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Antitumor Activity of the Novel Human Cytokine AIMP1 in an *in vivo* Tumor Model

Yeon-Sook Lee[1], Jung Min Han[2], Taehee Kang[2], Young In Park[1], Hwan Mook Kim[3], and Sunghoon Kim*

National Creative Research Initiatives Center for ARS Network, College of Pharmacy Seoul National University, Seoul 151-742, Korea;
[1] Division of Life Sciences, and Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea;
[2] Imagene Co. Ltd. Biotechnology Incubation Center, Seoul National University, Seoul 151-742, Korea;
[3] Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-333, Korea.

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Although AIMP1 (previously known as p43) is one of three auxiliary proteins bound to a macromolecular aminoacyl tRNA complex, it is also secreted as a cytokine controlling both angiogenesis and immune responses. Here we show that systemically administered purified recombinant human AIMP1 had anti-tumor activity in mouse xenograft models. In Meth A-bearing Balb/c mice, tumor volume increased about 28 fold in the vehicle treatment group, while an increase of about 16.7 fold was observed in the AIMP1-treated group. We also evaluated the anti-tumor activity of AIMP1 in combination with a sub-clinical dose of the cytotoxic anti-tumor drug, paclitaxel. The growth of NUGC-3 human stomach cancer cells was suppressed by 84% and 94% by the combinations of 5 mg/kg paclitaxel + 25 mg/kg AIMP1 ($p = 0.03$), and 5 mg/kg paclitaxel + 50 mg/kg AIMP1 ($p = 0.02$), respectively, while 5 mg/kg paclitaxel alone suppressed growth by only 54% ($p = 0.02$). A similar cooperative effect of AIMP1 and paclitaxel was observed in a lung cancer xenograft model. These results suggest that AIMP1 may be useful as a novel anti-tumor agent.

Keywords: AIMP1; Angiogenesis; Anticancer; EMAP II; p43; Tumor.

Introduction

AIMP1 was first identified as a factor associated with a macromolecular protein complex consisting of several different aminoacyl-tRNA synthetases in mammalian systems (Quevillon *et al*., 1997). However, we showed that AIMP1 was also secreted by intact cells and triggered a pro-inflammatory response (Ko *et al*., 2001) and proliferation of fibroblasts (Park *et al*., 2005). In addition, the secreted AIMP1 caused apoptosis of endothelial cells, leading to anti-angiogenic activity (Park *et al*., 2002). AIMP1 was found to interact with the α subunit of ATP synthase (Chang *et al.*, 2002), which was previously shown to mediate the anti-angiogenic activity of angiostatin (Moser *et al.*, 1999; 2001).

Since angiogenesis is thought to be essential for growth of solid tumors at primary and at secondary sites (Folkman, 2002; 2003), anti-angiogenic agents could be clinically useful for suppressing cancer progression (Folkman, 2002; Gervaz and Fontolliet, 1998; Hayes *et al*., 1999; O'Reilly, 2002). Thus, the administration of angiogenesis inhibitors might keep the tumor and its metastases dormant, while co-administration of cytotoxic drugs might lead to death of the tumor cells. Many studies have been conducted to evaluate the therapeutic effects of angiogenic inhibitors in combination with cytotoxic agents. Combined treatment with the anti-angiogenic agent TNP-470 and mitomycin-C, adriamycin, CDDP, and 5-FU greatly increased antitumor activity in mouse models (Kato *et al.*, 1994). Anti-VEGFR Mab DC101 and paclitaxel enhanced apoptosis of tumor cells and down-regulated tumor-induced neovascularization, prolonging survival of the treated animals (Inoue *et al.*, 2000). In this report, we tested whether AIMP1, with its anti-angiogenic cytokine activity, could suppress tumor growth. We also evaluated the combined effect of AIMP1 and paclitaxel against human NCI-H460

* To whom correspondence should be addressed.
Tel: 82-2-880-8180; Fax: 82-2-875-2621
E-mail: sungkim@snu.ac.kr

Abbreviations: AIMP1, ARS-interacting multi-functional proteins; EMAP II, endothelial-monocyte activating polypeptide II; Meth A fibrosarcoma, methylcholanthrene A-induced fibrosarcoma.

lung cancer and NUGC-3 gastric adenocarcinomas in animal models.

