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Original Article

Effects of liver depression and psychological stress on human uterine leiomyoma cells by an AR–cAMP–PKA signal transduction pathway

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ABSTRACT

Objective: Based on the emotional theory of Traditional Chinese Medicine, and combined with the modern medicine theory of psychological stress, a research model of human uterine leiomyoma cells (ULM) was cultured *in vitro* to determine the effectiveness of adrenergic receptor (AR) agonists in human ULM cell growth. In addition, we studied the functional influence of "liver depression and psychological stress theory" on fibroid formation by intervening in the AR–cAMP–PKA signaling pathway. The intention was to establish a new method to prevent and cure fibroids through "liver depression and psychological stress theory" and provide an experimental basis for the Traditional Chinese Medicine emotional theory.

Materials and methods: Primary human ULM cells were enriched by collagenase digestion. Immunohistochemistry and hematoxylin and eosin (HE) staining were used for cytological identification. Using this model, we studied intervention using specific AR agonists on ULM cells to observe the influence of "liver depression and psychological stress theory" on estrogen receptor (ER), progesterone receptor (PR), vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF).

Results: Norepinephrine (NE) and epinephrine (E) are adrenergic receptor agonists. They promoted ULM cell proliferation and increased the levels of ER, PR, VEGF and FGF. In contrast, isoproterenol (ISO) inhibited ULM cell proliferation and decreased the levels of ER, PR, VEGF and FGF. The protein expression of cAMP and PKA in ULM cells was reduced and the levels of ER, PR, VEGF and FGF were increased when co-treatment with the α-AR blocker (phentolamine). The β-AR blocker (metoprolol) displayed an opposite effect.

Conclusions: AR agonists modulated ER, PR, VEGF and FGF levels in ULM cells in an AR–cAMP–PKA-dependent signaling pathways to influence fibroid occurrence and development.

Introduction

Uterine leiomyoma is a very common benign gynecological tumor, which belongs to the abdominal mass in traditional Chinese medicine. Throughout the various opinions of many Chinese physicians, the pathogenesis of an abdominal mass includes internal causes and external causes. Often, when eating cold food or on exposure to exogenous wind-cold and damp-toxin during menstruation, or residuing the blood after delivery the human body can be potentially invaded by such external causes. The pathogenic qi is fighting with healthy qi and blood, which could obstruct the circulation of qi-blood and result in an abdominal mass. The internal cause, often provoked by a drastic and prolonged emotional stimulus that once exceeding a person's physiological regulatory ability, will cause qi stagnation to produce the blood-stasis setting.

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This will also lead to an abdominal mass. A person with "liver depression constitution" that easily succumbs to persistent anxiety and depression will be predisposed to blood stasis and abdominal mass, which is closely related to the onset of fibroid disease. As miraculous pivot record "if injured by anger, the qi will go upward and cause stagnation of qi and blood to result in abdominal mass", the Jing-yue's complete work also said "the sudden anger injure liver and cause regurgitation of qi and blood stasis result in abdominal mass; over-thinking and anxiety injury spleen and may make qi deficiency and blood stasis result in abdominal mass". So qi stagnation is the key pathogenesis of fibroid, for prolonged qi stagnation produce the blood-stasis and phlegm lead to fibroid.

Large sets of epidemiological data and clinical research indicate that psychological factors (including chronic stress, prolonged depression, social isolation, among others) [1], have a close relationship with the occurrence and development of tumors. Abnormal emotional activity leads to the occurrence and development of tumors by disturbing neuro-endocrine function and inhibiting immune function. However, positive emotional activity can prevent tumor formation and can improve the prognosis of tumor patients.

In the chronic psychological stress state, abnormal stress related neurotransmitter release stimulates the brain, pituitary gland, adrenal gland, sympathetic nerve endings and tumor cells to release multiple neurotransmitters and cytokines, which lead to prolonged production of those factors, and abnormally high levels in the blood and tissues. Thaker and Glaser's research indicated that chronic stress can improve the occurrence and development of tumors through interfering with neuro-endocrine and immune system functions [2,3].

Psychological stress may stimulate a neuro-endocrine-immunological regulatory network to modulate the body's response by generating the synthesis of cytokines and neurotransmitters. This function is similar to that of the liver in controlling dispersion (including regulating qi activity, smooth emotion, which is conducive to ejaculation in men, and promotes transformation and transportation in the spleen and stomach). At the same time, the traditional Chinese medicine emotional theory might cause disease, which could be reflected through a modern medicine psychological quantitative index.

