Inhibitory Effect of IPPV on the Angiogenesis of Mammary Hyperplasia in Rats

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Abstract **Objctive** To study the inhibitory effect of IPPV on the angiogenesis of mammary atypical hyperplasia and its underlying mechanism. **Methods** DMBA-induced animal model of mammary atypical hyperplasia was induced in rats. Fifty-five female SD rats were randomized into four groups and DMBA, DMBA with TAM, DMBA with IPPV and control diet had been administered into group A、B、C and D respectively for fourteen weeks. Samples of breasts were collected under light microscope observation with microvessel density(MVD) estimation. Their factor-VIII-related antigen, vascular endothelial growth factor(VEGF) and ras gene protein products were examined by immunohistochemical staining. **Results** The hyperplasia rate and number of atypical hyperplasia in group A (96/180, 68/96) were significantly higher than group B (46/180, 37/46) and group C (42/180, 32/42) (P<0.005), and there was no significant difference between group B and group C (P>0.05), which indicated IPPV had inhibitory effect on mammary hyperplasia. MVD and immunohistochemical staining of VEGF and ras showed significant difference between group A and group B, group A and group C (P<0.05), with no significant difference between group B and group C (P>0.05), which indicated that IPPV could inhibit the activity of VEGF and ras and reduce the MVD in breasts. **Conclusion** IPPV has inhibitory effect on mammary atypical hyperplasia and its angiogenesis.

Key words Intracellular polysaccharide of polystictus versicolor (IPPV); PSK; Mammary atypical hyperp lasia; Angiogenesis; Ras

Atypical hyperplasia of mammary epithelium is an important process of canceration, its risk of cancercation increases with the severity of atypical hyperplasia. In the development of canceration from atypical hyperplasia , a series of oncogene are activated to produce many kinds of cytokines and angiogenetic peptides, inducing angiogenesis in matrix and promoting the occurrence, development and migration of malignant neoplasms[1]. Intracellular polysaccharide of polysticus versicolor (IPPV), produced in China, is a kind of proteoglycan, extracted from a common polysticus versicolor. Its ingredient and structure is very similiar to PSK produced in Japan (kestin). PSK has been used to treat breast carcinoma and proven to be effective. In this experiment, with the animal model of cystic atypical hyperplasia of mammary gland induced by 7, 12-dimethyl benzanthracene(DMBA), we studied the inhibitory effect of IPPV on atypical cystic hyperplasia and angiogenesis, and its mechanism, providing theoretical basis for its clinical application to prevent breast cancer.

MATERIALS AND METHODS

Materials

Experimental Animals

Fifty-five female unpregnant SD rats of 38 to 45 days age (weighting from 180 to 220 gram), were purchased from Antibiotic Institute of Sichuan province , China.

Forage preparation

Forage was provided by the Animal Center of Chongqing University of Medical Sciences. Forage preparation: Generally, one rat eat about 20g forage per day; TAM(3.3mg/kg, d), IPPV (0.4% diet) were mixed in the forage for feeding.

Drug and experimental agent

TAM was the product of Finland Farmos Pharmaceutical Factory. IPPV was provided by DaXin Pharmaceutical Limited Campany: DMBA was purchased from Swiss Fluka Company. H-ras, Factor VIII related antigen (F Ⅷ -RA), vascular endothelial growth factor (VEGF) polyclone antibody (rabbit anti-human or rat) and other immunological agents were purchased from MaiXin Biologic Pharmaceutical Company

Methods

Animal grouping and experimental process

After adaptive feeding for 5 days, SD rats were randomized into the following four groups.

Group A: 10mg/100g of DMBA (dissolved in sesame oil according to 10mg/ml) were introduced into the stomach of 15 rats via stomach tube respectively, then feeding with the normal forage and natural lighting.

Group B: On the basis of group A, 0.33mg/100g TAM was mixed in the forage to feed the 15 rats.

Group C: On the basis of group A, 0.4% diet of IPPV was used to feed the 15 rats.

Group D: 10 rats were feeded with the normal forage and natural lighting

Specimen treatment

The rats were killed and dehaired with 8% Na2S, then the mammary gland were observed and examined and removed for detection .

Observations

Pathological examination of mammary gland

Samples undergone fixation with formaldehyde, paraffin imbedding, section and HE staining, were examined by light microscope. The diagnostic criteria for atypical hyperplasia of mammary gland has been described by page[2].

