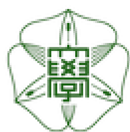

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Adrenomedullin antagonist suppresses *in vivo* growth of human pancreatic cancer cells in SCID mice by suppressing angiogenesis

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Running title: Adrenomedullin antagonist and pancreatic cancer

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ABSTRACT

Since it is reported that adrenomedullin (AM) upregulated by hypoxia inhibits hypoxic cell death, we examined the effects of AM antagonist (AM C-terminal fragment; AM(22-52)) on the growth of pancreatic cancer cells. We for the first time demonstrated that AM antagonist significantly reduced the *in vivo* growth of the pancreatic cancer cell line. Immunohistochemical analysis demonstrated that the mean diameter of blood vessels was significantly smaller in the tumor tissues treated with AM antagonist than in those treated with AM N-terminal fragment (AM(1-25)) and that the PCNA-labeling index was lower in the former than in the latter. Then we demonstrated that AM antagonist showed no effect on the *in vitro* growth of the pancreatic cancer cell line. These results showed that AM played an important role in the growth of pancreatic cancer cells *in vivo*, suggesting that AM antagonist might be

a useful tool for treating pancreatic cancers.

Pancreatic cancers are highly aggressive and one of the dominant causes of cancer death. Currently there is no effective therapy for this cancer; surgical resection is available only to a small fraction of patients and has only a marginal effect on overall survival rates. Chemotherapy and radiotherapy have very limited effects on patients' survival. Furthermore, even in patients with curatively excised pancreatic cancers, relapses involving hepatic metastases frequently result in death within a short period. In order to establish new approaches to such an aggressive disease, we need to understand detailed molecular basis of the pathogenesis.

We recently found that the constitutive expression of HIF-1α protein was implicated in the resistance of pancreatic cancer cells to apoptosis *in vivo* (Akakura et al., 2001), suggesting that HIF-1-mediated adaptation responses to hypoxia was important in the tumor formation of pancreatic cancer cells.

Also we have recently identified 38 genes as hypoxia-inducible genes with the use of a DNA microarray system (Niizeki et al., 2002); in those, we found adrenomedullin (AM), which has been reported to be induced by hypoxia and to promote vasodilation (Nuki et al. 1993; Zhao et al., 1996; Nakayama et al., 1998; Cormier-Regard et al., 1998; Hayakawa et al., 1999; Garayoa et al., 2000). Recent reports further demonstrated that AM acted as a growth factor for several cancer cells besides vasodilator, and that AM acted as a survival factor for several cancer cells and endothelial cells (Miller et al., 1996; Kato et al., 1997; Rocchi et al., 2001; Oehler et al., 2001). However, the function of AM in pancreatic cancer cells has not been determined. According to the previous findings, we hypothesized that hypoxia might give pancreatic cancer cells growth advantages by enhancing the expression of AM and that AM antagonist (AM(22-52); AMA) (Eguchi et al., 1994) might

inhibit the *in vivo* growth of pancreatic cancer cells. In this study, we first examined the effects of AMA on the *in vivo* growth of the pancreatic cancer cell line, PCI-43 in SCID mice. Intra-tumoral injection of AMA, but not AM N-terminal fragment, AM(1-25)(Watanabe et al., 1996), from day 8 to day 17 completely abrogated the tumor formation (Fig. 1a), suggesting that AM is essential for the *in vivo* tumor formation of pancreatic cancer cells. As recent reports demonstrated that AM acted as a growth factor for several cancer cells (Miller et al., 1996; Kato et al., 1997; Rocchi et al., 2001; Oehler et al., 2001), we then examined the expressions of AM and AM receptors and the effects of AMA on the growth of pancreatic cancer cells *in vitro*. All the pancreatic cancer cell lines expressed higher levels of AM mRNA under hypoxia than under normoxia (Fig. 1b). In contrast to our initial hypothesis, the expression levels of adrenomedullin receptors were low in the tested pancreatic

cancer cell lines (Fig. 1c). PCI-43 did not express CRLR or RAMP-2, both of which are receptors of AM (Muff et al., 2001). Among the tested cell lines, BxPC-3 and PCI-35 expressed both CRLR and RAMP-2. However, AMA did not show any effect on the growth of the BxPC-3 and PCI-35 cells *in vitro* (Fig. 1d and 1e). These results suggested that AM might not act in an autocrine fashion at least in pancreatic cancer cells and that the *in vivo* growth inhibition may be caused by indirect effects of AMA. In order to examine how AMA abrogated the tumor formation, we treated the tumors 4 times with intra-tumoral injection of AM(1-25) or AMA. Mean weights ± SD of 5 tumors treated with AM(1-25) and AMA were 0.0483 ± 0.0089 (g), and 0.0297 ± 0.0001 (g), respectively (Fig. 2a). In this experimental setting, treatment with AMA reduced the numbers of proliferating cells (PCNA-positive cells) in the tumor tissues compared with the treatment with AM(1-25) (Fig. 2b). Labeling indexes were

