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Full Length Research Paper

The effect of all-trans retinoic acid (ATRA) on the expression of vascular endothelial growth factor (VEGF) and VEGF receptors of human colon cancer LoVo cell line

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All-trans retinoic acid (ATRA) was found to inhibit cell growth, induce differentiation and enhance apoptosis in a variety of malignant solid tumors. Retinoic acid is effective in inhibiting the expression of vascular endothelial growth factor (VEGF) in some cancer. In this study, we investigated the effect of ATRA on the expression of VEGF and its receptors in LoVo cells, and its possible mechanisms. LoVo cells were treated with ATRA at different concentrations for different time, and with exogenous recombinant human VEGF₁₆₅ or VEGF₁₆₅ + ATRA. Cell viability was measured by microtitration (MTT) assay. Cell cycle and apoptosis were evaluated by flow cytometry (FCM). The expression of VEGF in LoVo cells were detected by ELISA technique and Western blot, and its receptors by flow cytometry. ATRA greatly inhibited the proliferation of LoVo cells in dose- and time-dependent manners; inhibition rate of the cells decreased significantly after treatment with ATRA. ATRA could dose-dependently block the VEGF₁₆₅-induced cell growth. FCM results show that ATRA induced apoptosis of LoVo cells with concomitant decrease of expressed VEGF and its receptors. The mechanism involved in down regulation of VEGF and its receptors may be related to apoptosis. ATRA could also disturb the stimulating effect of VEGF₁₆₅ on the growth of LoVo cells. These results suggest that ATRA can delay growth of LoVo cells by inhibiting the paracrine and autocrine pathways.

Key words: All-trans retinoic acid, LoVo cells, vascular endothelial growth factor, vascular endothelial growth factor (VEGF) receptors.

INTRODUCTION

The dependence of tumour growth and metastasis on blood vessels makes angiogenesis one of the fundamental hallmarks of cancer (Hanahan and Weinberg, 2000) and a rational target for (Carmeliet and Jain, 2000) several growth factor receptor pathways that have been implicated in the promotion of tumour angiogenesis. One of the major pathways involved in this

process is the vascular endothelial growth factor (VEGF) family of proteins, also known as vascular permeability factors, and its receptors. Finding a way to inhibit the expression of VEGF and its receptors has been of great interest. Recent studies have suggested that ATRA is effective in inhibiting the expression of VEGF in some cancer. However, there has been no research carried out on ATRA inhibition of the expression of VEGF and its receptors in colon cancer. Therefore, we investigated the effect of ATRA on the expression of VEGF and its receptors in LoVo cells, and its possible mechanisms in order that ATRA may be applied in the treatment of colon

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cancer.

MATERIALS AND METHODS

LoVo cells were obtained from ATCC (Rockville, MD, USA). ATRA, RPMI-1640, 25% pancrezyme, acridine orange (AO), ethidium bromide (EB), dimethyl sulfoxide (DMSO) and iodine were purchased from Sigma Co (USA); fetal bovine serum (FBS) was obtained from Sijiqing, China; methyl thiazolyl tetrazolium (MTT) was obtained from Fluka. Human VEGF ELISA kit, VEGF165 and phycoerythrin-conjugated mouse monoclonal anti-human VEGFR-1, VEGFR-2 and VEGFR-3 were obtained from R&D Co (USA). Flow cytometry was obtained from BD Biosciences, USA. Annexin V-FITC/PI staining kit was obtained from Bipeck Biopharma USA.

Cell culture

LoVo cells were cultured in RPMI-1640 supplemented with 10% FBS. The cultures were incubated at 37°C in a humidified 5% CO₂ incubator. Cells were harvested at mid-log phase and the culture medium was removed and then discarded.

MTT assay

LoVo cells were inoculated into three 96-hole culture plates when cells were in the denary logarithmic growth condition. The cells were divided into a control group, a 10⁻⁵ ATRA group, 10⁻⁶ ATRA group, and a 10⁻⁷ mol/L ATRA group, and the cells were cultured for 24, 48 and 72 h. MTT and DMSO were added to the cell cultures and the OD-value at 570 nm wavelength was measured for each treatment group using the MTT assay. Finally, in order to calculate the survival ratio of the cells, the following formula was used:

$$\text{Inhibition rate of LoVo} = 1 - \frac{\text{OD - Value of experimental group}}{\text{OD - Value of control group}} \times 100\%$$