Immunohistochemical determination of FVIII-RA in mammary tissues

Experimental steps:

Paraffin sections undergone deparaffin and hydration were washed by phosphate buffered saline (PBS, PH: 7.2). Added with peroxidase blockage solution and incubated in room temperature, then washed. Added with non-immune animal serum and incubated in room temperature and washed. Added with the first antibody (PBS as the control) and incubated in room temperature and washed. Added with biotin-labeled second antibody and incubated in room temperature and washed. Added with streptavidin peroxidase solution and incubated in room temperature and washed. Added with diaminobenzidine solution (DAB) and controled coloration under microscope, then washed. Finally the specimen undergone hematoxylin compound staining, dimethylbenzene transparency, neutral gum fixation. Vessel count methods: artifitial counting method of microvessel density (MVD) was accepted, it was counted in three densed vessel areas in each section by two phatologist under olympus microscope (×200 times). Single or brownish yellow stained areatus was taken as one vessel (except vessels containing eight red blood cells). The mean MVD=(n1+n2+n3)/3.

Immunohistochemical detection of VEGF in mammary tissues

Experimental steps were similiar to these of FVIII-RA. Fotanini creteria [3] the positive cells are stained brownish yellow. The expression of VEGF was taken as the percentage of positive cells in 100 atypical mammary hyperplasia cells. The positive rate less than 10% as negative(-), 10%-30% taken as (+), more than 30% as (++).

Immunohistochemical detection of ras gene protein product in mammary tissues.

The experimental steps were similiar to the above methods. The judgement criteria is based on whatWu xiaohuan has described. The positive cell was stained yellow brown, three dense areas in each section were counted. The positive cell less than 10% was taken as (-) and 10~50% (+), more than 50% (++).

Statistical treatment

The measurement data was represented with $\bar{x} \pm s$ and subjected chi-square test for comparison of rate and degree of hyperplasia, variance analysis for comparison

Table 1 Mammary hyperplasia in each group

group	Number	number of the whole mammary gland	Number of mammary hyperplasia	Hyperplasia rate	X^2	P
A	15	180	96	53.3%	34.325(A:C)	<0.005
B	15	180	46	25.5%	39.0477(A:C)	<0.005
C	15	180	42	23.3%	0.2406(B:C)	>0.05
D	10	120	0			

Table 2 Mammary hyperplasia degree in each group

group	Number of mammary hyperplasia	Simple epithelial hyperplasia	Moderate atypical hyperplasia	Active atypical hyperplasia	Total number of atypical hyperplasia1
A	96	10	18	68	86
B	46	9	21	16	37
C	42	10	17	15	32
D	0	0	0	0	

x^2=23.9085 A & B P<0.005, A & C P<0.005, B & C P>0.05

Table 3 MVD (FⅧ-RA) in each group of mammary hyperplasia tissue

group	number	$\bar{x}\pm s$(MVD)	Range
A	86	31.78±4.12	26.5~41.9
B	37	16.44±3.08	11.4~26.8
C	32	15.32±2.94	9.0~26.6

Variance analysis F=162.8986 A and B $P<0.01$, A and C $P<0.01$, B and C $P>0.05$

Table 4 Expression of VEGF in each group of atypical mammary hyperplasia tissue

group	Number	(-)	(+)	(++)	x^2	P
A	86	16	23	47	7.68104(A:B)	<0.025
B	37	14	12	11	6.32321(A:C)	<0.05
C	32	12	10	10	0.21588(B:C)	>0.05

Table 5 Expression of ras gene protein in each group of atypical mammary hperplasia

group	Number	(-)	(+)	(++)	x^2	P
A	86	24	22	40	8.1949(A:B)	<0.025
B	37	19	10	8	6.9695(A:C)	<0.05
C	32	16	9	7	0.01478(B:C)	>0.05

of MVD in atipical hyperplasia and rank test for comparison of other immunological results in each group, respectively. $P<0.05$ was considered significant difference.

RESULTS

The result of histological observation

The mammary gland in group A was a little more prominent than group B. A hard mammary lump was found in group A and histologically identified as type A mammary adenocarcinoma. The results of histological examination in each group are as follows:

From table 1 and 2, we can see that the hyperplasia rate of IPPV group and TAM group were obviously lower than that of model control group, the hyperplasia rate of IPPV group was a little lower than that of TAM group, but with no significant difference.

MVD in mammary hyperplasia tissue

From table 3, we can see that the number of microvessels in mammary hyperplasia tissue of IPPV group and TAM group was obviously less than that of model control group, implying that IPPV and TAM both have the effect to inhibit angiogenesis of mammary hyperplasia.

