Suppression of tumor growth by intra-muscular transfer of naked DNA encoding adrenomedullin antagonist

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Running title: Cancer gene therapy by adrenomedullin antagonist

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ABSTRACT

We have recently reported that the intra-tumoral injection of adrenomedullin (AM) antagonist (AMA; AM(22-52)) peptides significantly reduced the *in vivo* growth of a pancreatic cancer cell line in SCID mice. In the present study, we examined the effects of intra-tumoral and intra-muscular transfers of naked DNA encoding AMA on the *in vivo* growth of cancer cell lines. We demonstrate that these treatments induce the regression of a pancreatic cancer cell line and a breast cancer cell line inoculated in SCID mice. Furthermore CD31-positive cells disappear completely from tumor tissues, following treatment, indicating that neo-vascularisation is entirely inhibited. These results suggest that the intra-tumoral or intra-muscular transfer of naked DNA encoding AMA might be a promising alternative modality for treating human cancers.

INTRODUCTION

We have recently reported that the intra-tumoral injection of adrenomedullin (AM) antagonist (AMA) peptides induced the regression of pancreatic cancer cells in SCID mice [1]. Furthermore, we demonstrated that the mean diameter of blood vessels in the tumor tissues treated with AMA was smaller than that in the tumor tissues treated with AM [1]. Iimuro et al. recently showed that continuous infusion of AMA peptides suppressed the capillary development and growth of "sarcoma 1080" tumors [2]. These results suggest that AMA arrests the *in vivo* growth of cancer cells through the suppression of angiogenesis. A recent report states that AM gene transfer induced angiogenesis in a rabbit model and that AM enhanced the angiogenic potency of bone marrow transplantation in a rat model [3], indicating that AM is implicated in the angiogenic process *in vivo*, in accordance with our results.

Recent progress in gene therapy research demonstrates that naked DNA can be delivered to cells *in vivo* so that its gene will be expressed [4, 5, 6]. Since gene therapy using viral delivery systems has several aspects which still need to be resolved, like the evasion from helper virus contamination and avoidance of immunogenicity of the viral particle itself, gene therapy using non-viral delivery systems is now being anticipated [7]. However, the non-viral delivery systems, regrettably, also have several controversial

features, such as low efficiency in gene delivery and low immunogenicity of naked DNA [6, 7].

A recent report demonstrated that intra-muscular injection of naked DNA encoding the endostatin gene succeeded in suppressing both primary tumors and development of metastatic lesions [8]. This result prompted us to hypothesize that intra-muscular and intra-tumoral transfer of naked DNA encoding AMA might be a promising anti-angiogenic therapeutic approach. Here, we examined the efficacy of AMA in gene therapy for cancer, using naked DNA. We first examined the effects of intra-tumoral transfer of naked DNA encoding AMA on the *in vivo* growth of pancreatic cancer cells. Then we examined the effects of intra-muscular transfer of naked DNA encoding AMA on the *in vivo* growth of pancreatic and breast cancer cells.

MATERIALS AND METHODS

Cells and culture methods:

Pancreatic ductal adenocarcinoma cell lines (PCI-35 and PCI-43) were kindly supplied by Dr. Hiroshi Ishikura (The First Department of Pathology, Hokkaido University School of Medicine). BxPC3 (pancreatic cancer), MiaPaCa2 (pancreatic cancer), AGS (gastric cancer), HepG2 (hepatoma), A549 (lung cancer), MDA-MB-231 (breast cancer), HCT-116 (colon cancer) and HeLa cells were supplied by Dr. Jun-ichi Hamada (Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University). These cell lines were maintained in DMEM supplemented with 10 % fetal bovine serum (FBS). According to the previous definition of tumor hypoxia[13] (median pO_2 < 10 mmHg, approximately 1.25 %), the cells in the hypoxic group were incubated at 1 % O_2 in a hypoxic chamber gassed with 94 % N_2 and 5 % CO_2 (Wakenyaku Co. Ltd., Tokyo). As the tumor cells are exposed to hypoxia as well as glucose-deprived conditions, we cultured the cells under four different conditions, namely, under normoxic and normal glucose conditions (NN), hypoxic and normal glucose conditions (HN), normoxic and low glucose conditions (NL), and hypoxic and low glucose conditions (HL). Glucose-deprived cultures were kept in glucose-free DMEM supplemented with 10% FBS (final concentration of glucose: 13 mg/dl).

Real-time PCR:

Real-time PCR was done using the following primers: CCTTTTGAGGGCGACCTCCAAG and CTGGATGACGATGTCTGCGT for AMA, and GCTCCTCCTGAGCGCAAGT and TCGTCATACTCCTGCTTGCTGAT for β-actin.

