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A polysaccharide isolated from *Agaricus blazei* Murill inhibits sialyl Lewis X/E-selectin-mediated metastatic potential in HT-29 cells through down-regulating α-1,3-fucosyltransferase-VII (FucT-VII)

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ABSTRACT

In this research, one low molecular weight polysaccharide (LMW-ABP) was isolated from the fruiting bodies of *Agaricus blazei* Murill. We reported that the effect of LMW-ABP on inhibiting the interaction between E-selectin and sialyl Lewis X (sLe^x) by flow cytometry and real-time reverse transcription polymerase chain reaction (RT-PCR) technology. Different concentrations of LMW-ABP (5, 10, and 20 mg/L) could effectively dose-dependently inhibit adhesion of HT-29 cells to human umbilical vein endothelial cells (HUVECs) in static conditions, as well as down-regulating the expression of both α-1,3-fucosyltransferase-VII (FucT-VII) and sLe^x in transcription or translation level, respectively. These results suggest that LMW-ABP could suppress the metastasizing capacity of cancer cells through interfering with the interaction between E-selectin and sLe^x. Thus based on these findings, LMW-ABP is expected to be having enormous potential for use in treatment for neoplasm metastasis.

1. Introduction

Tumor metastasis is a multiple cascade process that involves malignant cells detaching from the primary tumor mass, escaping from recognition by the immunosurveillance system, infiltrating the extracellular matrix, migrating through the vascular endothelium, penetrating secondary host organs and finally resulting in new tumor colonies (Dymicka-Piekarska & Kemona, 2009). These interactions are mediated by cell-surface adhesion molecules (CAM) (Zetter, 1993). Among them, selectins appear to be of critical importance. Three members of the selectin family have been identified: L-selectin, E-selectin, and P-selectin. L-selectin is constitutively expressed on leukocytes. P- and E-selectins are expressed on activated endothelial cells. P-selectin is also expressed on thrombin-activated platelets (Vestweber & Blanks, 1999). Although they are differentially expressed, all selectins are kindly bind to the carbohydrate structure sLe^x, as well as its isomer sLe^a (Varki, 1994). Cell-surface antigen sLe^x [NeuAcα2-3Galβ1-4 (Fucα1-3) GlcNAc-R] is a constituent of the ligand for cell adhesion molecules, particularly expressed on the surface of all circulating leukocytes or carcinoma cells. The biosynthesis of sLe^x is mediated by FucT-VII, which catalyzes the transfer of fucose from GDP-β-fucose to the 3-OH of α-2,3-sialyl *N*-acetyllactosamine (SALN) (Miyashiro, Furuya, Fujishige, & Sugita, 2004; Zerfaoui, Fukuda, Sbarra, Lombardo, & EI-Battari, 2000). Many researchers believe that selectins, particularly E-selectin, are engaged in the growth of tumor and formation of metastasis, most probably through increased tumor angiogenesis and adhesion of tumor cells to endothelium (Alexandrakis, Passan, & Sfiridaki, 2004; Koch, Halloran, Haskell, Shah, & Polverini, 1995; Ley, 2003). For example, E-selectin mediates the metastasis of tumor cells to endothelium and elevated serum E-selectin levels indicate a high rate of liver metastasis in patients with colorectal cancer (Dymicka-Piekarska & Kemona, 2009).

Currently more and more attention was cast on polysaccharide by biochemical and nutritional researchers due to their various biological activities used in health-care food or medicine, especially anti-oxidant, immunostimulatory, and anti-tumor effects (Qiao et al., 2009; Sun & Liu, 2009a, 2009b; Tong et al., 2009; Yuan, Zhang, Fan, & Yang, 2008). To the best of our knowledge, most polysaccharides derived from plants are relatively nontoxic and do not cause significant side effects. Thus, plant polysaccharides are recognized as an effective biological response modifier with low toxicity. *A. blazei* is an edible mushroom distributed originally in Brazil and presently cultivated in other areas, including Japan, China, and Indonesia, which is an Agaricaceae fungus belonging