Expression of VEGF and ras in mammary hyperplasia tissue

From table 4 and 5, we can see that the expression of VEGF and ras in atyical mammary hyperplasia tissue of IPPV and TAM group was obviously lower than that of model control group, implying that IPPV and TAM both have the similiar inhibitory effect on the expression of VEGF and ras.

DISCUSSION

Atypical hyperplasia of mammary epithelium is an important precancerous lesion. With series of sections examination of the whole mammary gland of 200 cases of breast carcinoma, Zhang Zhendong et al found that 48% cases were accompanied with atypical hyperplasia. Kan Xiu et al found that the earlier the cancerous loci, the more frequency the atypical hyperplasia could be seen, and 91.7% cases of carcinoma in situ was accompanied with atypical hyperplasia. It was reported by Jiang Jun et al that the dynamic process of rat mammary carcinoma induced by DMBA was: epithelial hyperplasia of mammary duct→atypical hyperplasia→mammary carcinoma; the occurrence of mammary carcinoma could be prevented if the the development of precancerous disease of mammary gland was inhibited or precancerous atypial hyperplasia of mammary gland was reversed[5]. It was supported by many experiments that the occurrence, development and migration of solid carcinoma depended on angiogenesis, whose phenotype had been opened in atypical hyperplasia before malignancy.

Some hyperplasia cells had angiogenesis-induing oncogenes, which can automatically induce angiogenesis when the hyperplastic loci was 3 times larger than before. The breast hyperplasia tissue or breast cancer tissue could obviously induce angiogenesis when they were transplanted into the subcutaneous tissue of nude mice, while the normal breast tissue lack of this capacity [6]. Liu Shengchun, et al reported that the number of angiogenesis in simple mammary hyperplasia, atypical hyperplasia or carcinoma in situ was higher than that of normal tissue[1]. It was proved by immunohistochemical detection that there were many angiogenetic factors, such as VEGF, bFGF, IGF and PDECGF, which would be different according to different lesion degree or site. It is suggested that the development of precancerous lesion could be inhibited or reversed through antiangiogenesis.

The process of rat mammary canceration induced by DMBA is very similiar to that of human breast carcinoma, in which a series of oncogens are activated. The expression of ras oncogene protein product was detected in atypical hyperplasia tissue[5], and the mutant ras gene may not only promote cell proliferation, but also promote angiogenesis and provide nutrition for cell growth indirectly through upregulation of VEGF. Recently it was reported that mammary epithelium disposed by DMBA may produce VEGF and had angiogenesis capacity which progressively increased with the development of epithelial hyperplasia[7]. Jiang Xiaosan et al had studied the expression of MVD and VEGF in the tissue of mammary carcinoma induced by DMBA, and found that angiogenesis mediated by VEGF could take important role in the promoting migration of primary breast carcinoma[8]. The mammary tumor model induced by DMBA may be used to detect the dependence of tumor development on angiogenesis[7]. In this experiment, we found that there were more expression of MVD and VEGF in atypical hyperplasia tissue of rat mammary gland induced by DMBA at the same time, there were also expression of mutant H-ras in the atypical hyperplasia cell.

PSK is a kind of proteoglygan extracted from a common polysticus versicolor, which has many pharmacological activities and as an immunoenhancement agent, has been used in adjuvant treatment for cancer patient. Toi et al found that PSK combined with chemotherapy could improve the survival rate of breast cancer patient and prevent recurrence[9]. PSK could obviously enhance the therapeutic effect of TAM on rat mammary carcinoma. It is supported by many reserches that PSK may promote the proliferation reaction of lymph cell in the tumor, enhance the antitumor effects of natural kill cell, T kill cell and macrophage, induce expression of IL-1, IL-6, IL-8, TNF, chemotactic factor for monocyte and other cytokines, clear oxygen radical and protect chromosome. Kobayash et al studied the inhibition of PSK on tumor migration and believed that besides immunoregulating effect, PSK also had the inhibitory effect on the occurrence, development and migration of tumor[10].

In this experiment, we found that IPPV (i.e. PSK made in China) and TAM both have the inhibitory effect on the proliferation of mammary epithelium (especially severe hyperplasia) induced by DMBA, and the inhibitory effect of IPPV was a little higher than that of TAM, but with no significant difference. IPPV and TAM could both inhibit angiogenesis as well as the expression of VEGF and ras gene protein product in mammary hyperplasia tissue. IPPV and TAM could inhibit angiogenesis through inhibiting the expression of ras and VEGF, so that inhibit the development of atypical mammary hyperplasia and exhibit a good preventive effect on the occurrence of experiment mammary carcinoma.

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