25.8±3.9 and 6.3±5.2 in the tumor tissues treated with AM(1-25) and AMA, respectively ($p<0.01$)(Table 1). Then we examined the numbers of CD31-positive cells in the tumor tissues. There was no significant difference in the number of CD31-positive cells between the tumor tissues treated with AM(1-25) and those treated with AMA. However, a more number of large blood vessels (diameter>8 μm) were found in the former than in the latter (Fig. 2c and Table 2). The mean±SD of diameters of blood vessels in the tumor tissues treated with AM(1-25) and AMA were 6.454 ±5.313 (μm) and 2.276±1.120 (μm), respectively ($p<0.001$) (Table 2). These results suggest that AMA inhibited the growth of pancreatic cancer cells indirectly by suppressing the formation of large functional blood vessels in the tumor tissues, leading to the depletion of nutrient and oxygen supply. Our findings are consistent with the recent report demonstrating that neutralization of AM by intratumoral injection of the

anti-AM antibody suppressed the *in vivo* growth of a glioblastoma cell line partly by suppressing the formation of functional large blood vessels (Ouafik et al., 2002).

Whereas previous reports demonstrated that AM suppressed the apoptosis of endothelial cells and that AM was pro-tumorigenic either by stimulating angiogenesis alone or both angiogenesis and carcinoma cell growth directly (Kato et al., 1997; Oehler et al., 2002), the precise mechanism of AM in angiogenesis is yet to be determined. Angiogenesis is a morphogenetic process, which consists of a number of steps including endothelial cell invasion and capillary lumen formation controlled by various factors (Carmeliet, 2000). Although various factors involved in the angiogenesis have been identified (Folkman, 1995; Carmeliet, 2000; Browder et al., 2000; Kerbel, 2000; Yancopoulos et al., 2000), it is yet to be determined how capillary lumen formation is controlled.

Previous reports demonstrated that VEGF and angiopoietin-1 were involved in the regulation of lumen formation (Drake et al., 1995; Suri et al., 1998). A recent report further demonstrated that the disruption of HIF-1 pathway inhibited the formation of large blood vessels possibly via a lack of upregulation of VEGF expression (Carmeliet et al., 1998). From the facts that AM was upregulated by hypoxia in a HIF-1-dependent manner (Cormier-Regard et al., 1998; Garayoa et al., 2000) and that AMA inhibited the formation of large blood vessels, we suppose that AM might be involved in the lumen formation under the control of HIF-1. As we treated the tumors with AMA or AM 5 days before sacrificing the mice, it is unlikely that AMA inhibited the vasodilation promoted by AM. Although further studies will be required, our present result suggests that AMA would be a useful tool at least for the treatment of pancreatic cancers. While we injected AMA by one-shot injection in this

study, significant reduction of tumor growth was observed, suggesting that continuous infusion of AMA would bring about more significant anti-tumor effects. We are now planning to examine the effects of continuous injection of AMA and intra-tumoral injection of an AMA expression vector.

ACKNOWLEDGEMENTS

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Legends to Figures

Fig. 1. Effects of AM and AMA on the growth of pancreatic cancer cells. a) Growth curves of the tumors treated with AM(1-22) and AMA were shown. Five X 10^6 PCI-43 cells were inoculated subcutaneously into the right flanks of SCID mice (n=5, in each group). Tumor formation was observed every three days up to 3 weeks after inoculation. Adrenomedullin N-terminal fragment (AM(1-25)) and adrenomedullin C-terminal fragment (AM(22-52); AMA) were purchased from Peptide Institute Inc. (Osaka, Japan). Tumors were treated daily with intra-tumoral injection of AM(1-25) or AMA from day 8 to day 17 after the inoculation at 50 μg/tumor. b) AM mRNA expression in pancreatic cancer cell lines under normoxia and hypoxia was shown. Northern blot analysis was performed by the method described previously (Choi et al., 2000). Pancreatic cancer cell lines, PCI-10, PCI-19, PCI-35 and PCI-43 were kindly supplied by Dr. Hajime Ishikura

(The First Department of Pathology, Hokkaido University School of Medicine). BxPC-3 was kindly supplied from Dr. Jun-ichi Hamada (Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University). c) AM receptor mRNA expressions examined by RT-PCR were shown. HUVEC purchased from KURABO (Osaka, Japan) is used as a positive control. PCR was performed in a DNA thermal cycler (Barnstead/Thermolyne, Dubuque, Iowa, USA) for 35 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min). The PCR product (9 µl) was subjected to electrophoresis on 1% agarose gels and stained with ethidium bromide. PCR primers for amplification of calcitonin receptor-like receptor (CRLR) and receptor activity-modifying protein-2 (RAMP-2), both of which were reported to be receptors for AM, were as follows: CRLR forward, 5’-ctcctctacattaccatgg-3’; reverse, 5’-cctccctctgcaatctttcc-3’, RAMP-2 forward,

5'-cgtctccctaggacccgac-3'; reverse, 5'-tggtggggatgagagagtgaga-3'. β-actin forward, 5'-gctcctcctgagcgcaagt-3'; reverse, 5'-tcgtcatactcctgcttgctgat-3'. d) Growth of PCI-35. e) Growth of BxPc-3. Growth of the cells was estimated by a colorimetric MTS assay using a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS) (Promega, Madison, MI) according to the manufacturer's instruction. AMA 1: AMA was added at 1 μg/ml. AMA 2: AMA was added at 2 μg/ml. Control: AM(1-25) was added at 2 μg/ml. The cultures were incubated for the first 24, 48 and 72 hours and then incubated with MTS for the final 1-4 hours. Absorbance at 490 nm was read on an MTP-100 microplate reader (Corona Electric, Tokyo).