Based on these observations, we hypothesized that liver depression and psychological stress might stimulate sympathetic nerve endings and the adrenal medulla to release catecholamine hormones, like NE, E, Dopamine and others, by excitation of the sympathetic nervous system. This will activate the α/β-AR–cAMP–PKA signaling pathway of ULM cells to mediate ER and PR and improve ULM cell growth by intervening in VEFG and FGF expression.

Materials and methods

Chemicals and reagents

E, NE (they are adrenergic receptor agonists, E is epinephrine, NE is norepinephrine), and metoprolol were obtained from Jin Yao Amino Acids, Co. Ltd, Tianjing, China. Isoproterenol was obtained from Hefeng Pharmaceutical Co., Ltd, Shanghai, China. Phentolamine was obtained from Haipu Pharmaceutical Co., Ltd, Xudong of Shanghai, China. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were both purchased from GIBCO, USA. Trypsin–EDTA and Collagenase I were purchased from Sigma, USA. The agents and cytokines PR, ER, VEGF, and FGF ELISA kits were obtained from R and B Co. USA. ^{125}I-cAMP free kits were obtained from Phoenix Pharmaceuticals, Inc, USA. The BCATM protein analysis kit was obtained from Pierce Co. USA.

Cell culture

Leiomyoma tissues were randomly obtained from patients undergoing Laparoscopic Uterine fibroids removal surgery at Nan Kai Hospital, Tianjin, China in May 2012 to December 2013, with the permission of patients' informed consent. This study included women with TCM syndrome of qi stagnation and blood stasis type caused by emotional stimulation, who did not receive any type of hormonal or drug therapy at least three months before the surgery and the pathological diagnosis showed uterine fibroids after the surgery.

The size of the tumors was about 2 cm^3 in diameter. All tissue samples used in this study were confirmed as histologically ordinary leiomyomas, with no cellular, epithelioid, bizarre, or plexiform variants present. Tissues were rinsed several times with saline and minced into about 1 × 1 cm^3 small pieces in a sterile petri-dish, then digested in Collagenase I solution (DMEM 20 mL, Collagenase I at 1 mg) in 50 mL Erlenmeyer flasks in which there was a 3 cm magnet. The flask was then incubated at 37 °C with a slowly rotating magnetic stirrer for 40–50 min. The digested tissue was passed through a 200 mesh sieve, and the uterine leiomyoma cells were collected by centrifugation (1000 rpm, for 10 min). The isolated cells were seeded in a 75 cm^2 culture flask in culture medium (DMEM) supplemented with 15% FBS and 1% antibiotics and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Cells were collected between passages three and four for the experiments, which were performed with 70–80% confluent cultures, and identified by hematoxylin–eosin (HE) staining and immunocytochemistry.

Cells (1 × 10^5 cells/mL) were seeded in 12-well plates in which there was culture medium (DMEM) that was supplemented with 15% FBS, following which they were cultured in a humidified atmosphere with 5% CO_2 in air. After adhering to the culture plates, cells were cultured in DMEM with 4% FBS for 24 h before starting the studies.

In the first experiment, the cells treated with E, NE or isoproterenol at 0, 0.1, 1 and 10 μM were harvested for the culture supernatants, which were removed at 3, 6, 12 and 24 h. According to the cell proliferation assays, we determined the optimal timepoints and the optimal drug concentration for subsequent experiments. In the second experiment, the cells were treated in three separate groups as follows: (i) control, (ii) NE and (iii) NE plus phentolamine. The cells were treated with 1 μM NE (except for the control group), and the supernatants were harvested at 12 h, centrifuged, and frozen at −80 °C until assayed. For the blocking experiments, phentolamine was added to the cell cultures for 1 h in the NE plus phentolamine group, before adding NE. In the third experiment, the cells were treated in three separate groups as follows: (i) control, (ii) iso, and (iii) iso plus metoprol, following which, the three groups of cells were treated with 1 μM iso, while the control group was left untreated. Supernatants were harvested at 24 h, centrifuged, and frozen at −80 °C until assayed. For the blocking experiments, metoprol was added to the cell cultures for 1 h in the iso plus metoprol group before adding the iso. Each experiment was repeated at least three times in duplicate with unstimulated/naïve to treatment cells.

Identification of uterine tumor cells

HE staining

Uterine leiomyoma cells were cultured in DMEM supplemented with 10% FBS on the cell monolayer cover-slides. The slides were treated with formalin for 30 min, and stained with the