LoVo cells were seeded in another 96-well plate. When cells were in the denary logarithmic growth condition, exogenous recombinant human VEGF₁₆₅ (2.5, 5, 15 and 20 ng/ml) or VEGF₁₆₅ (2.5, 5, 15 and 20 ng/ml) + 10⁻⁵ mol/L ATRA or VEGF₁₆₅ (2.5, 5, 15 and 20 ng/ml) + 10⁻⁶ mol/L ATRA or VEGF₁₆₅ (2.5, 5, 15 and 20 ng/ml) + 10⁻⁷ mol/L ATRA were added to the medium in three parallel wells each, and cultured for a further 48 h. For control wells, an equal volume of medium was added. The methods for the MTT assay and calculation of the percentage of viable cells were the same as aforementioned.

Cell cycle assessment of LoVo cells

LoVo cells were seeded in a 24-well plate (1 × 10⁶ cells/well). After 48 h seeding, cells were treated with ATRA (10⁻⁷, 10⁻⁶ and 10⁻⁵ mol/L) for 48 h, and untreated cells served as controls. Then the cells were washed with phosphate buffer saline (PBS), re-suspended in 500 μl of PBS and fixed in 500 μl of ice-cold absolute ethanol at -20°C. After incubation for 30 min, the cell pellets were collected by centrifugation, re-suspended in 0.5 ml of PBS containing 100 μg/ml RNase and incubated at 37°C for 30 min. Then 0.5 ml of propidium iodide solution (50 μg/ml in PBS) was added and the mixture was allowed to stain on ice for 30 min. The cells were analyzed with FACScan flow cytometer. The cell cycle

phase distribution was analyzed using ModFit LT software.

Cell apoptosis assessment of LoVo cells by annexin V-FITC / PI staining

The methods for treating the LoVo cells were the same as aforementioned. By 48 h, the cells were washed twice with cold PBS and then re-suspended in a binding buffer at a concentration of 1 × 10⁶ cells/ml, and the 100 μl solution (1 × 10⁵ cells) was transferred to 5 ml culture tubes. 5 μl of annexin V-FITC and 10 μl propidium iodide (PI) (μg/ml) were added to each 100 μl solution, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark. The samples, to which 400 μl PBS was added, were analyzed by FACSCalibur flow cytometer. Early apoptosis was estimated by the relative amount of FITC + PI – cells.

VEGF ELISA assay

LoVo cells were inoculated into three 6-hole culture plates when cells were in the denary logarithmic growth condition. The cells were divided into a control group, a 10⁻⁵ ATRA group, 10⁻⁶ ATRA group and a 10⁻⁷ mol/L ATRA group. After the cells were cultured for 24, 48 and 72 h, 5 × 10⁶ cells were transferred to 5 ml RPMI- 1640 and cultured for 48 h. Then the culture-liquid was collected, centrifuged at 2000 r/min for 8~10 min, and stored at -20°C. ELISA experiment was done according to the instruction of the reagent and OD-value was measured at 450 nm wavelength for each group.

Western blotting

LoVo cells treated with different concentrations of ATRA for 72 h were harvested and lysed. The protein concentration was determined by the method of Bradford with Coomassie blue R-250 using bovine serum albumin as standard. Western blotting was performed according to a method previously described with a modification. Proteins were electrophoresed on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane. After pre-blocking with PBS containing 0.05% Tween 20 (PBS-T) and 5% non-fat milk for 30 min at normal temperature, the membrane was incubated with a 1:100 dilution of rabbit anti-VEGF serum for 1 h at normal temperature. Horseradish peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) was applied as a secondary antibody using a 1:800 dilution. Immunoreactive bands were detected using the 3, 3'-diaminobenzidine as substrate. As an internal control, the membrane was probed with anti-beta-actin antibody. This experiment is representative of three separate determinations.