Fig. 2. Immunohistochemical analysis for CD31-positive cells

and PCNA-positive cells in the tumor tissues. a) Growth of the tumors treated with 4 times injection of AM or AMA. In order to examine the mechanisms by which AMA inhibited the growth, we treated the tumors 4 times with AM(1-25) or AMA. Twenty-one days after inoculation, tumor weights were measured. b) PCNA-positive cells. c) CD31-positive cells. Expressions of CD31 and PCNA were analyzed by immunohistochemical staining using the streptavidin-biotin technique (Histofine SAB-PO kit, Nichirei, Tokyo). Snap-frozen tissue specimens and paraffin-embedded tissue specimens were used for the analysis of CD31 and PCNA, respectively. The tissue sections were pre-incubated for 30 minutes with PBS containing 1% bovine serum albumin, and endogenous peroxidase was inactivated with 3% H_2O_2 in methanol for 15 minutes. The sections were then incubated overnight at 4°C with anti-mouse CD31 antibody (BD Pharmingen, San Diego, CA) and anti-PCNA antibody (BD Pharmingen, San Diego,

CA) at concentrations of 5 μg/ml in PBS. After washing with PBS, the sections were incubated for 1 hour at room temperature with the biotin-conjugated anti-rat second antibody (DAKO, Tokyo), followed by the avidin-biotin-peroxidase reaction. DAB was used as a chromogen to visualize the reaction products. Finally, all the sections were counter-stained with hematoxylin. PCNA-labeling index was determined by counting the number of PCNP-positive cells in 100 tumor cells in each tumor tissue. The mean blood vessel size was obtained by measuring diameters of 100 blood vessels in each tumor tissue.

Table 1. Diameter of blood vessels in the tumor tissues

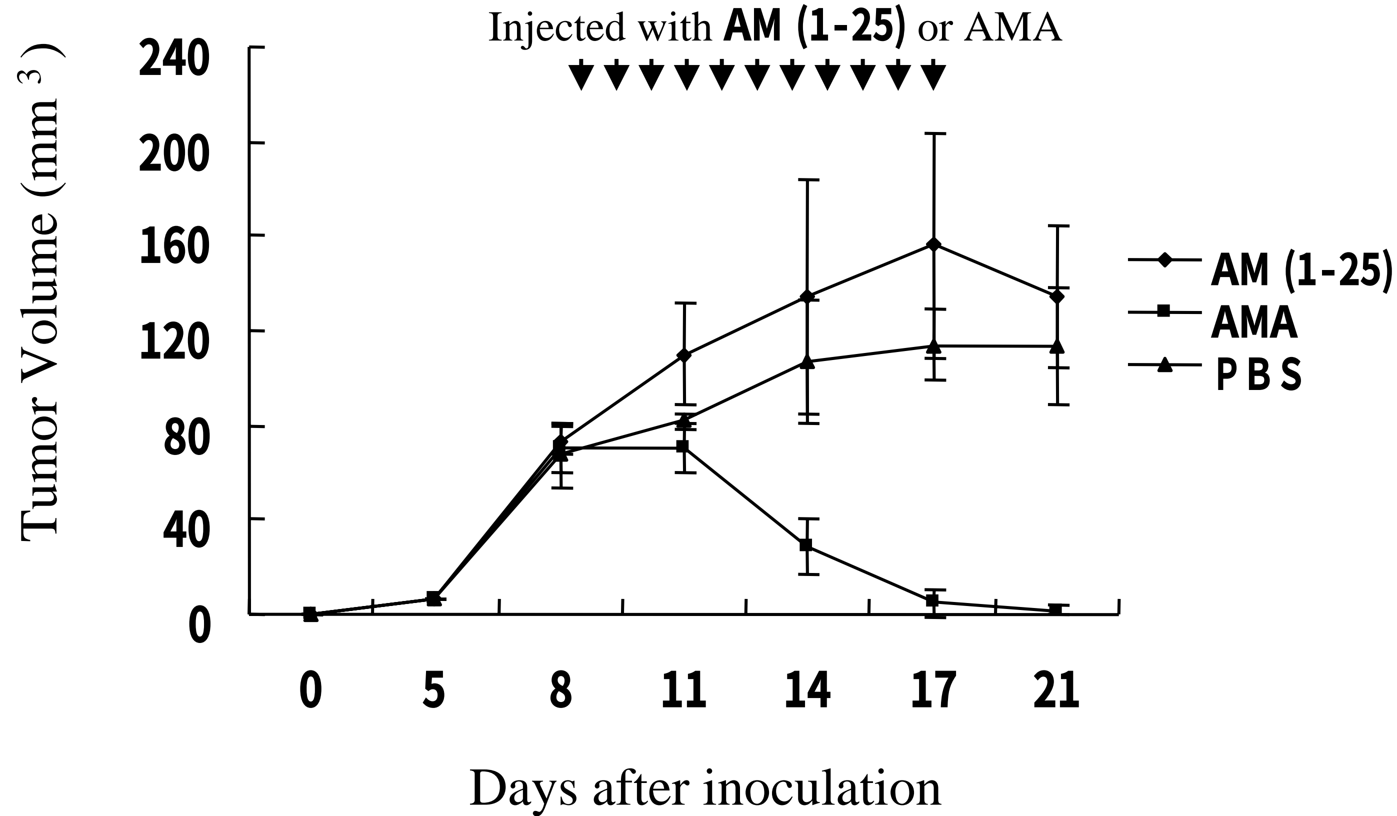
Treatment	Numbers of vessels			Mean±SD of diameter
	Diameter ～2μm	2～8μm	8μm～	
AM(1-25)	15	54	31	6.454±5.313 *
AMA	55	44	1	2.276±1.120 *

