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Effects of retinoic acid on metastasis and its related proteins in gastric cancer cells *in vivo* and *in vitro*[1]

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KEY WORDS tretinoin; neoplasm metastasis; stomach neoplasms; nude mice

ABSTRACT

AIM: To investigate the effects of *all-trans* retinoic acid (ATRA) on metastasis and its related proteins in human gastric cancer cells *in vivo* and *in vitro*. **METHODS:** Gastric cancer cells, MGC80-3 and SGC-7901, were inoculated into spleen subcapsule of nude mice, respectively. Nude mice were administered with ATRA (0.7 mg/kg, ig) every other day. Six weeks later, nude mice were sacrificed. All the tumors formed in spleen and in liver were removed. Some of them were fixed, and then embedded. Others were kept in liquid nitrogen for further use. Expression level of proteins in tumor and in cell was analyzed by Western blot. Microvessel in tumor section was shown by immunohistochemistry and adhesive ability of cell to amnion was measured by adhesion assay. **RESULTS:** When inoculated nude mice were treated with ATRA, the xenograft tumors in spleen and metastatic tumors in liver were suppressed by 50 % respectively, and inhibition of microvessel formation in xenograft and metastatic tumors was also observed obviously. Although ATRA regulated expression of nm23 and mts1/p16 proteins at different patterns *in vivo* and *in vitro*, high ratio of nm23:mts1/p16 was in association with low adhesive activity of cells. In addition, ATRA induced ICAM-1 protein expression *in vivo* and *in vitro*. **CONCLUSION:** ATRA inhibits the growth of xenograft tumors and their metastasis to liver. This process may be associated with regulation of metastatic related proteins, including nm23, mts1/p16, and ICAM-1 *in vivo* and *in vitro*.

INTRODUCTION

Although significant progress has been made in the surgical treatment of tumors, as well as in diagnostic techniques, more than 50 % patients still die from malignant tumors. It is partially due to distant metastasis at the time of diagnosis. Metastasis is known to consist of a cascade of multiple steps. Tumor cell migrates from the primary lesion, invades the tumor border and hosts tissues, initiates angiogenesis, and colonizes at distal sites[1]. Thus, blocking one of these steps is of clinical significance in prevention of tumor metastasis.

Metastatic process is regulated by activation and

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inactivation of several specific genes and proteins. The nm23 gene, an anti-metastatic gene, was found originally to be correlated with tumor metastatic potential in murine melanoma cell[2] and was associated with a good prognosis in breast cancer[3]. However, the relationship between expression of nm23 protein and prognostic value was not found in colon cancer, neuroblastoma, or pulmonary carcinoma[4-6]. Another gene mts1/p16, together with its protein, was also found to be related to high metastatic potential and growth of some human tumors[7]. It was reported recently that there was a close relationship between nm23 and mts1/p16 in regulation of metastatic behavior[8]. Since mts1/p16 mutations occur preferentially in metastatic tumors, they might be important events in late phases of tumor progression and could represent useful markers of tumor aggressiveness in non-small cell lung carcinomas[9].

Angiogenesis has been shown to be critical for tumor growth. It facilitates the infiltration of reparative cells, enhances the delivery of oxygen, nutrients, growth factors, and cytokines, and allows the removal of waste products[10]. Primary and metastatic tumors can not grow beyond 2 mm in diameter without an enhanced vascularity. The newly formed blood vessels are more penetrative to tumor mass and may contribute to metastasis[11]. Thus, anti-angiogenesis has been studied as one of the major approaches for tumor treatment.

Retinoids play an important role in proliferation and differentiation of a variety of cell types. *In vitro* and animal studies have suggested that retinoids are promising agents in the prevention and treatment of human cancers. Recently, we have developed an *in vivo* model for estimating the ability of tumor metastasis[12], in which tumor cells were inoculated into spleen subcapsule of nude mice, and xenograft tumors formed in spleen metastasized to liver. In the present study, we investigated the effects of *all-trans* retinoic acid (ATRA) on metastasis and its related proteins in human gastric cancer cells *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cell lines and culture condition Human gastric cancer cell line SGC-7901 was purchased from the Institute of Cell Biology, Shanghai, China. MGC80-3 cell line was established by Cancer Research Center in Xiamen University. Both of cell lines were maintained in RPMI-1640 medium supplemented with 10 % FCS, glutamine 1 mmol/L, and benzylpenicillin 100 U/L.

Inoculation of nude mice with cancer cells Athymic nude mice (BALB/c, SPF Grade, 18-21 g, 7 weeks old, Certificate № 23-007 approved by Administration Committee of Medical Experimental Animal, Fujian Province) were housed in a laminar flow under sterilized condition. The temperature was maintained at 28 ℃. The light was controlled at 12 h light and 12 h dark. Mice were fed with autoclaved mouse chow.