Measurement of VEGF-Receptors

LoVo cells were inoculated into three 6-hole culture plates when cells were in the denary logarithmic growth condition. The cells were divided into a control group, a 10⁻⁵ ATRA group, 10⁻⁶ ATRA group and a 10⁻⁷ mol/L-ATRA group. After the cells were cultured for 24, 48 and 72h, cells of each bottle were collected and centrifuged at 3000 r/min for 5 min and washed three times in an isotonic PBS buffer (supplemented with 0.5% BSA). Cells were then re-suspended in the same buffer to a final concentration of 4 × 10⁶ cells/ml and 25 μl of cells (1 × 10⁵) were transferred to a 5 ml tube for staining. The following experimental procedures were then carried out; 1) cells were Fc-blocked by treatment with 1 μl of human IgG/105 cells for 15 min at room temperature prior to staining; 2) 25 μl of the Fc-blocked cells (1 × 10⁵ cells) was

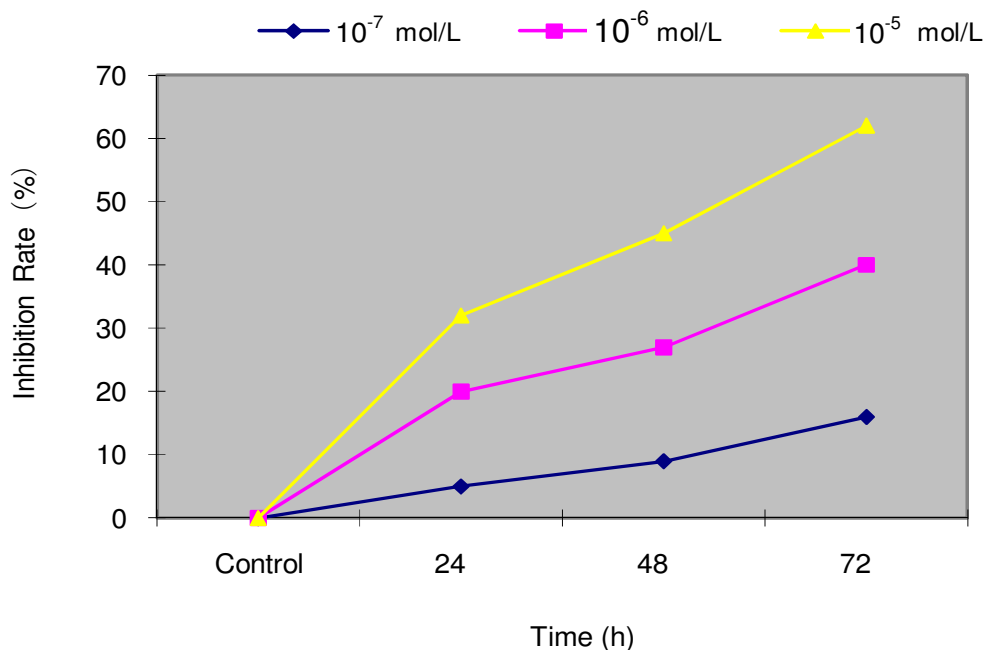


Figure 1. ATRA inhibition of cell proliferation of LoVo cells.

transferred to a 5 ml tube; 3) 10 μ l of PE-conjugated anti-VEGF R1 reagent was added; 4) Incubation was done for 30 to 45 min at 2 to 8°C; 5) following the incubation, unreacted anti-VEGF R1 reagent was removed by washing the cells twice in 4 ml of the same PBS buffer; 6) the cells were re-suspend in 200 to 400 μ l of PBS buffer for final flow cytometric analysis; 7) as a control for analysis, cells in a separate tube were treated with PE-labeled mouse IgG1 antibody. The same procedures were done for anti-VEGF R2 or anti-VEGF R3 reagent and the effect was explored on the other two receptors. The VEGF receptors were measured using FCM.

Statistical analysis

Differences in data between the groups were compared by one and two-way ANOVA. If the analysis was significant, then each mean was compared with each other mean using Student-Newman-Keul's method. The frequencies of positive cells were compared using the Chi-square test. Statistical significance was defined as $P < 0.05$. SPSS 11.0 for Windows was used for all the analyses.

RESULTS

Effect of ATRA on cell growth in LoVo cells

The cell proliferation was determined using the MTT assay performed with logarithmically growing LoVo cells treated with ATRA. Within 24 h of the addition of 10^{-7} mol/L ATRA on the LoVo cells, the inhibition rate was $5.58 \pm 0.03\%$ ($P < 0.05$). ATRA inhibited the growth of LoVo cells in time-dependent and dose-dependent inhibitory fashions at concentrations of 10^{-7} ~ 10^{-5} mol/L (Figure 1). These data imply that ATRA has a significant growth inhibitory effect on LoVo cells *in vitro*.