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to the Basidiomycotina. In China its fruiting body is generally called "Baxi Mogu" and used as health food and home remedy. In our previous investigation, one new water-soluble polysaccharide (LMW-ABP), with a molecular mass of 4.8×10^4 Da, was obtained from the fruiting bodies of *A. blazei*. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Total carbohydrate content was determined to be 94% according to Phenol–H_2SO_4 method. Structure elucidation of LMW-ABP was investigated by a combination of chemical and instrumental analysis. The results indicated that the backbone of LMW-ABP is mainly (1 → 3)-linked-β-D-glucan. Preliminary pharmacology test showed that LMW-ABP possesses very potent anti-tumor and anti-metastasis activities (Niu, Liu, Zhao, Su, & Cui, 2009; Niu, Liu, Zhao, & Wu, 2009). So in this study, LMW-ABP was investigated whether it can inhibit neoplasm metastasis initiated by the adhesion of HT-29 cells to HUVECs through suppressing the expression of FucT-VII. Selective inhibitors of FucT-VII are expected to be potential therapeutics for the treatment of neoplasm metastasis.

2. Materials and methods

2.1. Materials

The fruiting body of *A. blazei* was purchased from Zhengjiang Bo Rui Pharmaceuticals Co., Ltd.. (Zhejiang, China). HT-29 human colon cancer cells and HUVECs were from ATCC. EDTA and Ribonuclease A was from Amresco Inc. Medium RPMI 1640, fetal bovine serum, and trypsin were from Gibco Invitrogen Co. SYBR®-ExScript™RT-PCR Kit, FucT-VII primer, E-selectin primer, GAPDH primer, agarose, DNA Marker DL2000, RNAiso Reagent, and Rnase-free H_2O were from Bao biotechnology Co., Ltd. HECA-452, R4-22, 68-5H11, MOPC-21, and CSLEX1 were from BD Biosciences. KM231 and KM93 were from Calbiochem Co., Ltd. McCoy's 5A culture medium, Rose Bengal, and 6-carbonoxyluciferin acetate were from Sigma Chemical Co. DiI was from BiYunTian biotechnology Co., Ltd. DEPC was from Ruixing Chemical Co., Ltd. All other reagents were of analytical grade.

2.2. Extraction and purification of LMW-ABP

The fruiting bodies of *A. blazei* were extracted 3× with 80% EtOH at 75 °C for 6 h to defat and remove some colored materials, oligosaccharides, and some small molecule materials under reflux. The residue was then extracted 4× with 10 vol. of distilled water at 75 °C for 3 h. After centrifugation (1700 *g* for 10 min, at 20 °C), the supernatant was concentrated 10-fold, and precipitated with 95% EtOH (1:5, v/v) at 4 °C for 12 h. The precipitate collected by centrifugation was deproteinated by a combination of proteinase and Sevag method (Sun et al., 2008) and exhaustively dialyzed against water for 48 h. Dialysis solutions was precipitated by adding 5 vol. of 95% ethanol to obtain the crude polysaccharide, named as CABP.

The CABP was dissolved in distilled water, centrifuged, and then the supernatant was applied to a column of DEAE-650M equilibrated with 0.9% NaCl. After loading with sample the column was eluted with different concentrations of NaCl aqueous solution (0 and 1 M) stepwise at 8 ml/12 min. Test tubes (containing 8 ml eluant each) were collected using an automated step-by-step fraction collector. Then the eluted solution of 0.1 M NaCl was purified on Toyopearl HW-65F and Toyopearl HW-50S columns (90 × 2 cm) one after another, loading 100 mg the above-purified fraction for each run. The columns were eluted with 0.2 M NaCl with a flow rate of 0.5 ml/min. The eluted fractions containing a large amount of sugar were only separated into one fraction and precipitated by 5 vol. of 95% ethanol to obtain purified polysaccharide, named as LMW-ABP. Total carbohydrate and protein content of each tube were measured by Dubois's (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and Lowry's (Lowry, Rosebrough, Farr, & Randall, 1951) method respectively. A stock LMW-ABP solution was prepared by dissolving in PBS and sterilized by passing it through a 0.22 μm Millipore filter.

