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Effect of α1-Acid Glycoprotein Expressed in Cancer Cells on Malignant Characteristics

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The α1-acid glycoprotein (AAG) is a prototypical serum acute phase reactant in most mammalian species; it is synthesized mainly in liver parenchymal cells. Recently, we found that mRNAs of AAG were expressed in non-hepatic cancer cells, and the expression levels were regulated by the cytokines - IL-1, IL-6, and TNF-α. The functional role of AAG in non-hepatic cancer cells has not yet been established. In order to understand the functional role of the AAG expressed in HT-29 cells, the cancer cells were transfected with cloned cDNA for AAG, or exposed to antisense oligodeoxynucleotide (ODN) for AAG. The colony-forming capacity, invasion, and adhesion to laminin of these transformed cancer cells were measured. Overexpression of AAG by transfection, and inhibition of the AAG expression by antisense ODNs were identified by Western blot as well as nested reverse transcriptase-polymerase chain reaction (nested RT-PCR), respectively. Results showed that the overexpression of AAG by transfection reduced colony-forming capacities, invasion, and adhesion to laminin of the cancer cells; on the other hand, the antisense ODN for AAG elevated colony-forming capacities, invasion, and adhesion to laminin of the cancer cells. These results suggest that AAG, expressed in cancer cells inhibited proliferation, invasion, and metastasis of the cancer cells.

Keywords: α1-Acid Glycoprotein (AAG); Acute Phase Reactant; Antisense; Gene Overexpression; Invasion; Metastasis; Proliferation.

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Introduction

The α1-Acid glycoprotein (AAG) is a 44 kDa plasma protein. AAG is one of positive acute phase proteins and its concentration in plasma is known to increase approximately two to four fold following tissue injury (Kushner, 1990; Stadnyk and Gauldie, 1991). It has been speculated that AAG plays an important role in inflammation and cancer, but its exact biological function is still unclear, despite extensive studies of AAG regarding its immunoregulatory role and binding to a number of diverse drugs (Ades *et al*., 1982; Alam and Papaconstantinou, 1992; Baumann *et al*., 1990; Hussain *et al*., 1995; Niewiarowski *et al*., 1975). The acute phase proteins are known to be synthesized mainly in the liver. The synthesis of the proteins is regulated by IL-1, IL-6, TNF-α, IFN-γ, as well as by other stimulatory factors and cofactors.

In a recent study, we identified the expression of mRNA for AAG. The expression of the AAG mRNA in HT-29 human colon carcinoma cells is regulated by cytokines (IL-1, IL-6, and TNF-α) in a manner that is characteristic of the acute phase response. The expression of AAG mRNA is up-regulated in well-differentiated HT-29 cells. The functional role of the AAG expressed in non-hepatic cancer cells has not yet been established.

In this paper, we report that the AAG may weaken the malignant potencies, the characteristics of proliferation, invasion, and metastasis of the HT-29 human colon carcinoma cells using transfection experiments.

Materials and Methods

Cell and cell culture HT-29 cells, derived from a colon adenocarcinoma, were purchased from the American Type Culture Collection (USA), and maintained in RPMI 1640 that was supplemented with 10% FBS.

Construction of the AAG expression vector A construct of AAG was generated by a polymerase chain reaction amplification of the full-length human AAG cDNA using oligonucleotides 5′-ACGTGCCTCCTGGTCTCAGTAT-3′ (sense) and 5′-TTAGCT GTTCCAAACACAGAAG-3′ (antisense). The amplified product of 764 bp was purified from an agarose gel and directionally cloned in a pCR3-unit expressing vector (Invitrogen, USA). *E. coli* (TOP10F') were then transformed by the plasmid, selected colonies were harvested, the bacteria were lysed by alkali treatment, and the plasmid were purified for transfection in HT-29 cells.