Cells were trypsinized and washed twice with phosphate buffered saline (PBS). The inoculation dosage was 0.05 mL cell suspension per mouse (cell density was 2.5×10^{10}/L). Before inoculation, nude mouse was injected with 0.1 mL of 0.5 % CCl_4, then 24 h later, anesthetized with 0.625 % sodium pentothal (0.2 mL, ip) and subjected to a routine surgical operation. Cells were inoculated into spleen subcapsule of nude mice. The inoculated nude mice were randomly separated into experimental and control groups (6 mice for each group, 3 male and 3 female). For experimental group, nude mice were administered with ATRA 0.7 mg/kg every other day (ATRA was dissolved in ethanol and Me_2SO with a ratio of 1:1). For control group, nude mice were administered with the corresponding dissolvent without ATRA. After 6 weeks, nude mice were sacrificed and all the tumors formed in spleen and in liver were removed. Some of them were fixed and embedded for immunohistochemistry and others were kept in liquid nitrogen for Western blot.

Immunohistochemistry Sections from spleen tumor and liver tumor were deparaffinized. Endogenous peroxidase was blocked by 0.3 % hydrogen peroxide in methanol for 30 min. After washed with PBS, sections were incubated with 10 % goat serum in PBS for 20 min at room temperature to block non-specific binding of the second antibody, then incubated overnight at 4 ℃ with rabbit anti-human factor-VIII-related-antigen antibody (Zymed) 1:100 in PBS containing 1 % bovine serum albumin. Rinsed three times in PBS, sections were treated with biotinylated anti-rabbit immunoglobulin for 1 h at room temperature, then washed again and reacted with streptoavidin-biotin system using 0.04 % 3,3'-diaminobenzidene tetrahydrochloride for 1 min as chromogen. To calculate inhibitory rate of

microvessel density (MVD), at least four sections, including those from spleen tumors and liver tumors, were observed under microscope and microvessels were counted within 20 hot spots randomly.

Western blot and quantitative analysis Cells or tumors kept in liquid nitrogen were lysed in NaCl 150 mmol/L, Tris-HCl 10 mmol/L (pH 7.4), edetic acid 5 mmol/L, 1% TritonX-100, and phenylmethylsulphonyl fluoride. Total protein (50 μg) was separated by 10 % SDS-PAGE and transferred to nitrocellulose. Protein was visualized using ECL (enhanced chemiluminescence) detection system (Amersham), after incubation for 2-4 h at room temperature with primary antibodies, including ICAM-1, nm23, and mts1/p16 (Santa Cruz), and their corresponding secondary antibodies, respectively. To quantify relative expression level of nm23 or mts1/p16 protein, each band revealed by ECL was scanned by densitometer.

Adhesion assay Amnion, obtained from fresh placenta, was washed twice with PBS and immersed in NH_4OH 0.25 mol/L for 2 h to remove the epithelial cells. When treated with ATRA for 30 d and 80 d respectively, cells were trypsinized and suspended in RPMI-1640 medium to a final density of 5×10^7/L. Cell suspension 2 mL was added to the upper level of Boyden chamber, and RPMI-1640 medium was filled to the bottom level of chamber. After incubated for 4 h and 6 h respectively at 37 ℃ in CO_2 incubator, the upper chamber was removed and the cells attached to amnion were trypsinized and counted under microscope. Result represented the mean of two independent experiments.

Statistical analysis Tukey's procedure was applied to estimate the difference among groups. Statistical significance was defined as $P<0.05$.

RESULTS

Effects of ATRA on growth of xenograft tumor in spleen and its metastasis to liver Gastric cancer cells were inoculated into spleen subcapsule of nude mice. Inoculated nude mice in experimental group were administered with ATRA every other day. After 6 weeks, nude mice were sacrificed. Xenograft tumors formed in spleen were found in all of mice except one mouse in experimental group. However, number of spleen tumors in experimental group was fewer than that in control group, the mean weight of spleen tumors in experimental group was also lighter than that in control group significantly ($P<0.05$, Tab 1). Metastatic tumors found in liver were multiple and noncapsuled in both experimental and control groups. However, the occurrence of metastatic tumors was obviously decreased by 50 % in experimental groups, number of metastatic tumors reduced much more and the mean weight of liver was also lighter (Tab 1), compared with the control group.

Effect of ATRA on microvessel formation Immunohistochemistry analysis indicated a strong staining for microvessel in all of the tumor sections, including those in spleen tumors and liver tumors. However, microvessel density (MVD) in experimental group was lower than that in control group by statistical analysis.