2.3. Detection of sLe^x by fluorescence microscopy

The expression of sLe^x on the surface of cells was examined by mAbs specific for its epitope using fluorescence microscope according to the method previously described by Zeisig, Stahn, Wenzel, Behrens, and Fichtner (2004), with some modifications. In brief, the 6-well glass slide was coated with 50 μL of the cells (1×10^5), and detached with 2 mmoL/L EDTA at 37 °C for 30 min. The precipitated cells were fixed for 5 min with 5% formalin–PBS solutions, then adding 50 μL of PBS in each slide, incubated at 37 °C for 15 min. After three washing with PBS, 50 μL of different titer of antibodies solutions, namely monoclonal antibodies CSLEX1 (anti-sLe^x, 1:500), KM93 (anti-sLe^x, 1:100), KM231 (anti-sLe^a, 1:100) or PBS as control were seeded into each well for 24 h at 4 °C. Three washes later, the supernatant was abandoned. The precipitated cells were incubated for 1.5 h at room temperature with 50 μL of FITC-conjugated goat anti-mouse IgM/IgG antibody (1:200), followed by three washes with PBS. After removal of the supernatant, the results were recorded and imaged by fluorescence microscopy (BX-60F-3, OLYMPUS).

2.4. Cell adhesion experiments under static conditions

HT-29 cells were assayed for adhesive force to HUVECs expressing E-selectin stimulated by TNF-α as previously described (Devaraj, Li, & Jialal, 1996; Schuldes et al., 2003). Briefly, 100 μL of the HUVECS (3×10^4 cells/well) in exponential phase of growth was incubated 1–2 days in 96-well microtiter plate with ternary duplication, until the cell monolayer was formed. The fresh culture medium was replaced before the HUVECs were activated by TNF-α (5 ng/mL) at 37 °C for 4 h. Each aliquot of 200 μL HT-29 cells (5×10^4/well), treated by LMW-ABP (5, 10, and 20 mg/L) for 48 h, were added to the above 96-well microtiter plate at 37 °C for 30 min in a shaking way (120 rpm). After two washes by F-12K medium containing 10% fetal bovine serum, the non-adhesion cells were rinsed and stained by 100 μL of 0.25% Rose Bengal for 5 min. Getting rid of the excess staining solution with three washes again, the reaction was terminated by adding 200 μL of 95% ethanol/PBS (1:1) in each well at room temperature for 30 min. The absorbance at 570 nm was measured on an ELISA reader (Safire 2, TECAN). Each sample had three dishes, and each dish was measured for 3 min. The mean value was calculated from five trials. The HT-29 cell not treated by LMW-ABP was the control. Data were expressed as the mean OD value of the samples minus the mean OD value of the control. The rate of adhesion and inhibition were calculated according to the formula below:

$$\text{Adhesion rate } (\%) = \text{the mean OD value of the samples} / \text{the mean OD value of the control} \times 100\%$$

$$\text{Inhibition rate } (\%) = (1 - \text{the mean OD value of the samples} / \text{the mean OD value of the control}) \times 100\%$$

2.5. Determination of cell migration and invasion

The cell migration and invasion assay was performed using 24-well transwell cell culture chambers (Costar, Cambridge, MA) with

polycarbonate filters of 8 μm pore size according to the method as previously documented (Barreiro et al., 2005; Julien et al., 2001; Yao et al., 2003), with some modifications. Briefly, the film polycarbonate filters was coated with 5×10^4 cell/well for 1–3 days in order to from a continuous thin layer, which can mimic the extracellular matrix in vivo. Before the test, the HUVECs were activated by TNF-α (5 ng/mL) at 37 °C for 4 h. The HT-29 cells were treated by different of LMW-ABP (5, 10, and 20 mg/mL) for 48 h, followed by staining with fluorescent dye DiI for 30 min, and then were added to the upper compartment of the transwell units (1×10^6 in 100 μL/well). Meanwhile each 100 μL of laminin (10 mg/L) in F-12K complete medium as chemoattractant was filled with each lower compartment. This system was incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2, and then fixed with formaldehyde for 30 min. After the cells on the film of polycarbonate filters were softly wiped off, the number of the HT-29 cells that had migrated to the lower side of the polycarbonate filter was counted in five randomly selected fields by fluorescence microscope (BX-60F-3, OLYMPUS) based on five independent experiments. The groups not be treated by LMW-ABP were the control. The rate of migration and invasion was calculated according to the formula below:

$$\text{Inhibition rate } (\%) = (1 - \text{the mean OD value of the samples} / \text{the mean OD value of the control}) \times 100\%$$