Transfection The HT-29 cells were transfected in serum-free media using the calcium phosphate precipitation method (Graham and Vander, 1973) with the plasmid DNA inserted AAG full-length cDNA. Forty-eight hours after transfection, the cells were cultured in RPMI 1640 containing 10% FBS for 24 h. The cultured cells was diluted to ten-fold and inoculated to a culture flask with RPMI 1640 that was supplemented with 10% FBS and 1 mg/ml neomycin (Sigma, USA). The colonies that were resistant to neomycin were expanded, and the AAG protein expression was identified from conditioned mediums and cell lysates using Western blot (Hong and Kang, 1998).

Antisense oligodeoxynucleotide (ODN) to human AAG mRNA A phosphorothioate analogue of antisense ODN to AAG mRNA, which was designed to bind the translation initiation codon (ATG), was synthesized by a DNA synthesizer (Millipore, USA). The sequence of antisense ODN was 5′-CAGG-ACAGCGCCATACTGAG-3′, and the sequence of sense ODN (used as a control) was 5′-CTCAGTATGGCGCTGTCCTG-3′. Cancer cells were cultured with a serum-free medium containing DOTAP (Boehringer Mannheim, Germany) and 20 μM of sense or antisense ODN. Sixteen hours after culture, the total RNA was extracted from the cancer cells. A nested reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for identification of the AAG mRNA expression (Sense: 5′-CCTTCTTTTACTTCACCCCCAAC-3′ Antisense: 5′-CATAG-ACAGACAGCCCCCAGTTC-3′).

Colony forming assay The 10^4 cancer cells were resuspended in 1 ml of a serum-free medium containing 0.3% agar. The cells was then inoculated into a 33 mm plate coated with 0.6% agar. After 21 d, the number of colonies containing more than 100 cells were counted.

Invasion assay A 24-well transwell unit (12 μm pore size; Sigma, USA) was used for the motility assay. The filters in the transwells were coated with 30 μg (0.6 mg/ml) of Matrigel per filter for 16 h at room temperature. Prior to the addition of the cells, an excess medium was removed from the upper compartment. The lower compartment contained 0.5 μl of a serum-free medium that was supplemented with a platelet-derived growth factor (PDGF; 10 ng/ml) as a chemotactant. The 2×10^4 cells were resuspended in 100 μl of a serum-free medium, and placed in the upper compartment of the transwell unit for 5 h at 37°C in a humidified 95% air/5% CO_2 atmosphere. The cells were fixed with methanol for 2 h and stained with the Wright method. The cells on the upper surface of the filter were removed by wiping with a cotton swab, and invasion was determined by counting the cells that migrated to the lower side of the filter with a phase contrast microscope at ×160 magnification.

Adhesion assay A 96-well culture plate was coated with 100 μl of laminin (10 μg/ml) at 4°C overnight. The cells were then harvested and plated with 2×10^4 cells in 200 μl of serum-free medium. The wells were washed 3 h after incubation at 37°C and the numbers of adherent cells were estimated using spectrophotometric absorbance following uptake of 3-[4,5] dimethylthiazol-2-yl-2,5-Diphenyl-tetrazolium bromide (MTT).

Statistical analysis The statistical analyses were performed using the SAS program. The average, and the S.D of the triplicate experiments, are shown. A p-value less than 0.05, based on the Student's t-test, was considered to indicate statistical significance.

Results

Overexpression of AAG in HT-29 cells In order to identify insertion of AAG cDNA into the plasmid vector, the plasma vectors isolated from transformed *E. coli*, were digested with *Eco*R1 and *Hin*dIII, followed by agarose gel electrophoresis. As shown in Fig. 1A, one single band of 764 bp was identified, indicating that the full length of cDNA of AAG was inserted into the plasmid vector.

To confirm the secretion of the AAG from the HT-29 cancer cells, overexpressed by means of the transfection with the pCR3-AAG plasmid vector, a Western blot was performed on the concentrates of the culture medium. The result showed a AAG single band, indicating that the transfected HT-29 cancer cells secrete AAG. In order to verify the validity of the inhibitory effect of the antisense ODN on AAG synthesis, the concentrates of the medium from the HT-29 cells (16 h after treatment of the antisense ODN) were examined by Western blot. The antisense treatment decreased the expression of the AAG in the HT-29 cells (Fig. 1B).