•p<0.001. Diameteres of 100 blood vessels were measured in each group.

Table 2. PCNA-labeling index

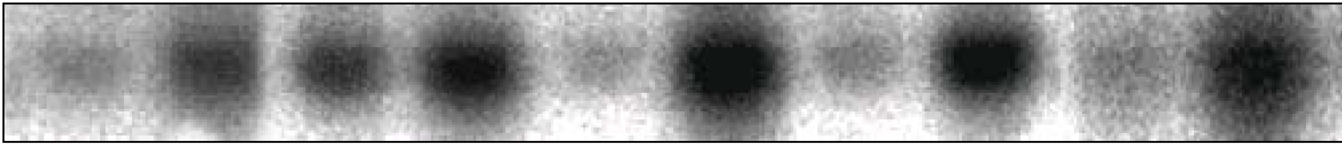
Treatment	PCNA-labeling index	
AM (1-25)	25.8±3.9	*
AMA	6.3±5.2	

$p < 0.001$.
Five tumor tissues were examined in each group.

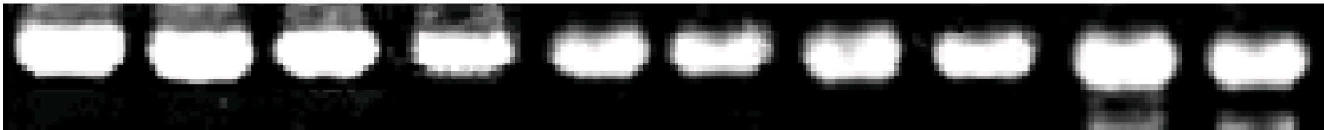

Injected with AM (1-25) or AMA
Tumor Volume (mm 3)
240
200
160
120
80
40
0
0
5
8
11
14
17
21
Days after inoculation
AM (1-25)
AMA
P B S