2.6. Flow cytometry analysis

The expression of sLe^x on the surface of HT-29 cells influenced by various LMW-ABP samples was determined according to the method as previously described (Barreiro et al., 2005; Julien et al., 2001; Yao et al., 2003). A 12 h of incubation was finished by replacing the McCoy's 5A incomplete medium as soon as HT-29 cells (1×10^6/well) in logarithmic phase adhered to bottom of 6-well culture plates, and then this system was incubated with different LMW-APB samples (5, 10, and 20 mg/mL) for 48 h. Thereafter HT-29 cells (1×10^6) were collected, washed, and cultivated with mAbs to sialyl Lewis antigens CD15s or PBS (control) for 1 h, 4 °C. Finally the cells (1×10^6) were marked by FITC-conjugated goat anti-mouse IgM/IgG antibody for 30 min at 4 °C. Then the cells were subjected to fluorescence analysis performed on a FACS Calibur flow-cytometric apparatus after suitable washing. Data were expressed as the mean fluorescence intensity or the number of positive cells. Each experiment was performed in triplicate.

2.7. Analysis of FucT-VII mRNA expression by real-time RT-PCR

Real-time RT-PCR was performed to determine changes in FucT-VII gene expression as described by Miyashiro et al. (2004), with some modification. First, HT-29 cells were pretreated by different LMW-ABP samples (5, 10, and 20 mg/mL) for 48 h. Second, total cellular RNA was isolated from HT-29 cells using an Trizol reagent kit (Invitrogen, MD) according to the supplier's instruction manuals. The purity and concentration of isolated RNA were calculated by UV–vis absorption at 260 or 280 nm. The specific value of sample required for RT-PCR must be among 1.8–2.0. One unit of absorption is an equivalent weight of 40 μg/mL RNA. The concentrations of RNA (μg/mL) = ($A_{260} \times$ Dilution $\times$ 40)/1000. And third, reverse transcription (RT) of the RNA was performed using a SYBR®ExScript™RT-PCR Kit (TaKaRa, Japan) with 1 μg of total cellular RNA. The standard curves for the FucT-VII and *GAPDH* cDNAs were generated by serial dilution. Finally, based on the template of single-stranded cDNA and SYBR Premix Ex Taq kit, FucT-VII and GAPDH mRNA were amplified on ABI-7300 FQ-PCR instrument using the primer sets (forward: 5′-GCCCAGTACCGCTTCTACCT-3′; reverse: 5′-GCCTGAAACCAACCCTCAA-3′ for FucT-VII; forward: 5′-GAAGGT GAAGGTCGGAGTC-3′; reverse: 5′-GAAGATGGTGATGGGATTTC-3′ for GAPDH). PCRs were performed in a final volume of 50 μL with 2.5 U of *Taq* polymerase (TaKaRa, Japan) and 4 μL of single-stranded cDNA from the RT reaction as template. The reactions were carried out one initial cycle at 95 °C for 3 s, and then 40 cycles at 95 °C for 5 s and 60 °C for 30 s for GAPDH and FucT-VII. When the reaction was finished, the threshold cycle (C_T) value of each sample was analyzed by sequence detection software version 1.2.3 (Applied Biosystems). The C_T value decreased with the rise of templete's concentration. The reaction specificity of PCR was determined by the melting curve.

2.8. Statistical analysis

The data were expressed as means ± standard errors (SE). Data were analyzed by one-way ANOVA using SPSS 13.0 for windows. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Expression of sLe^x antigen on the surface of HT-29 cells

To determine whether cultured HT-29 cells express sLe^x oligosaccharides, cells (1×10^5) were exposed to different mAbs at 37 °C for 30 min followed by washings and addition of FITC-conjugated goat anti-mouse IgM/IgG secondary antibody. The quantitation of fluorescent cells was determined by fluorescence microscopy with an electron microscope. From Fig. 1, we can see that cells strongly express sLe^x and sLe^a epitopes, recognized by CSLEX1 (anti-sLe^x), KM93 (anti-sLe^x), and KM231 (anti-sLe^a), respectively.