Materials and Methods

Purification of AIMP1 Human AIMP1 was expressed as a His-tagged fusion protein in *Escherichia coli* BL21 (DE3) cells and purified by nickel affinity and Mono S ion-exchange chromatography (Ko *et al*., 2001). To remove lipopolysaccharide (LPS), the protein was dialyzed against pyrogen-free buffer (10 mM potassium phosphate buffer, pH 6.0, and 100 mM NaCl). After dialysis, it was loaded onto polymyxin resin (Bio-Rad) pre-equilibrated with the same buffer, incubated for 20 min, and eluted. To remove residual LPS, the protein was dialyzed against PBS containing 20% glycerol, and filtered through a Posidyne membrane (Pall Gelman Laboratory, Ann Arbor, USA). The concentration of LPS in the final AIMP1 preparation was below 20 pg/ml, as determined with a Limulus Amebocyte Lysate QCL-1000 kit (BioWhittaker, USA).

Mice and cell lines 6-week-old specific pathogen-free female BALB/c mice (Meth-A fibrosarcoma assay) and 5-week-old male BALB/c-*nu/nu* nude mice (human tumor xenograft experiments) were obtained from Harlan Co. Ltd. (USA). The mice were housed in a pathogen-free barrier facility with ambient light controlled automatically to produce 12-h light and dark cycles. The Meth-A (murine tumor), NCI-H460 (human lung carcinoma) and NUGC-3 (human gastric adenocarcinoma) lines were obtained from the Cell Bank Facility, Korea Research Institute of Biotechnology (KRIBB).

Meth A fibrosarcoma assay The effect of AIMP1 was evaluated at the indicated doses after implantation of Meth-A tumor cells. Briefly, 6-week-old female BALB/c mice were implanted subcutaneously (*s.c.*) with 2×10^5 cells. When the tumors were about 50 mm^3 in volume, the mice were administered AIMP1 every other day from day 0 to day 12 by intratumor injection.

Antitumor activity was assessed by the tumor growth inhibition rate (TGI) 14 d after the start of treatment. TGI was calculated as: % TGI = $(1-T/C) \times 100$, where T is the mean final tumor weight of the treated group, and C the mean final tumor weight of the control group.

Anti-tumor experiments in human xenografted mice Two separate sets of experiments were carried out, one for each of the cell lines, NCI-H460 and NUGC-3. We injected human NCI-H460 (lung cancer) or NUGC-3 (gastric cancer) cells adjusted to 3×10^7 cells/ml, subcutaneously into the right scapular region of each mouse in a total volume of 300 μl of PBS. The tumors were monitored for growth before the mice were randomly assigned to the various treatment and control groups.

In the NCI-H460 experiments, 40 mice bearing xenografts were randomized into 5 groups, comprising control, low dose AIMP1 (25 mg/kg), high dose of AIMP1 (50 mg/kg), combination of paclitaxel (5 mg/kg) + low dose AIMP3 (25 mg/kg), and combination of paclitaxel (5 mg/kg) + high dose AIMP1 (50 mg/kg). In the NUGC-3 set of experiment, 48 mice bearing xenografts were randomized into 6 groups, as a paclitaxel alone (5 mg/kg) group was included. The drugs were administered by intraperitoneal injection. Treatment was started when tumor size was about 50–100 mm^3. Recombinant human AIMP1 was diluted in 20% glycerol containing PBS and injected daily over 4 weeks, whereas paclitaxel (Genexol, Samyang Genex Co., Korea) was diluted in saline and injected five time, 0, 2, 5, 8, and 11 days after initiation of treatment. Control mice received vehicle solution alone.

Measurement of tumor size Tumors were measured in three dimensions with calipers, and tumor volume (in mm^3) was calculated as:

$$Volume\ (mm^3) = Length \times Width \times Depth \times 1/2$$

Tumor volumes were measured on alternate days. Relative tumor volume (RTV) was calculated as RTV = V_i/V_o, where V_i is the tumor volume at any given time and V_o is the volume at the start of treatment. Tumor growth inhibition data were analyzed by Student's *t*-test.

Results

Antitumor activity in a murine Meth A fibrosarcoma model We evaluated the antitumor activity of AIMP1 using the BALB/c mouse-Meth A fibrosarcoma system. AIMP1 was injected into the tumors in two doses of 1 μg/dose and 10 μg/dose every other day from day 0 to 12. The tumor volume had increased about 28 fold in the vehicle treatment group on day 12 whereas it had increased only 16.7 fold in the 10 μg AIMP1 group ($p < 0.01$); the 1 μg/dose did not cause a significant reduction in relative tumor size (Fig. 1A). The treatments did not induce a loss of body weight in any of the tested animals (Fig. 1B). The Meth-A tumors were excised and weighed on day 14. The 10 μg AIMP1 group showed a 32% reduction of tumor weight ($p < 0.04$) compared to the vehicle treatment group, and the 1 μg AIMP1 treated group showed a 29% reduction ($p < 0.04$); the relative reductions in tumor volumes were not statistically significant (Table 1).

