.16*
pLXSN	58.62 ± 5.58	59.12 ± 5.70#
PBS	59.89 ± 5.86	56.76 ± 6.13#

*P < 0.01 compared with before transfection; #P < 0.01 compared with pLXSN-hBD2 group.

ence between pLXSN group and PBS group (P > 0.05) (**Figure 3**).

VEGF levels in serum before and after transfection

VEGF levels in serum in pLXSN-hBD2 group, pLXSN group and PBS group before transfection were compared. The difference was not statistically significant among them 4 weeks before transfection (P > 0.05). Two weeks after transfection, VEGF level in serum of rat model in pLXSN-hBD2 group was obviously lower than those in pLXSN and PBS groups. The differences were both statistically significant (P < 0.01). The latter two groups were compared and the difference was not statistically significant (P > 0.05). In addition, VEGF level in serum of rat model in pLXSN-hBD2 group after transfection was obviously lower than that before transfection. The difference was statistically significant (P < 0.01). VEGF levels in serum before and after transfection in pLXSN group and PBS group were compared respectively. The differences were not statistically significant (P > 0.05) (**Table 2**).

Ovarian hormones levels in serum after transfection

Two weeks after transfection, the changes of ovarian hormones (E_2, P, FSH and LH) levels in serum were detected. There was no significant difference of each index among three groups (P > 0.05) (**Table 3**).

Pathological changes of ovary after transfection

Two weeks after transfection, the ovarian tissue sections of rats in each group were observed. All levels of follicle and corpus luteum could be seen in every section, and the structure of corpus luteum was basically normal.

Discussion

VEGF is one of the most critical stimulating factors. It is verified that VEGF may increase in the early stage of EMS. The concentration of VEGF in peritoneal fluid of patients with EMS is obviously higher than that of patients without EMS. The increase level is positively correlated with the disease severity [17]. Other polypeptide factors, mostly, such as IL-1β, IL-6 and TNF-α, indirectly play a role in promoting angiogenesis and resisting angiogenesis via VEGF [18]. The concentration of IL-1β in ectopic endometrial tissue of patients with EMS is obviously higher than those in endometrium of patients with EMS and normal endometrium in control group. Besides, the IL-1β level in peritoneal

Table 3. Ovarian hormones levels in serum after transfection

Group	E_2 (pg/ml)	P (ng/ml)	LH (IU/L)	FSH (IU/L)
pLXSN-hBD2	11.58 ± 5.86	19.23 ± 7.50	6.78 ± 2.26	2.83 ± 1.38
pLXSN	11.30 ± 5.25	18.30 ± 4.86	7.17 ± 1.82	4.11 ± 1.13
PBS	13.96 ± 6.07	18.89 ± 6.88	5.96 ± 3.27	3.83 ± 1.23

E_2, estradiol; P, progesterone; LH, luteinizing hormone; FSH, follicle stimulating hormone.

fluid increases apparently. IL-1β level in ovarian endometriosis cyst is significantly higher than that in other diseased regions [19]. TNF-α in peritoneal fluid and serum of patients with EMS increases obviously as well and its increase is correlated with disease severity. In addition, TNF-α synchronously increases with the increase of VEGF in the body [20, 21]. MVD in foci of patients with EMS is obviously higher than that in normal endometrial [22], which suggests that occurrence and development of EMS are closely related to abnormal expression of micro vessels. Moreover, MVD is a quantitative indicator for judging angiogenesis. MVD is frequently observed to evaluate focus activity and treatment effect.

HBD-2 was initially discovered by Harder in 1997. It is reported that hBD-2 can resist tumor by anti-angiogenesis effect [8-10]. This study has observed the effect of pLXSN-hBD2 on angiogenesis and inflammatory factors of rat model EMS. Results found that, the VEGF level in serum of rat model transfected with hBD-2 decreases. mRNA expression levels of IL-1β, TNF-α and NF-κB in ectopic endometrial tissues were all significantly lower than those in the two control groups, as well as MVD. In addition, the volume of ectopic focus apparently reduces, compared with control groups and the volume before transfection. Meanwhile, it is observed under electron microscope that the number of glands in ectopic foci of rats transfected with hBD-2 reduces. In gland cells, mitochondrial swelling, chromatin condensation and margination, disappearance of microvilli and apoptosis are observed as well. Obvious decrease of ectopic endometrial tissues, lumens stenosis, sparsity and atrophy of cells and disappearance of peripheral vessels are observed under optical microscope. The above results all show that hBD-2 has a certain inhibitory effect on ectopic foci of rat model of EMS. In addition, two weeks after transfection, there was no significant difference of E_2, P, FSH or LH among three groups, and the structure of corpus luteum was basically normal. This indicated that hBD-2 did not affect the ovarian function.

In this study, the reason for inhibitory effect of hBD-2 on rat model of EMS may include various pathways and factors. The overexpression of hBD-2 may have the following effects: i) VEGF level in serum of rats is directly reduced and thus angiogenesis is resisted. ii) mRNA expressions of IL-1β and TNF-α in rats are directly inhibited, which leads to the improvement of local abnormal immune environment in abdominal cavity and ectopic foci. Local immune inflammatory response of rats with EMS is relieved. Plantation and growth of ectopic endometrium are directly inhibited. iii) mRNA expression of NF-κB is directly inhibited. Positive feedback pathway, which further produces inflammatory cytokines, such as IL-lβ, TNF-α, IL-6 and IL-8, in abnormal immune environment, is inhibited. That further reduces VEGF level in rats and indirectly plays a role in anti-angiogenesis. iv) hBD-2 can effectively play a role in anti-angiogenesis for EMS, which leads to the decrease of MVD. Eventually, the growth of ectopic foci is limited and the volume of foci is reduced.

At present, the effect of hBD-2 on tumor treatment has shown good application prospects. But malignant tumor cells have a relatively strong ability of abnormal proliferation. Therefore, tumors cannot be cured radically with hBD-2. Though EMS has some malignant characteristics, it does not have the ability of abnormal proliferation. As aautograft, ectopic endometrium is more dependent on the support of vessels. If angiogenesis is stopped, ectopic endometrium will not be planted successfully. Therefore, it is concluded by us that treatment for EMS with hBD-2 should have better application prospects compared with treatment for malignant tumors. Treatment for EMS via anti-immune inflammatory response and anti-angiogenesis may be the effective treatment for EMS in the future. However, the application in clinical treatment will face some difficulties, such as the best route of administration, the preferred dosage, side effects, the cost of the synthesis of hBD-2 and etc. All of those require numerous long-term animal experiments and

clinical researches for deep exploration and verification.

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Disclosure of conflict of interest

None.

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