In order to determine the optional condition of infusion of antisense ODN, a nested RT-PCR was carried out from total RNA that was separated from the HT-29 cancer cells that were coincubated with 5-20 μM of antisense ODN, sense ODN, and DOTAP. The AAG mRNA expression was prominently suppressed when incubated with 10 μM antisense ODN and 13 μM DOTAP for 16 h (Fig. 2).

Functional role of overexpressed AAG by transfection in HT-29 cancer cells In order to examine the functional role of overexpressed AAG in HT-29 cancer cells, the

malignant characteristics (i.e. proliferation, invasion and

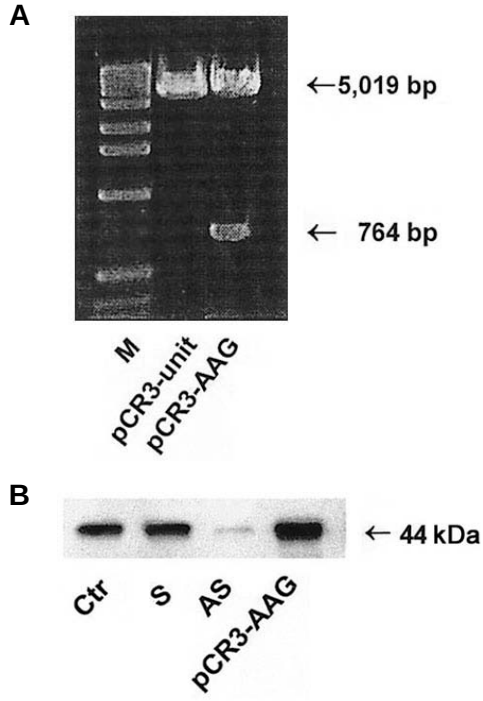

Fig. 1. A. Agarose gel electrophoretic pattern of *Eco*RI and *Hin*dIII-digested pCR3-unit, or pCR3-AAG plasmid vector. M, DNA molecular weight marker. **B.** Western blot analysis on the concentrate of the culture supernatant from HT-29 cells transfected with the pCR3-AAG plasmid vector. When cultured cells were exponentially grown, sense or antisense ODN was treated at 37°C for 16 h. S, HT-29 cells exposed to sense ODN; AS, HT-29 cells exposed to antisense ODNA; Ctr, control HT-29 cells transfected with pCR3-unit plasmid vector only; pCR3-AAG, HT-29 cells transfected with pCR3-AAG plasmid vector.

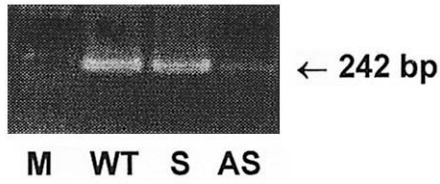

Fig. 2. Nested RT-PCR analysis of the expression of AAG mRNA in HT-29 cells exposed to antisense or sense ODN. M, DNA molecular weight marker; WT, wild type HT-29 cells; S, HT-29 cells exposed to sense ODN; AS, HT-29 cells exposed to antisense ODN.

metastasis of the HT-29 cancer cells) were investigated.

To determine the proliferation of the HT-29 cancer cells, the *in vitro* colony forming ability was examined. As shown in Fig. 3, the colony forming capability of the HT-29 cancer cells that were transfected with the AAG-expressed vector was markedly reduced, 267 ± 47, compared with that of the control group, 470 ± 57 ($P < 0.001$).