LoVo cells were incubated with varying concentrations of VEGF165 (2.5, 5, 15 and 20 ng/ml) or VEGF165 (2.5, 5, 15 and 20 ng/ml) + 10 to 5 mol/L ATRA or VEGF165 (2.5, 5, 15 and 20 ng/ml) + 10 to 6 mol/L ATRA or VEGF165 (2.5, 5, 15 and 20 ng/ml) + 10 to 7 mol/L ATRA. Its effects were measured using the MTT assay (Table 1). The results show that VEGF165 accelerated the growth of LoVo cells, but ATRA disturbed the stimulatory effect of VEGF165.

Effect of ATRA on cell cycle in LoVo cells

The effect of ATRA on cell cycle phase distribution was analyzed by flow cytometer. The results showed that the percentage of cells treated with ATRA was higher in the G0/G1 period, lower in the sub-S period, and lower in the G2/M period when compared to the controls. Apoptotic cells were respectively 8.23 ± 1.28 , 26.52 ± 1.67 and $39.79 \pm 3.96\%$, in the 10^{-7} , 10^{-6} and 10^{-5} mol/L ATRA groups (Table 2). These results suggest that one of the mechanisms of the growth inhibitory effect of ATRA on LoVo cells is through an arrest in the G0/G1 phase of the cell cycle and apoptosis induction.

Inhibition of VEGF protein expression in LoVo cells cultural supernatant by ATRA

To determine whether ATRA had effect on the expression of VEGF protein in LoVo cells, VEGF expression level was detected by ELISA. There was no significant difference between 10^{-7} ATRA group and the control

Table 1. Effect of ATRA on VEGF-stimulated growth of LoVo cells (%).

Group (mol/L)	Concentration (ng/ml)				
	2.5	5	10	15	20
VEGF ₁₆₅ + 0 ATRA	105.23	106.30	108.23	109.85	112.19
VEGF ₁₆₅ + 10 ⁻⁷ ATRA	89.74*	91.24*	90.89*	97.56*	100.4*
VEGF ₁₆₅ + 10 ⁻⁶ ATRA	75.43*	84.57*	85.67*	89.49*	95.62*
VEGF ₁₆₅ + 10 ⁻⁵ ATRA	67.25*	70.63*	73.49*	80.56*	86.53*

* *P*<0.05 compared among varying concentrations of ATRA.

Table 2. The effects of ATRA on cell cycle in LoVo cells.

ATRA (mol/L)	Cell cycle (%)			Apoptosis rate (%)
	G0/G1	S	G2/M	
0	60.10±1.27	24.35±1.73	15.55±1.12	0
10 ⁻⁷	75.69±1.81*	20.83±1.31	3.48±0.50	8.23±1.28*
10 ⁻⁶	85.88±1.51*	6.89±1.12	6.13±0.40	26.52±1.67*
10 ⁻⁵	84.80±1.40*	12.14±1.30	3.06±1.00	39.79±3.96*

* *P*<0.05 compared with the control group.

Table 3. VEGF level in LoVo cells cultural supernatant.

ATRA (mol/L)	VEGF level in LoVo cells cultural supernatant (pmol/ml, $\bar{x} \pm s$)		
	24 h	48 h	72 h
0	139.6925 ± 3.97	143.345 ± 6.46	145.7948 ± 3.65
10 ⁻⁷	134.1528 ± 2.13 ^b	110.1325 ± 1.97 ^{ab}	88.1725 ± 3.62 ^{ab}
10 ⁻⁶	113.765 ± 2.21 ^{ab}	92.27 ± 4.38 ^{ab}	75.5075 ± 4.78 ^{ab}
10 ⁻⁵	99.0675 ± 5.16 ^{ab}	62.32 ± 2.90 ^{ab}	54.3675 ± 5.41 ^{ab}

^a*P*<0.05 compared with the control group; ^b compared among the experimental groups.

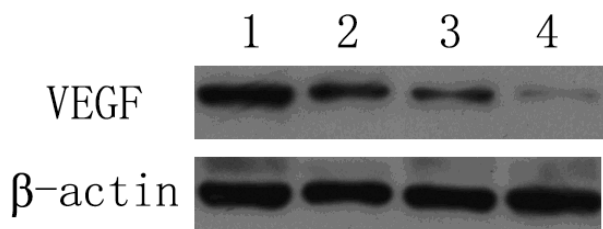


Figure 2. Western blotting analysis of the expressions of VEGF. Lane 1, control; Lane 2, 10⁻⁷ mol/L ATRA; Lane 3, 10⁻⁶ mol/L ATRA; Lane 4, 10⁻⁵ mol/L ATRA.

group at 24 h (*P* = 0.1002) but with increase of the concentration of all-trans retinoic acid and prolonging of time, the ATRA groups had statistical prominence compared to the control group (*P*<0.05). As shown in Table 3, these results indicate that ATRA inhibited VEGF protein expression of LoVo cells in time-dependent and dose-dependent inhibitory fashions.