Tab 1. Effect of ATRA (0.7 mg/kg, ig every other day for 6 weeks) on xenograft and metastatic tumors in nude mice. n=6. Mean±SD. $^bP<0.05$, $^cP<0.01$ vs control.

Cell line	Group	Spleen			Liver		
		Rate of tumor occurrence/%	Number of tumors	Weight/g	Rate of tumor occurrence/%	Number of tumors	Weight/g
MGC[1)]	Control	100 (5/5)[2)]	13	1.6±1.0	100 (5/5)	134	3.2±1.0
	ATRA	100 (6/6)	5	0.4±0.5[b]	50 (3/6)	8	1.7±0.3[c]
SGC	Control	100 (6/6)	7	0.6±0.2	100 (6/6)	48	1.8±0.4
	ATRA	83.3 (5/6)	6	0.3±0.1[b]	50 (3/6)	18	1.3±0.4[c]

[1)] Some data of MGC80-3 cell were cited from reference 13.

[2)] One of the MGC80-3 inoculated mice in control group died at the 40th day due to the development of tumor.

The inhibitory rate of MVD showed significant decrease in MGC80-3 experimental group ($P<0.05$, Tab 2) and more significant decrease in SGC-7901 experimental group ($P<0.01$, Tab 2).

Tab 2. Effect of ATRA (0.7 mg/kg, ig every other day for 6 weeks) on microvessel density (MVD) *in vivo*. $n=4$. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

Cell line	Tumor	MVD		Inhibitory rate of MVD/%
		Control	ATRA	
MGC	Spleen	11.2±3.0	8.0±2.9^b	28.6
	Liver	10.9±2.4	8.4±2.4^b	22.9
SGC	Spleen	8.6±2.0	6.6±1.5^b	23.7
	Liver	13.3±2.3	8.3±1.6^c	37.6

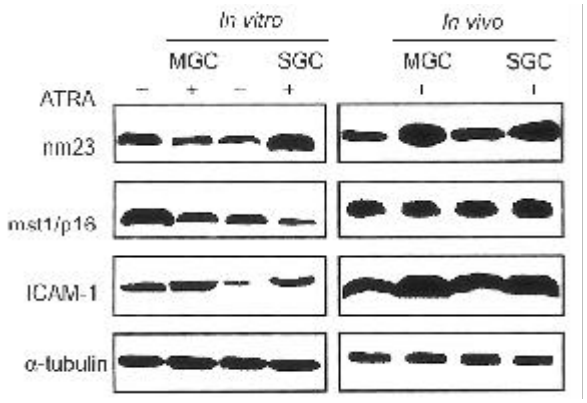

Fig 1. Expressions of nm23, mts1/p16, and ICAM-1 proteins shown by Western blot *in vivo* and *in vitro*. α-Tubulin was used to quantify the amount of protein used. Protein *in vivo* was extracted from several liver tumors, and protein *in vitro* was extracted from cells treated with ATRA 1.0 μmol/L for 24 h.

Effects of ATRA on regulation of nm23, mts1/p16, and ICAM-1 proteins *in vivo* and *in vitro* To investigate the molecular mechanism of ATRA in affecting metastatic progress, the expression level of nm23, mts1/p16, and ICAM-1 proteins correlated with metastasis were detected by Western blot. Protein *in vivo* was extracted from liver tumors that represented metastatic tumor lesion. Protein *in vitro* was obtained from cells. The results showed that regulation of ATRA in these two types of protein (*in vivo* and *in vitro*) differed obviously. Expression of nm23 and ICAM-1 proteins *in vivo* was up-regulated by ATRA, and expression of mts1/p16 protein was not changed obviously (Fig 1). In contrast to the result shown *in vivo*, after treatment of cells with ATRA for 24 h *in vitro*, expression of nm23 protein was decreased in MGC80-3 cells and increased in SGC-7901 cells. Expression of mts1/p16 protein was down-regulated by ATRA in both MGC80-3 and SGC-7901 cells. However, ICAM-1 showed the similar trend as that *in vivo* (Fig 1). The expression level of nm23 and mts1/p16 proteins was further quantified by densitometer, respectively. The values of relative abundances were shown in Tab 3. Although ATRA treatment led to increase or decrease in nm23 and mts1/p16 protein levels in different groups respectively, nm23:mts1/p16 ratio always kept higher in experimental group than that in control group *in vivo* and *in vitro*.