Anti-tumor activity of AIMP1 in mice xenografted with human cancers We asked whether the anti-tumor activity of AIMP1 could be increased by combining it with cytotoxic drugs, since non-cytotoxic agents are often administered in combination with conventional chemotherapeutics. To explore this possibility, we injected NCI-H460 human pulmonary cancer and NUGC-3 gastric cancer cells into nude mouse, and, once the tumors had grown to about 50–100 mm^3, we administered AIMP1 either alone (25 or 50 mg/kg) or in combination with a sub-clinical dose (5 mg/kg) of paclitaxel. AIMP1

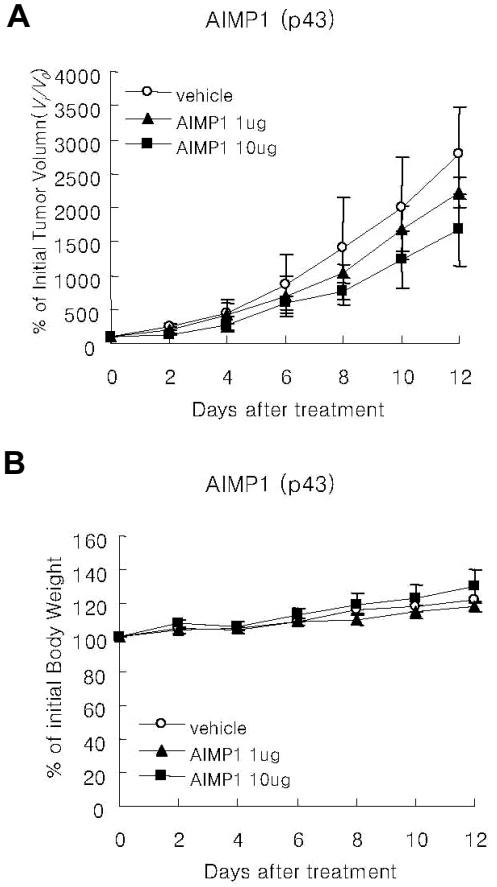

Fig. 1. Inhibition of growth of Meth-A fibrosacoma xenografts by AIMP1. 6-week-old male BALB/c mice were implanted subcutaneously (s.c.) with 2×10^5 cells. When the tumors were about 50 mm^3 in volume they were pair-matched into groups and treatments were initiated (day 0). **A.** Mice were treated by intratumor injection with vehicle (○), or 1 μg/day (▲) or 10 μg/day (■)AIMP1, every other day from day 0 to 12. Tumor size was measured every other day and relative tumor volume (RTV) was calculated as RTV = *Vi/Vo*, where *Vi* is the tumor volume at a given time and *Vo* is the tumor volume at the start of treatment. **B.** The body weights of the treatment groups are given as percentages of the initial body weights. Values are means; bars, standard deviations; $n = 6$.

alone had little impact on NUGC-3 tumor growth up to day 12, while paclitaxel alone significantly reduced tumor growth. At the same time, tumor volume increased to 446 % of its initial value, whereas a 1011% volume increase was observed in the control group ($p = 0.02$). Tumor suppression by paclitaxel was strongly boosted by combining it with AIMP1 (Fig. 2A). The combination of paclitaxel (5 mg/kg) and low dose AIMP1 (25 mg/kg) gave a 157% increase in tumor volume ($p = 0.03$) and the combination of paclitaxel (5 mg/kg) and high dose AIMP1 (50 mg/kg) resulted in a 66% increase, that is, a slight reduction in tumor volume ($p = 0.02$). We only compared NUGC-3 tumor volumes up to day 12, because the survival of the vehicle only control group fell below 50% from day 14. However we continued the AIMP1 treatment according to schedule and compared the survival of the treatment groups on day 28. All 16 animals were still alive in the two paclitaxel + AIMP1 combined therapy groups, whereas only two of the 8 control animals (25%) and 5 of the 8 animals (63%) receiving paclitaxel alone survived (Fig. 2B).

Table 1. Tumor weight of Meth-A xenografted Balb/c mice treated with AIMP1 at day 14.