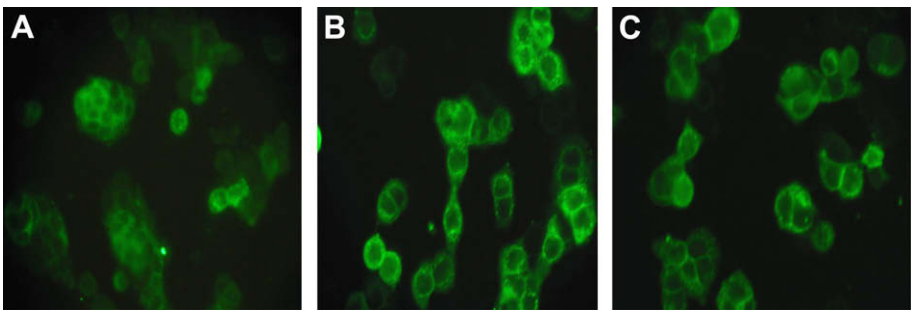

Fig. 1. The positive expression of sLe^x and sLe^a epitopes recognized by various monoclonal antibodies CSLEX1 (A, anti-sLe^x), KM93 (B, anti-sLe^x), and KM231 (C, anti-sLe^a) in HT-29 cells.

Table 1
The effect of different LMW-ABP samples on the adhesion of HT-29 cells to HUVECs.

Groups	Concentrations (mg/mL)	OD_{570}	Inhibition ratio (%)
HUVECs + HT29 + TNF-α (control)	0	0.575 ± 0.060	–
HUVECs + HT29 + TNF-α + LMW-ABP	5	0.419 ± 0.027*	27.13
HUVECs + HT29 + TNF-α + LMW-ABP	10	0.439 ± 0.039*	23.65
HUVECs + HT29 + TNF-α + LMW-ABP	20	0.415 ± 0.038*	27.83

The values are represented as means ± SD (n = 5). Significant differences with saline group were designated as *P < 0.05.

3.2. Inhibition of adhesion of HT-29 cells to HUVECs by LMW-ABP

To assess whether the effects of LMW-ABP on neoplasm metastasis are due to the inhibition of the interaction of E-selectin with its ligands sLe^x, we performed adhesion assays using HT-29 cells, which are human colon cancer cells expressing sLe^x ligand, to HUVECs by colorimetric method. As shown in Table 1, the maximum adhesion effect of was obtained after the HUVECs were stimulated by TNF-α (5 ng/mL). A panel of LMW-ABP exhibits a significant inhibitory effect on adhesion of HT-29 cells to HUVECs from 23.65% to 27.83%.

3.3. Decrease of cell migration and invasion by LMW-ABP

The abilities of cells migration and invasion play a key role in the metastasis of malignant cells. We adopt 24-well transwell cell culture chambers to identify whether LMW-ABP can suppress the HT-29 cells metastatic potential. As seen from Fig. 2, there was a decrease in the fluorescence intensity in groups treated by LMW-ABP especially at high dose, and accordingly inhibition rate was enhanced from 24.17 ± 5.22 to 41.89 ± 5.36% in Table 2.

3.4. Inhibition of sLe^x expression of HT-29 cells by LMW-ABP

Tumor cells, particularly carcinoma and leukemic cell lines, can express large amounts of sLe^x and sLe^a ligands on their surfaces.

Table 2
The effect of different LMW-ABP samples on the migration and invasion of HT-29 cells, using 24-well transwell cell culture chambers.

Groups	Concentrations (mg/mL)	The number of cells	Inhibition ratio (%)
Control	0	31.20 ± 6.04	–
LMW-ABP	5	23.53 ± 2.82*	24.58 ± 3.21
	10	23.66 ± 3.24*	24.17 ± 5.22
	20	18.13 ± 2.29*	41.89 ± 5.36

The values are represented as means ± SD (n = 5). Significant differences with saline group were designated as *P < 0.05.

These oligosaccharides can mediate direct interaction between tumor cells and endothelia, followed by tumor extravasation. In the past decade, many antagonists that block or interfere with the E-selectin had been designed for inhibiting neoplasm metastasis (Fei et al., 2008). Flow cytometric analysis indicated that all groups of LMW-ABP at three concentrations possess powerful attenuating effect on the expression of sLe^x on the surface of HT-29 cells compared with the control in Fig. 3. Accordingly from Fig. 4, we can see the decrease of sLe^x expression was accompanied with the raise of concentration of LMW-ABP. The present study clarified that LMW-ABP could suppress cancer metastasis by its inhibition of cell adhesion mediated by blocking binding of sLex to E-selectin on the HUVECs.