Result of Western blotting

Protein bands were photographed and quantified by densitometry in Western blot analysis. ATRA decreased the expression of VEGF protein in a dose-dependent manner, as shown in Figure 2. The actin protein was used as an internal control for normalization of protein loading. Right panel, relative levels of mRNA expression normalized to actin as determined by densitometry using UVI image analysis software. This experiment is representative of three separate determinations.

The effect of ATRA on expression of VEGF receptors in LoVo cells by FCM

To determine whether ATRA played a rule in expression of VEGF receptors in LoVo cells, we assessed the VEGF receptors of the LoVo cells before and after treatment with ATRA, using FCM. As shown in Figure 3, expression of VEGFR-1 and VEGFR-2 was confirmed in LoVo cell

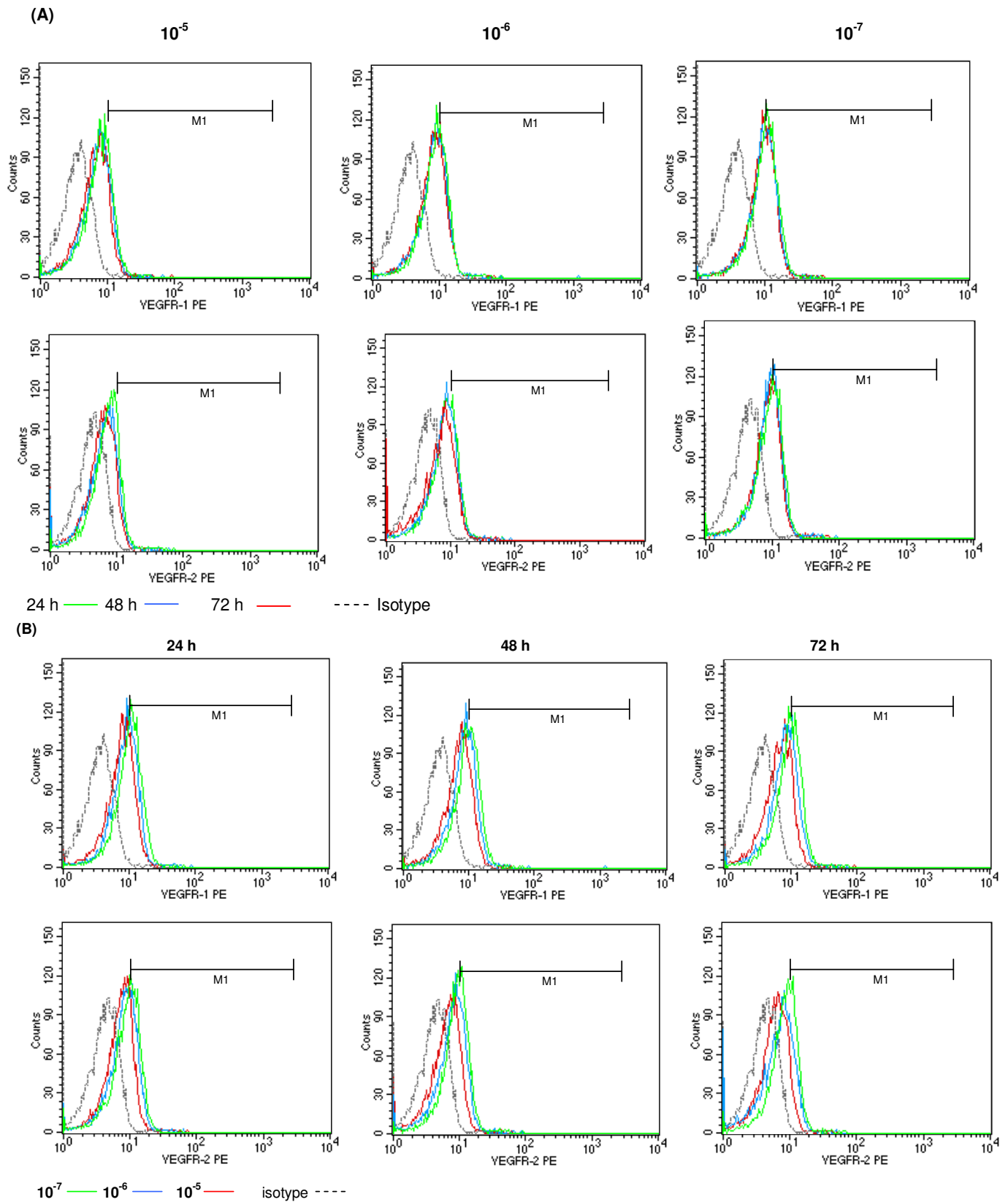


Figure 3. The effect of ATRA on expression of VEGF receptors in LoVo cells by FCM. (A) ATRA could reduce the expression of VEGF receptors in LoVo cells in time-dependent inhibitory fashions; (B) ATRA could reduce the expression of VEGF receptors in LoVo cells in dose-dependent inhibitory fashions; (C) the percentages of VEGFR-3 and isotype IgG-1 for 48 h.

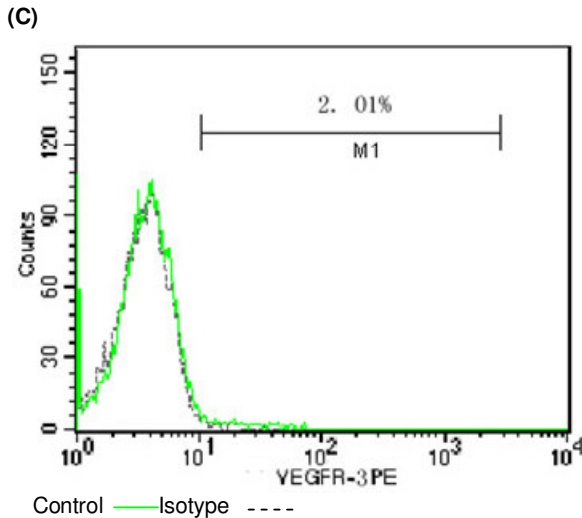


Figure 3. Contd.

lines. The percentages of VEGFR-3 and isotype IgG-1 for 48 h were 2 ± 0.2 and $1.62 \pm 0.8\%$ ($P = 0.5003$). It meant that LoVo cells had no expression of VEGFR-3. These findings suggested that ATRA could reduce the expression of VEGF receptors in LoVo cells in time-dependent and dose-dependent inhibitory fashions.

DISCUSSION