PCI-10 PCI-19 PCI-35 PCI-43 BxPC-3

N H N H N H N H N H

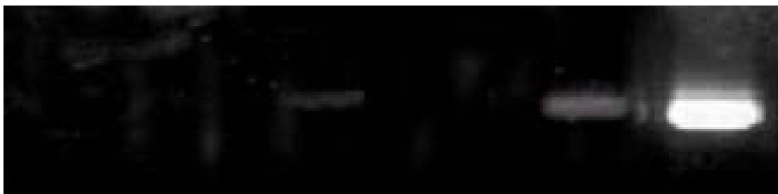


ADM



28S

PCI-10
PCI-19
PCI-35
PCI-43
BxPC-3
HUVEC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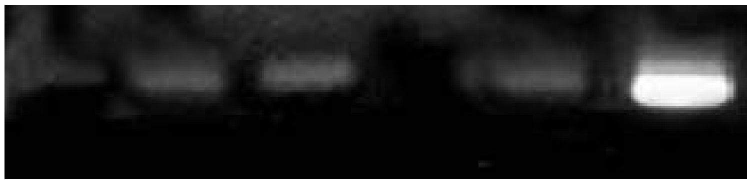
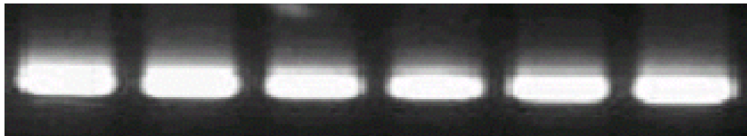
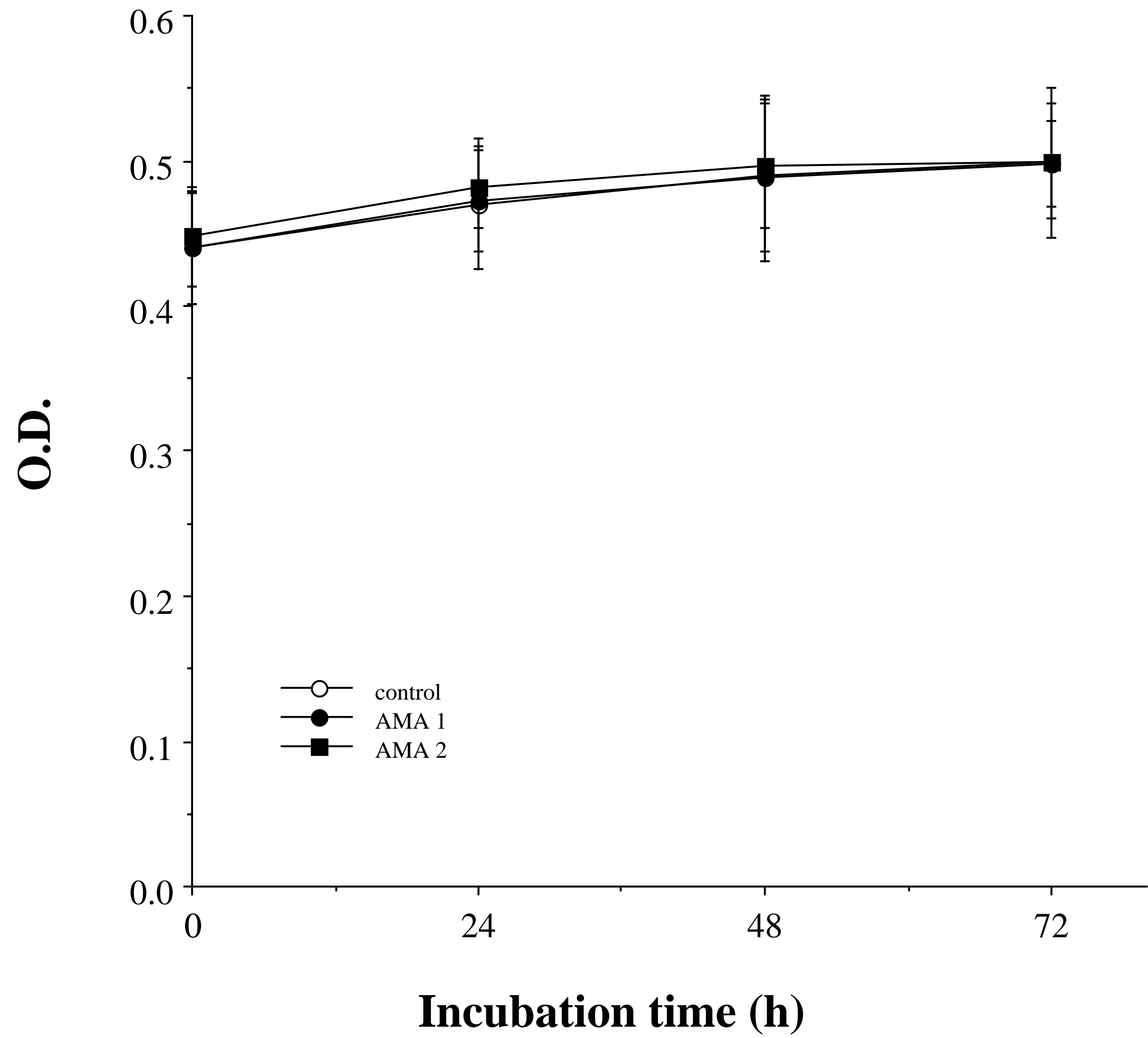
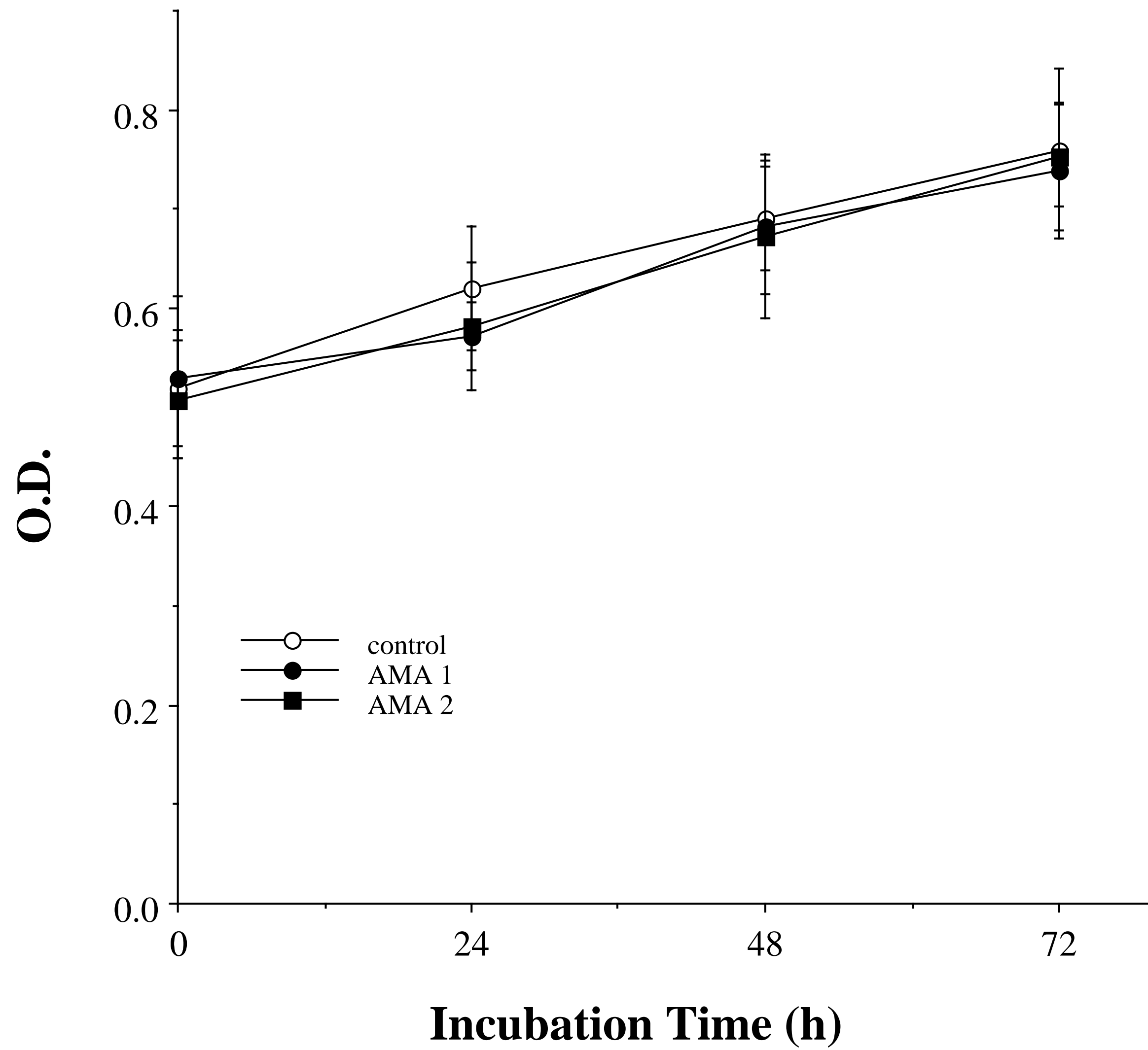