3.5. Inhibition of FucT-VII mRNA expression of HT-29 cells by LMW-ABP

The biosynthesis of the carbohydrate antigen sLe^x in cancer cell is mediated by FucT-VII, which catalyzes the transfer of fucose from GDP-β-fucose to the 3-OH of α-2,3-sialyl *N*-acetyllactosamine (SALN). Therefore, the inhibition of FucT-VII activity could suppress the expression of selectin ligands, thereby reducing selectin-dependent adhesion of HT-29 cells to endothelial cells. Thus, selective inhibitors of FucT-VII are expected to be potential therapeutic method for weakening neoplasm metastasis. The difference of expression of FucT-VII was demonstrated by the value of $2^{-\Delta\Delta C_T}$. From the Fig. 5, the FucT-VII mRNA in the groups treated with LMW-ABP were significant decreased compared with the control group.

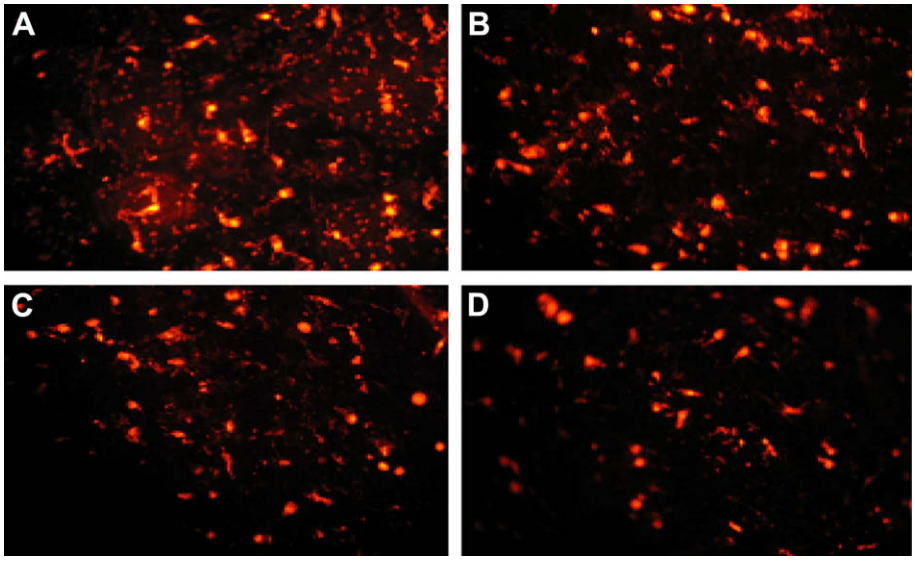

Fig. 2. The effect of different LMW-ABP samples (A, control; B, LMW-ABP of 5 mg/L; C, LMW-ABP of 10 mg/L; D, LMW-ABP of 20 mg/L) on the migration and invasion of HT-29 cells, using 24-well transwell cell culture chambers.

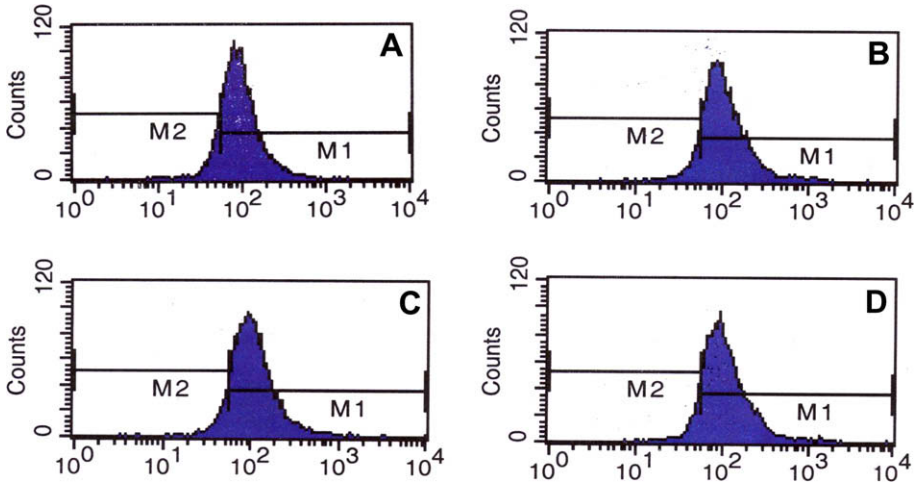

Fig. 3. Flow cytometry analysis of the expression of sLe^x on the surface of HT-29 cells treated by different concentrations of LMW-ABP (A, control; B, LMW-ABP of 5 mg/L; C, LMW-ABP of 10 mg/L; D, LMW-ABP of 20 mg/L).