Colorectal cancer (CRC) is one of the most common malignancies in the world (Mehmet and Turkan, 2009). Approximately 50% of patients who undergo potentially curative surgery alone ultimately relapse and die of metastatic disease (Manfredi et al., 2006). The dependence of tumour growth and metastasis on blood vessels makes angiogenesis one of the fundamental hallmarks of cancer (Hanahan and Weinberg, 2000) and a rational target for (Carmeliet and Jain, 2000) several growth factor receptor pathways that have been implicated in the promotion of tumour angiogenesis. One of the major pathways involved in this process is the VEGF family of proteins, also known as vascular permeability factors, and its receptors. The VEGF pathway plays a crucial role in normal and pathologic angiogenesis, triggering multiple signalling networks that result in endothelial cell survival, migration, mitogenesis, differentiation, and vascular permeability (Ferrara and Davis-Smyth, 1997). Finding a way to inhibit the expression of VEGF and its receptors has been of great interest.

ATRA is a derivative of vitamin A, and has attracted researchers due to the favorable effects of this agent in the treatment of acute promyelocytic leukemia. In additional studies, ATRA was found to inhibit cell growth (Xiao et al., 2006), induce differentiation and enhance

apoptosis (Xu and Gong, 2004; Mirza et al., 2006; Huss et al., 2004) in a variety of malignant solid tumors through its influence on the expression of certain genes closely related to inhibition of tumor invasion and metastasis (García-Alonso et al., 2005; Day et al., 2006; Suzuki et al., 2006).