To further determine the possible link between nm23 and mts1/p16, various concentrations of ATRA were used to treat cells for 24 h *in vitro*. Although expression of nm23 and p16 proteins, revealed by Western blot, was down-regulated in an ATRA

Tab 3. Quantitative analysis of nm23 and mts1/p16 protein level by ATRA *in vivo* (0.7 mg/kg, ig every other day for 6 weeks) and *in vitro* (10 μmol/L, for 24 h).

	In vivo				*In vitro*			
	MGC		SGC		MGC		SGC	
ATRA:	–	+	–	+	–	+	–	+
nm23	1.758	1.234	1.000	2.375	1.550	3.326	2.199	3.078
mts1/p16	3.676	1.676	1.778	1.000	2.587	2.510	2.917	3.146
nm23:mts1/p16	0.478	0.736	0.562	2.375	0.599	1.325	0.754	0.978

concentration-dependent manner in both MGC80-3 and SGC-7901 cells (Fig 2), nm23:mts1/p16 ratio displayed an increase also in an ATRA concentration-dependent manner after quantitative analysis by densitometer (Tab 4).

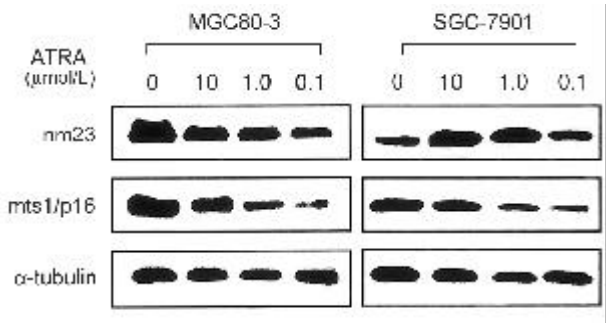

Fig 2. Western blot was performed to examine the levels of nm23 and mts1/p16 protein expression. a-Tubulin was used to quantify the amount of protein used. Protein was extracted from cells treated with various concentrations of ATRA for 24 h.

Effect of ATRA on cell adhesion To elucidate effect of ATRA on cell adhesion, the adhesive ability of cell to amnion was measured by adhesion assay. As shown in Tab 5, the adhesive ability of cell to amnion was suppressed remarkably with extension of ATRA treatment. When cells were treated with ATRA for 30 d and 80 d respectively, the rate of SGC-7901 cell adhesion (4 h and 6 h attachment) and that of MGC80-3 cell adhesion (6 h attachment) showed more significant decrease ($P<0.01$, Tab 5), compared with the control group.

Tab 5. Effect of ATRA (1.0 μmol/L) on adhesive capability of cells. $n=2$. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

Cell line	Day of ATRA treatment	Rate of adhesion/% 4 h	6 h
MGC[1)]	0	29±4	40±4
	30	28±6	38±4
	80	12.2±2.1[b]	18±3[c]
SGC	0	42±5	58±4
	30	26±3[c]	38±5[c]
	80	20±4[c]	29±6[c]

[1)] Some data of MGC80-3 cells were cited from reference 13.

DISCUSSION

In this animal model, nude mice were injected with CCl_4 and some growth factors were secreted to facilitate tumor cell proliferation and mass formation in liver. Autopsy and statistics showed that when gastric cancer cells were inoculated into spleen subcapsule of nude mice, xenograft tumors were formed in spleen, and then metastasized to liver. However, after nude mice were administered with ATRA every other day for 6 weeks, it apparently caused the suppression in growth of xenograft tumors in spleen. More importantly, tumor metastasis was inhibited obviously in experimental group. Only 50 % of the splenic tumor-bearing individuals was developed into the metastasized tumors in liver, as compared with 100 % metastasis occurred in the control group. These results suggested that ATRA might be a promising agent for tumor prevention and treatment *in vivo*. In addition, the fact that ATRA inhibited angiogenesis formation in xenograft splenic tumor and in metastasized liver tumor implied that ATRA might be one of the valuable candidates clinically used for anti-angiogenesis. This result was in accordance with other reports from breast and vagina cancer cells[14].

Low expression of nm23 protein has been found

Tab 4. Quantitative analysis of nm23 and mts1/p16 protein level at various concentrations of ATRA (μmol/L, for 24 h).