Treatment	Dose	Average tumor weight (g)	TGI (%)	*t*-test
Vehicle	-	5.11 ± 1.15		
AIMP1	1 μg/dose	3.62 ± 0.81	29	*P = 0.04*
	10 μg/dose	3.47 ± 1.30	32	*P = 0.04*

Statistical significance was evaluated by comparing the mean tumor size of vehicle-treated groups with drug-treated groups using two-tailed Student's *t*-tests. All treatments were at daily intervals by intratumor injection of doses formulated in PBS with 1% bovine serum albumin. Tumor growth inhibition was calculated as the difference between the average tumor weight in control group and drug-treated group (T/C × 100) on day 14.

With the NCI-H460 tumors, a statistically significant reduction in tumor volume was observed from day 4 of the AIMP1 + paclitaxel treatment. Although AIMP1 on its own was effective in reducing the rate of growth of the tumors, its anti-tumor effect was augmented in combination with paclitaxel. The relative mean tumor volume of the control tumors was 67.95 ± 22.22 on day 28, and the relative mean tumor volumes were 64.02 ± 23.45 (6% reduction), 58.36 ± 12.84 (14%), 54 ± 13.8 (21%), and 39.58 ± 16.86 (42%) at the end of the treatments with 25 mg/kg AIMP1, 50 mg/kg AIMP1, 25 mg/kg AIMP1 + paclitaxel, and 50 mg/kg AIMP1 + paclitaxel, respectively. The drug-treated groups had lost body weight after 2 days and their weights recovered gradually in the AIMP1 only group whereas in the AIMP1 + paclitaxel groups body weight did not returned to within the control range up to the end of the experiment (Table 2).

Discussion

Although AIMP1 has complex extracellular effects, its C-terminal domain spanning 22 kDa (previously named EMAP II) has potent anti-tumor activity probably due to multiple effects on inflammatory and vascular cells within the tumors (Kao *et al.*, 1994, Schwarz *et al*., 1999). In previous reports, we detected pro-inflammatory and anti-angiogenic activity of full length AIMP1 and suggested that it was a true cytokine secreted by an active mechanism (Ko *et al.*, 2001; Park *et al.*, 2002). Here, we inves-

Table 2. Anticancer efficacy of AIMP1 in human xenografted mice. All dosing was by intraperitonial injection formulated in PBS with 20% glycerol.

Tumor	Treatment	Schedule	Dose	Route	Average relative tumor volume (*Vi/Vo*)		% of initial body weight	
					Day 12	Day 28	Day 12	Day 28
NUGC-3	Vehicle	Day 0–27		*i.p.*	13.91 ± 6.04	-	74.1 ± 6.5	-
	Low dose AIMP1	Day 0–27	25 mg/kg	*i.p.*	10.21 ± 7.73	-	83.4 ± 12.8	-
	High dose AIMP1	Day 0–27	50 mg/kg	*i.p.*	9.17 ± 6.20	-	85.4 ± 9.9	-
	Low dose AIMP1 + paclitaxel	Day 0–27 Day 0, 2, 5, 8, 11	25 mg/kg 5 mg/kg	*i.p.*	11.00 ± 4.78	-	96.9 ± 4.2	-
	High dose AIMP1 + paclitaxel	Day 0–27 Day 0, 2, 5, 8, 11	50 mg/kg 5 mg/kg	*i.p.*	0.71 ± 0.28	-	100.4 ± 3.1	-
	Paclitaxel	Day 0–27	5 mg/kg	*i.p.*	6.02 ± 4.78	-	104.7 ± 3.1	-
NCI-H460	Vehicle	Day 0–27		*i.p.*	18.85 ± 4.76	67.95 ± 22.22	-	127.1 ± 18.8
	Low dose AIMP1	Day 0–27	25 mg/kg	*i.p.*	18.43 ± 6.38	64.02 ± 23.45	-	129.3 ± 5.5
	High dose AIMP1	Day 0–27	50 mg/kg	*i.p.*	15.87 ± 6.22	58.36 ± 12.84	-	130.4 ± 8.6
	Low dose AIMP1 + paclitaxel	Day 0–27 Day 0, 2, 5, 8, 11	25 mg/kg 5 mg/kg	*i.p.*	13.57 ± 3.59	54.00 ± 13.80	-	124.3 ± 10.6
	High dose AIMP1 + paclitaxel	Day 0–27 Day 0, 2, 5, 8, 11	50 mg/kg 5 mg/kg	*i.p.*	10.67 ± 3.56	39.58 ± 16.86	-	121.7 ± 6.2

Day 12 and day 0 data were compared for NUGC-3 cancers, and day 12 and day 28 for NCI-H460 cancers. *i.p.*: intra peritoneal injection. *Vi*, tumor volume on any day; *Vo*, tumor volume on day 0.