RAMP2


β-actin



0.6
0.5
0.4
0.3
0.2
0.1
0.0
O.D.
0
24
48
72
Incubation time (h)
control
AMA 1
AMA 2


O.D.
0.0
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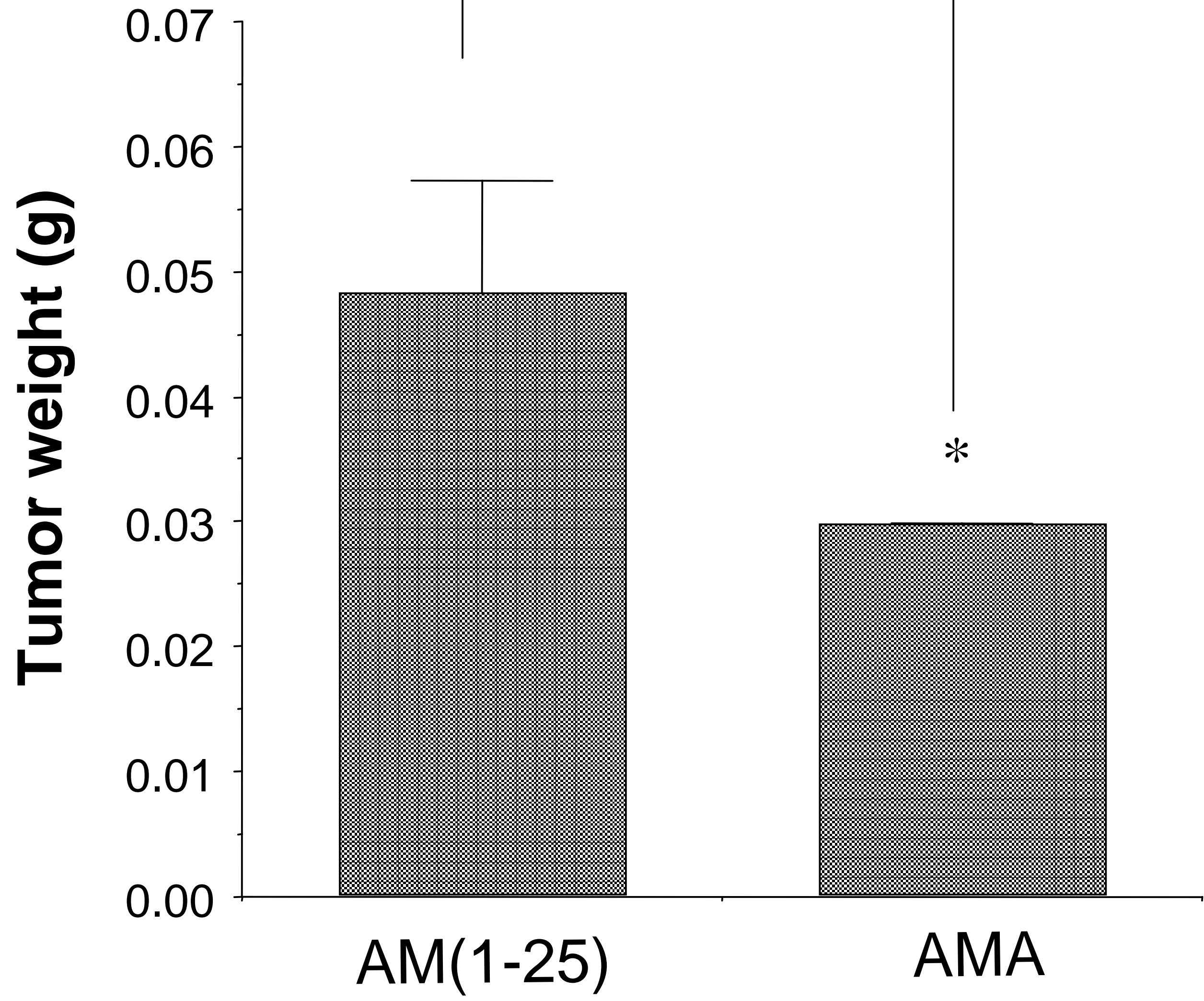
0.6
0.8
0
24
48
72
Incubation Time (h)
control
AMA 1
AMA 2