In our study of the effect of ATRA on growth and proliferation of LoVo cells, the MTT assay showed that ATRA inhibited the growth of LoVo cells in a dose-dependent manner. Flow cytometry assays showed that the percentage of the cells treated with ATRA were higher in the G0/G1 and lower in the G2/M period when compared to the controls. These results show that ATRA inhibited the viability of LoVo cells and arrested the cells in G1 phase, blocked or delayed their entry into S phase, disturbed DNA synthesis, and induced apoptosis in the G1 phase. The results of ELISA show that there was no significant difference between 10^{-7} ATRA group and the control group at 24 h ($P > 0.05$) but with increasing concentration of all-trans retinoic acid and prolonged time of exposure, the ATRA groups had statistical prominence compared to the control group ($P < 0.05$). These results indicate that ATRA inhibits VEGF protein expression of LoVo cells in time-dependent and dose-dependent inhibitory fashions. In Western blot analysis, ATRA decreased the expression of VEGF protein in a dose-dependent manner,

All types of specific receptors for VEGF, VEGFR-1, VEGFR-2 and VEGFR-3, are commonly distributed in endothelial cells. VEGFR-1 and VEGFR-2 are expressed predominantly on endothelial cells, but a few additional types of cells express one or both of these receptors (Ferrara, 2004; Ferrara et al., 2003). VEGFR-3 is initially expressed throughout the embryonic vasculature and is limited to lymphatic endothelial cells in later stages of development (Veikkola et al., 2001). VEGFRs belong to the receptor tyrosine kinase (RTK) family and have a characteristic structure with 7 Ig-like domains in the extracellular domain and a cytoplasmic tyrosine kinase domain with a long kinase insert region (Ferrara, 2004; Gu et al., 2003; Shibuya, 2002). VEGF acts by binding to the receptors and prompting endothelial cells proliferation. VEGF and its receptors are the most important pathways in tumor angiogenesis. Inhibition of VEGF/VEGFR pathways may suppress angiogenesis and tumour growth. However, the expression of VEGFR is not endothelial cells-specific, and recent emerging evidence has shown that VEGFRs are expressed in several types of non-endothelial cells, especially in tumour cells, which indicates that there is an autocrine pathway of VEGF in tumor cells (Jackson et al., 2002; Zhang et al., 2002; Dias et al., 2001). VEGFR may play an important role in paracrine and autocrine pathways of VEGF, and VEGFR inhibitors can inhibit tumor angiogenesis and growth (Ciardiello et al., 2004).

In the study, we examined further, the effect of ATRA on VEGFR expression and intended to confirm the

anticancer activity of ATRA, which may block the paracrine and autocrine VEGF/VEGFR pathways thereby delaying new tumour blood vessel formation and tumour cell growth. Our results show that VEGFR-1(Flt-1) and VEGFR-2(KDR) except VEGFR-3 were expressed in the LoVo cells and their expression in tumour cell control were higher than that in ATRA-treated group. The percentage of VEGFR-1(Flt-1) and VEGFR-2(KDR) in tumor cells were significantly reduced in the ATRA-treated groups ($P < 0.05$). The percentage of VEGFR-1(Flt-1) and VEGFR-2(KDR) in the 10^{-5} mol/L group were less than those in the 10^{-7} mol/L group ($P < 0.05$). These results suggest that ATRA can result in significant down-regulation of VEGFR-1(Flt-1) and VEGFR-2(KDR) in a dose-dependent manner. We showed the absence of VEGFR-3 expression in colorectal tumor LoVo cell lines. VEGFR-3 does play an important role in colon cancer proliferation and metastasis, but this is likely due to its presence on endothelial cells within the tumour (Bruns et al., 2000), rather than on the tumour cells themselves.

The results of further experiments *in vitro* showed that exogenous VEGF₁₆₅ could stimulate the growth of LoVo cells, and ATRA may have disturbed the stimulatory effect of VEGF₁₆₅. It indicates that the autocrine or paracrine pathway of VEGF through VEGFRs is possible in colon carcinoma, and ATRA inhibits expression of Flt-1 and KDR in endothelial and tumor cells. ATRA may block new blood vessel formation through the paracrine pathway and affect growth of tumor cells through the autocrine pathway of VEGF/VEGFRs, and delay tumor growth.

Conclusion

The results of our study showed that ATRA delayed growth of LoVo cells, inhibited their proliferation and induced apoptosis, which resulted in down-regulation of VEGFR-1(Flt-1) and VEGFR-2(KDR) expression in a dose-dependent manner. ATRA may block new blood vessel formation through the paracrine pathway and affect growth of tumor cells through the autocrine pathway of VEGF/VEGFRs in colorectal carcinoma, which if confirmed by further experiments, would provide a new therapeutic option for the treatment of colorectal cancer.

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