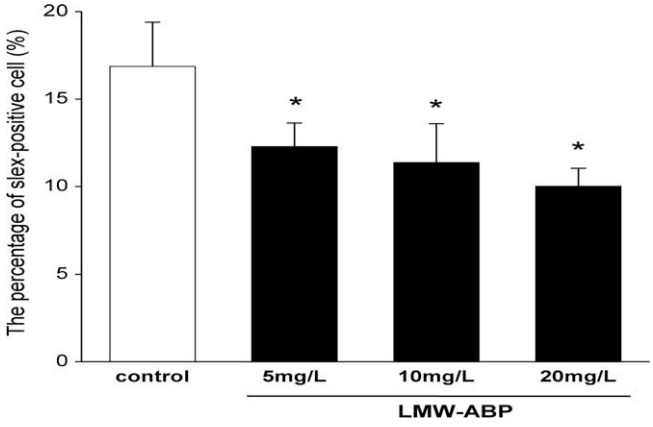

Fig. 4. The percentage of sLe^x-positive cell on the surface of HT-29 cells treated by different concentrations of LMW-ABP (A, control; B, LMW-ABP of 5 mg/L; C, LMW-ABP of 10 mg/L; D, LMW-ABP of 20 mg/L) in flow cytometry analysis. The values are represented as means ± SD ($n = 5$). Significant differences with saline group were designated as $^*P < 0.05$.

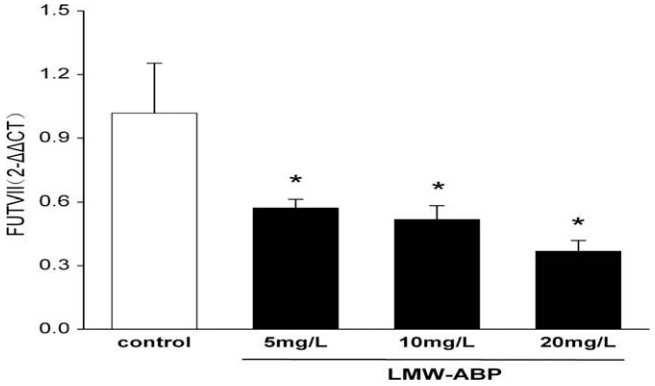

Fig. 5. The relative magnitude comparison of FucT-VII gene expression on the surface of HT-29 cells treated by different concentrations of LMW-ABP (A, control; B, LMW-ABP of 5 mg/L; C, LMW-ABP of 10 mg/L; D, LMW-ABP of 20 mg/L) in flow cytometry analysis. The values are represented as means ± SD ($n = 5$). Significant differences with saline group were designated as $^*P < 0.05$.

4. Conclusion

Nowadays three conventional oncotherapy means, namely surgery methods, chemotherapy and radiotherapy, are still far from satisfactory for treatment of neoplasm metastasis. In addition, during the evolution of neoplastic diseases, the body's natural defenses are usually attenuated, and this immunodeficiency is enhanced by conventional cancer treatments (Salgaller & Lodge, 1998). To overcome this situation, alternative medicine has been successfully applied to the patients suffered from cancer to enhance the immunological function by various national scientists. Among the alternative medicine, immunomodulating polysaccharides from mushrooms as a biological response modifier (BRM) had been drawn much attention by many research groups (Han et al., 2006a, 2006b). However every few people have notice that polysaccharides as immunopotentiators also can affect adhesion of cancer cell to endothelial cell. According to our previous research, we have isolated one anti-tumor polysaccharide from the fruiting bodies of *A. blazei*. Whether LMW-ABP could interfere in adhesion of HT-29 cell to HUVECs had been investigated here. Based on the above experimental results, the expression of sLe^x and FucT-VII mRNA on the surface of HT-29 cells were forcefully suppressed by different concentrations of LMW-ABP (5, 10, and 20 mg/mL) by the methods of fluorescence microscopy detection, cell adhesion experiments, the cell migration and invasion assay, flow cytometry analysis and real-time RT-PCR. The overall results implied LMW-ABP could be considered as a potential candidate for developing a novel low toxicity therapeutical agent for neoplasm metastasis.

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