Tumor weight (g)
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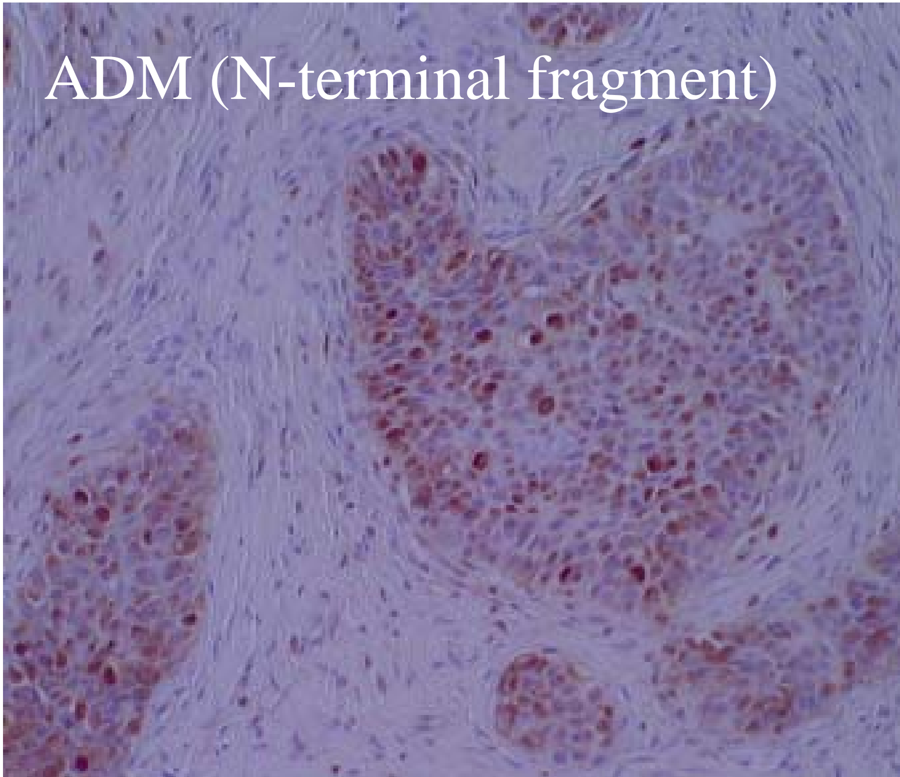
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
AM(1-25)
AMA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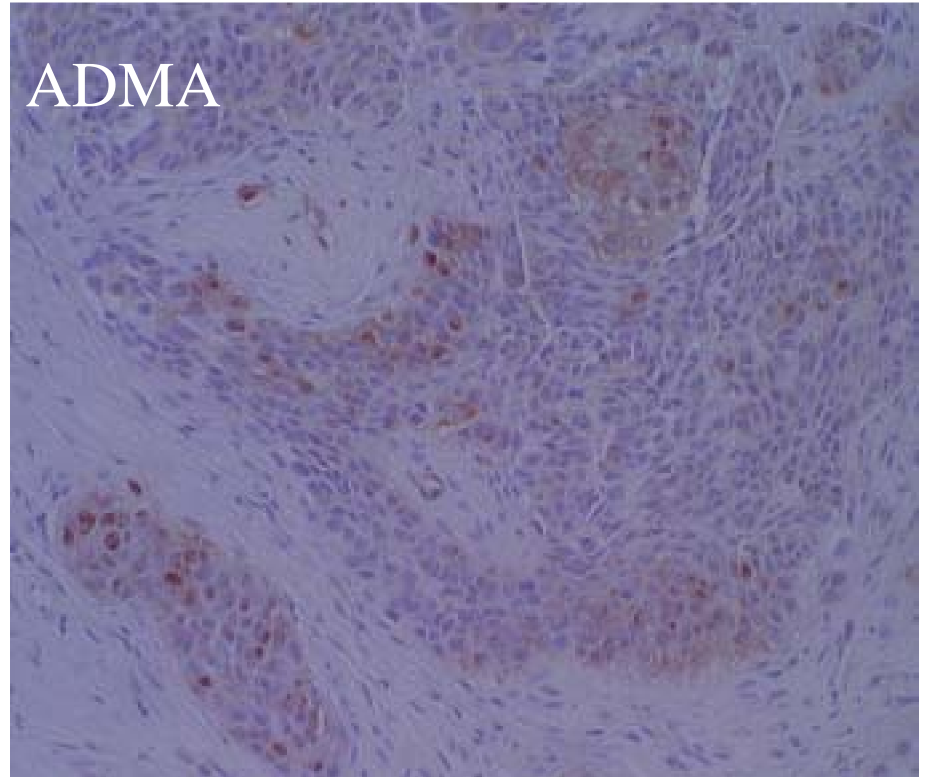
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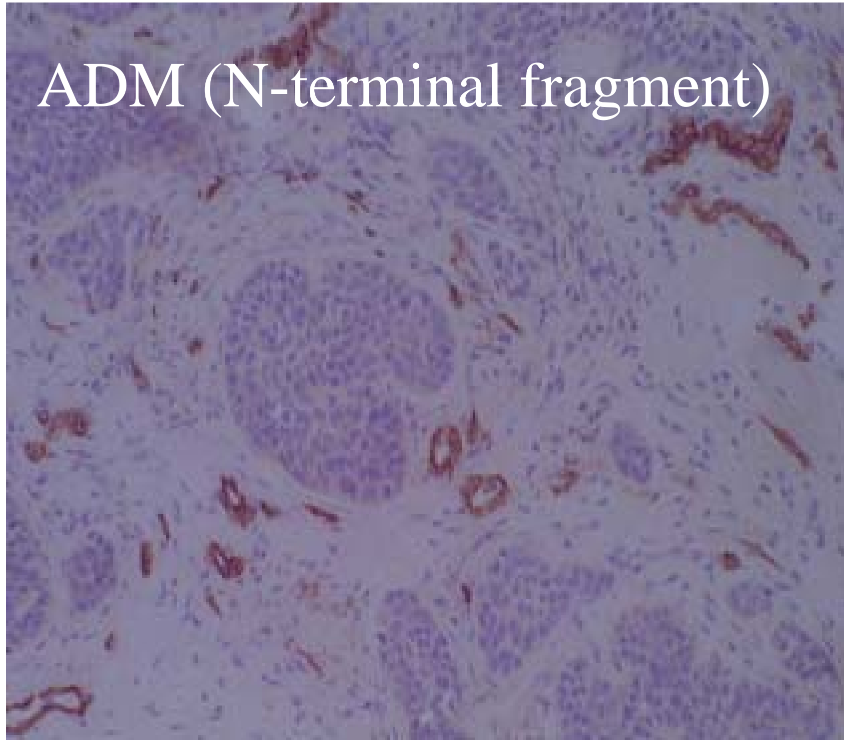
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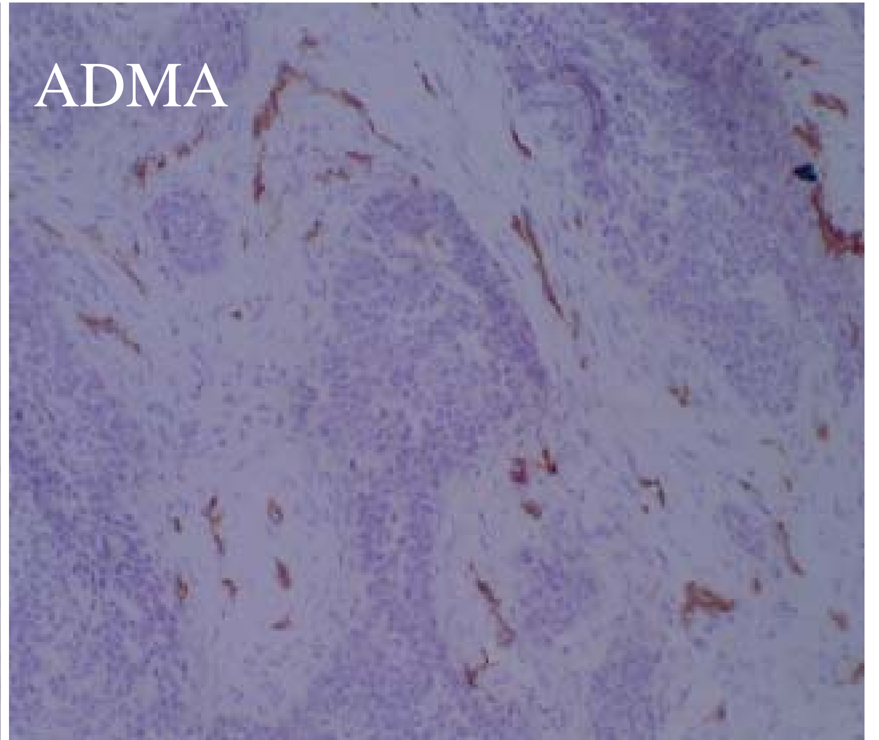
ADMA