However, the colony-forming capability of the HT-29

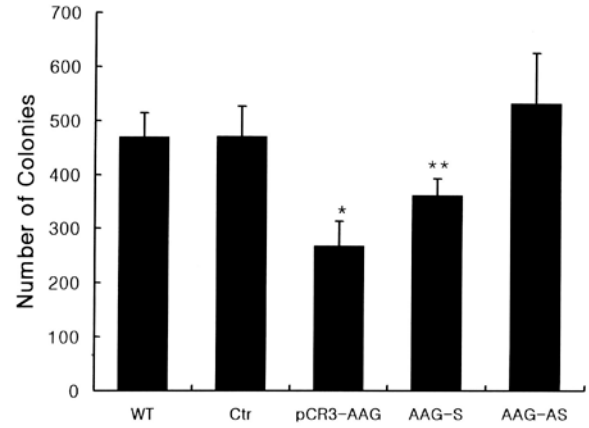

Fig. 3. Colony-forming capability of HT-29 cells transfected with the AAG-expression vector, exposed to 20 μM antisense or sense ODN for 16 h at 37°C. Mean ± S.D., $^{*}P < 0.001$ vs Ctr; $^{**}P < 0.05$ vs AS. WT, wild type HT-29 cells; Ctr, control HT-29 cells transfected with pCR3-unit plasmid vector only; pCR3-AAG, HT-29 cells transfected with pCR3-AAG plasmid vector; S, HT-29 cells exposed to sense ODN; AS, HT-29 cells exposed to antisense ODN.

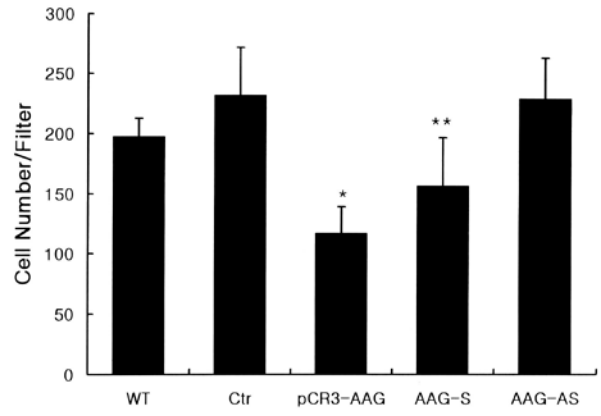

Fig. 4. Invasion of HT-29 cells transfected with the AAG-expression vector, exposed to 20 μM antisense or sense ODN for 16 h at 37°C. Mean ± S.D., $^{*}P < 0.005$ vs Ctr; $^{**}P < 0.05$ vs AS, WT, wild type HT-29 cells; Ctr, control HT-29 cells transfected with pCR3-unit plasmid vector only; pCR3-AAG, HT-29 cells transfected with pCR3-AAG plasmid vector; S, HT-29 cells exposed to sense ODN; AS, HT-29 cells exposed to antisense ODN.

cancer cells that were exposed to 20 μM of sense for 16 h at 37°C significantly decreased, 362 ± 33, compared with the antisense ODN group, 533 ± 93 ($P < 0.05$).

As shown in Fig. 4, invasion (i.e. cell number per filter) of the HT-29 cancer cells that were transfected with the AAG-expressed vector prominently decreased, 117 ± 23

cells, compared with the control cells transfected with the

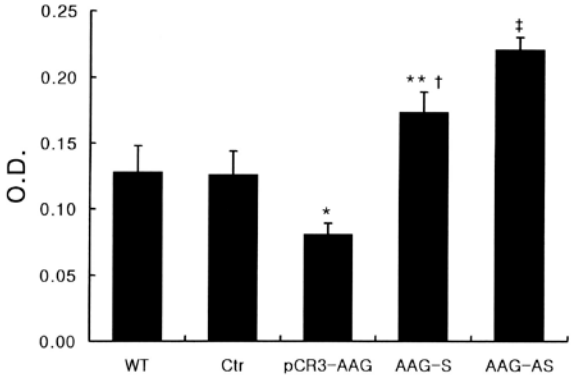

Fig. 5. Adhesion of HT-29 cells transfected with the AAG-expression vector, exposed to 20 μM antisense, or sense ODN for 16 h at 37°C to laminin. Mean ± S.D. * P < 0.005 vs Ctr; ** P < 0.005 vs AS; † P < 0.05 vs WT; ‡ P < 0.005 vs WT; WT, wild type HT-29 cells; Ctr, control HT-29 cells transfected with the pCR3-unit plasmid vector only; pCR3-AAG, HT-29 cells transfected with the pCR3-AAG plasmid vector; S, HT-29 cells exposed to sense ODN; AS, HT-29 cells exposed to antisense ODN.