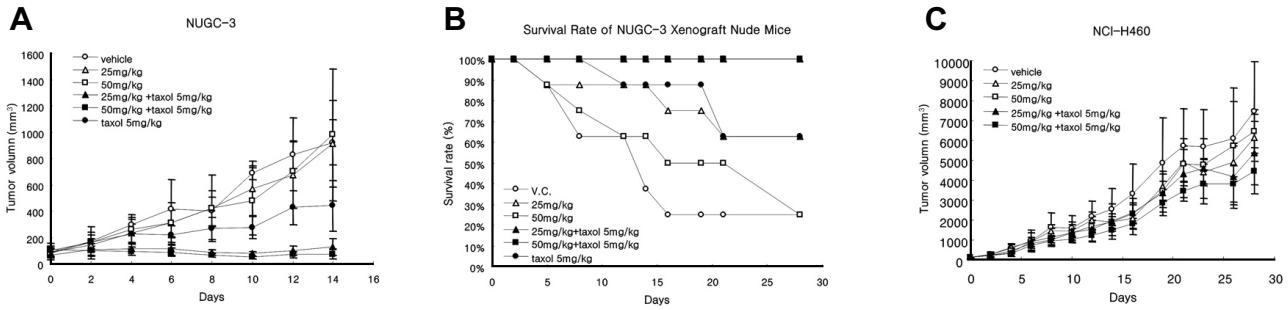

Fig. 2. Effect of AIMP1 alone or in combination with paclitaxel on growth of human tumor xenografts. Growth curves of human xenografts treated with AIMP1 and paclitaxel. Human NUGC-3 stomach (A) and H460 lung (C) cancer cells were implanted subcutaneously into male nude mice and allowed to grow to a size of 50–100 mm^3, at which time they were pair-matched into groups, and treatment was initiated (day 0). Drugs were formulated in PBS with 20% glycerol and administered by intraperitonial injection once a day for AIMP1, and 5 times in total for paclitaxel (on day 0, 2, 5, 8, 11). Mice were treated with vehicle (○), 25 mg/kg AIMP1(△), 50 mg/kg AIMP1 (□), 5 mg/kg paclitaxel + 25 mg/kg AIMP1 (▲), 5 mg/kg paclitaxel + 50 mg/kg AIMP1 (■) or 5 mg/kg paclitaxel alone (●). Tumor size was measured every other day. Values are means; bars, standard deviations. B) Survival rates as percentages of animals remaining alive at the end of the study.

tigated whether full-length AIMP1 has anti-tumor activity like its C-terminal domain. In fact, it had tumor suppressive activity against Meth A tumors. In addition, recombinant AIMP1 alone inhibits the growth of MKN-45 and SNU-16 stomach cancers, as well as A-549 lung cancers, and HCT-116 colon cancers, although its efficacy depends on the type of cancer (data not shown), implying that it has potential use as a primary anti-tumor agent against certain tumors.

The anticancer effect of AIMP1 on the stomach and lung cancer models was synergistically increased by combination with the cytotoxic anticancer drug, paclitaxel. In mice grafted with NUGC-3 stomach cancer cells, combined AIMP1 and paclitaxel treatment suppressed tumor growth completely or caused regression, whereas paclitaxel alone did not completely eradicate tumor growth. Although AIMP1 alone did not suppress NUGC-3 tumor growth significantly, it prolonged survival of the host mice. In the control group, only 25% of the animals survived until day 28 and 44% and 63% of the animals in the

AIMP1 and paclitaxel alone groups, respectively, survived. In contrast survival was 100% when AIMP1 was combined with a sub-clinical dose of paclitaxel.

It is not clear whether the anti-angiogenic activity of AIMP1 is solely responsible for its antitumor effect because, as described previously, it also affects immune responses. It is now accepted that biological agents such as IFN-α, epidermal growth factor, and Her-2/neu receptor antagonists (Perrotte *et al.*, 1999; Slaton *et al.*, 1999; Ye *et al*., 1999) can modulate host responses and enhance the efficacy of standard chemotherapy. In this context, our data suggest that AIMP1 may be of use either as a primary antitumor agent or as a supplement to primary cytotoxic anticancer drugs.

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