	MGC				SGC			
ATRA:	0	0.1	1.0	10	0	0.1	1.0	10
nm23	2.473	1.598	1.369	1.108	1.000	2.050	1.750	1.350
mts1/p16	4.537	2.754	1.579	0.916	3.141	2.258	1.427	1.000
nm23:mts1/p16	0.545	0.580	0.867	1.210	0.318	0.908	1.226	1.350

to be correlated with high metastatic potential in several tumors[2,3]. However, some data revealed the contrast results in other tumors, suggesting that the function of nm23 in cancer cells remained unclear. More recently, it was reported that expression of nm23 gene alone was not related to metastatic behavior, but the relative ratio of nm23:mts1/p16 protein correlated with metastatic potential[15]. This evidence implied that they might act as a co-regulator on tumor metastasis. Consistent with this observation, our data showed that not only the ratio of nm23:mts1/p16 protein in experimental groups was increased obviously in an ATRA concentration-dependent manner, but always kept higher than that in control group, no matter whether expression of nm23 and mts1/p16 proteins was decreased or increased by ATRA *in vivo* or *in vitro*. According to the reports, SGC-7901 cell line was established from the metastatic lesion of lymph node in gastric cancer[16], and MGC80-3 cell line from the mass of gastric adenocarcinoma with lower metastasis[17]. Thus some of special characteristics associated with metastasis should be shown in SGC-7901 cells markedly after treatment of ATRA. They were displayed *in vivo* and *in vitro*: highest inhibitory effect on tumor microvessel density, more significant suppression rate of adhesion, as well as induction of nm23 protein expression. These features might explain partially why ATRA regulation on expression of nm23 and mts1/p16 proteins displayed distinct patterns *in vivo* and *in vitro* between MGC80-3 and SGC-7901 cells. It was likely that in gastric cancer cells, expression of nm23 protein along *in vivo* was associated with metastatic behavior, and the relative ratio of nm23:mts1/p16 expression *in vitro* correlated with metastatic potential. Accordingly, it was quite possible that (1) phenomena observed *in vitro* were not relevant *in vivo*, since ATRA might be quickly metabolized and what was observed *in vivo* might be mediated by a metabolite and differed significantly from effects by ATRA *in vitro*. (2) Role of nm23 with regard to prognosis was cell-type specific, which was associated with metastatic capability of cells. Thus inhibitory effect of ATRA on metastasis might lie in its concurrent regulation on expression of two proteins, nm23 and mts1/p16. Of course, it should be necessary to further address this possibility by using different types of cell, including high and low metastatic cancer cell lines.

Lakshimi *et al* pointed out that one possible link between nm23 and mts1/p16 and their target effects might be microtubule assembly[8]. Parket *et al* reported that nm23 and mts1/p16 expressions affected the invasive properties of tumor cells[18]. Present results, combining with our other results[13], further confirmed that high ratio of nm23:mts1/p16 was in association with low adhesive activity, such as decrease of adhesion, disappearance of microvilli at surface of tumor cell, reassembly of microfilament[13].

In cancer therapy, retinoic acid (RA) has been used to increase ICAM-1 expression to render tumor cell more susceptible to immune attack[19]. Thus, it was suggested that up-regulation of ICAM-1 by RA played a major role in cell adhesion, cell movement, and even tissue development. Induction of ICAM-1 expression by ATRA depended on retinoic acid response element (RARE) located at 266 bp upstream of the 5' translation start site in the ICAM-1 promoter[20]. Our previous study has shown that effect of RA was mainly mediated by its receptors, which bound to RARE and regulated positively or negatively transcriptional activities of target genes[21]. Thus, it was implied that in gastric cancer cells, ATRA might up-regulate ICAM-1 expression directly through the mediation of retinoic acid receptors that bound to RARE located in the ICAM-1 promoter. Surely, the detail, especially on function of ICAM-1 in gastric cancer cells, should be studied further.

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视黄酸在体内外对胃癌细胞转移及其相关蛋白的影响[1]

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关键词 维甲酸；肿瘤转移；胃肿瘤；裸小鼠

目的：从体内外探讨全反式视黄酸(ATRA)对人胃癌细胞转移及其相关蛋白影响．方法：胃癌细胞接种到裸鼠脾包膜，每隔两天灌胃给予 ATRA 0.7 mg/kg，6周后处死裸鼠，取出所有在脾和肝形成的肿瘤，一些肿瘤被固定和包埋，另一些肿瘤保存在液氮中用于后续实验．用蛋白质印迹法测定蛋白水平；通过免疫组化显示微血管；采用粘附实验测定细胞粘附能力．结果：ATRA 灌胃后，脾移植瘤和肝转移瘤受到明显抑制(50 %)，血管生成也受到抑制．尽管 ATRA 在体内外调节 nm23 和 mts1/p16 蛋白的方式不同，但高比值的 nm23:mts1/p16 与低粘附性相关．ATRA 在体内外诱导 ICAM-1 蛋白表达．结论：ATRA 显著抑制移植瘤生长及其向肝转移，这一过程可能与对转移相关蛋白 nm23，mst1/p16 和 ICAM-1 的调控有关．

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