pCR3-unit plasma vector, 231 ± 41 (P < 0.005). However, the cell numbers per filter of the HT-29 cancer cells that were exposed to 20 μM of sense for 16 h at 37°C significantly decreased, 157 ± 41 cells, compared with the antisense ODN group, 229 ± 34 cells (P < 0.05).

To determine the metastasis of the HT-29 cancer cells, the adhesion of the cancer cells to laminin was examined. As shown in Fig. 5, O.D. of the HT-29 cancer cells that were transfected with the AAG-expressed vector was profoundly reduced, 0.081 ± 0.009, compared with that of control group, 0.126 ± 0.018 (P < 0.05). However, the O.D. of the HT-29 cancer cells that were exposed to 20 μM of sense for 16 h at 37°C was significantly reduced, 0.174 ± 0.016, compared with the antisense ODN group, 0.221 ± 0.010 (P < 0.05). The O.D. of the sense, or antisense ODN group, was elevated compared to the wild type cells, 0.128 ± 0.020 (P < 0.05, < 0.005, respectively).

Discussion

This present study demonstrates that the overexpression of the AAG in the HT-29 cancer cells weakens the malignant potencies, but the reduced expression of the AAG in the HT-29 cancer cells by antisense ODN strengthens the malignant characteristics, proliferation, invasion, and metastasis. Also, the overexpression of the AAG in the HT-29 cancer cells increases the secretion of AAG, but the antisense treatment decreases the expression of the AAG. This study is thought to be the first to examine how the AAG of the human colon carcinoma cells regulates the malignant potencies.

In order to examine the metastasis of the HT-29 cancer cells *in vitro*, the adhesion of the cells to laminin, adhesion assay, was carried out in this study. It has been well known that malignant cells have laminin receptors on the cell surface to bind laminin, one-component of the extracellular matrix. In order to metastasize, the laminin receptors of the cells for cancer cells first bind to laminin and are then intravasated (Gui *et al*., 1997; Lukacs *et al*., 1993; Tuszynski *et al*., 1997; Ziober *et al*., 1996). Lukacs *et al.* (1993) reported that the gene encoding a highly immunogenic mycobacterial heat-shock protein (hsp65) was transfected into the murine macrophage tumor cell line J774, and the resulting hsp65-expressing cells (J774-hsp65) were no longer able to produce tumors in syngeneic mice. The results clearly showed that transfection of the tumor cells with the bacterial hsp65 results in the induction of immunity against the parent tumor cell line; this immunity was reflected in the generation of cytotoxic T cells, and protective immunity against the parent tumor. Currently, there is a great deal of interest in the use of gene transfer; for example, using IL-2- or TNF-encoding genes to increase the immunological recognition of cancer cells (Gutieerez *et al*., 1992; Miller, 1992).

When the HT-29 cells were cultured on polycarbonate coated chambers for 17 d in order to differentiate, or when exposed to a well-known differentiating agent (*all-trans* retinoic acid), the AAG mRNAs were upregulated (Lee *et al.*, 2001).

This result provides evidence that the upregulation of the AAG mRNAs weaken the malignant potency by an unknown intracellular mechanism without the action of the secreted AAG. The significantly elevated adhesiveness of the tumor cells (following sense or antisense ODN treatment), when compared to the wild type cells in the present study, remains to be elucidated.

A plausible explanation for the role of AAG could be that the AAG may weaken the malignant potencies of the HT-29 human colon carcinoma cells, the characteristics of proliferation, invasion, and metastasis. Further studies on the roles of the other acute phase proteins in cancer cells are under investigation in our laboratory.

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