Total RNA was extracted using Trizol® Reagent (Invitrogen) and cDNA was synthesized by the use of M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Each cDNA (10 ng) was amplified in triplicates with the use of Quantitect^{TM} SYBR® Green PCR assay kit (QIAGEN) and then detected on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). The conditions for real-time PCR were as follows: 50 ^{o}C for 2 min and 95 ^{o}C for 15 min for the initial incubation; 94 ^{o}C for 15 sec for denaturing, 60 ^{o}C for 30 sec for annealing, 72 ^{o}C for 1 min for extension, for 40 cycles; and the last dissociation step was 95 ^{o}C for 15 sec and 60 ^{o}C for 15 sec and 95 ^{o}C for 15 sec. β-actin was used to standardize the total amount of cDNA in the real-time PCR. Relative mRNA levels were determined by comparing the PCR cycle thresholds of the cDNA of the gene of interest to that of β-actin.

Expression vector:

Full length human adrenomedullin precursor cDNA was isolated by RT-PCR using the total RNA from hypoxia-treated K562 cells. Adrenomedullin antagonist (AMA) expression vectors were generated by PCR combined with site-directed mutagenesis. AMA214 lacked 27-115 amino acids, and the threonine at the 116 amino acid position was changed to serine. AMA224 lacked 95-115 amino acids and the threonine at the 116 amino acid position was changed to serine, which retained the N-20 terminal peptide. AMA214 and AMA224 cDNAs were cloned respectively into p3xFLAG-CMV-14 (SIGMA), both of which carried three repeats of FLAG-tag at the C-terminus of the AMA. The plasmids used for transfection were p3Xflag AMA214, p3Xflag AMA 224 and p3Xflag control vector.

In vivo treatment:

Five million cells were inoculated subcutaneously into the right flanks of SCID mice (n=5, in each group). After 9 and 5 days of inoculation of PCI-43 and MDA-MB231 cells, respectively, 500μg of p3Xflag AMA214, p3Xflag AMA 224 or p3Xflag control vector were inoculated into tumor tissues or left femoral muscles. To minimize the doses of naked DNA, we used the ultrasound-microbubble method.

Different amounts of expression vectors mixed with microbubble (Optison, Mallinckrodt, St. Louis, Mo., USA) were applied to the tumors. For ultrasound irradiation, we used a sonication machine (Sonitron 2000, Nepa Gene, Tokyo, Japan) and probe (3 mm diameter, Nepa Gene). The skin on the target tumors was smeared with echo gel and the sterilized probe was put in contact with the skin, delivering the expression vectors. Tumor size was observed every three days up to 3 weeks after inoculation.

RESULTS

We previously demonstrated that the expression of AM mRNA was enhanced under hypoxia, when compared to normoxia [1]. Since solid tumor tissues are under glucose-deprived conditions as well as hypoxic conditions, we examined the expression levels of AM mRNA under hypoxic and/or glucose-deprived conditions. Hypoxia alone or glucose-deprivation alone enhanced the expression of AM mRNA in several cancer cell lines; moreover, hypoxia plus glucose-deprivation additively, and often synergistically, enhanced the expression of AMA mRNA in pancreatic cancer cell lines as well as in non-pancreatic cancer cell lines (Fig. 1a and 1b). The expression of AM mRNA was increased more than ten-fold under hypoxic and glucose-deprived conditions than under normoxic and normal glucose conditions. These results suggest that AM mRNA expression is regulated by glucose concentration and its sensor/s, as well as hypoxia-inducible factor-1 (HIF-1). We are now focusing on identifying how glucose concentration regulates AM mRNA expression. Our preliminary data suggest that AMPK is implicated in the regulation of AM mRNA expression by glucose concentration (data not shown), in accordance with previous reports [9, 10] and that stability of AM mRNA may not be involved in this regulation, in contrast to a previous report [11].

Tumor cells were implanted subcutaneously in the back of SCID mice and grown to ~5 mm in diameter. On day 9, 500 μg naked DNA encoding AMA was injected into the tumor tissues. Twenty-one days after treatment, the tumors treated with only one injection of naked DNA encoding AMA regressed, whereas those treated with the control vector continued growing (Fig. 2a). In some experiments, the tumor tissues were removed on day 16 and stained with anti-CD31 antibody. A number of CD31-positive cells were observed in the tumor tissues treated with the control vector; however, no CD31-positive cells were observed in the tumor tissues treated with naked DNA encoding AMA (Fig. 3a).

We next examined the effects of intra-muscular injection of naked DNA encoding AMA into a femoral muscle on the growth of pancreatic cancer cells implanted *s.c.* in the back of SCID mice. The intra-muscular injection was effective against the growth of pancreatic cancer cells implanted in the distal site (Fig. 2b). Immunohistochemical examination of the tumor tissues demonstrated that this treatment completely eliminated CD31-positive cells from the tumor tissues (Fig. 3b). To determine whether the AMA being secreted in the femoral muscle was reaching the tumor tissues, we stained tumour sections with anti-Flag antibody. Both the tumor tissues and neighboring normal tissues, in mice treated with AMA, stained positively with anti-Flag antibody (Fig. 3c).

We finally examined the effects of naked DNA encoding AMA on the *in vivo* growth of a breast cancer cell line. Intra-muscular injection of naked DNA encoding AMA was effective against the growth of breast cancer cells implanted in the distal site (Fig. 2c). In this experiment, we used the ultrasound-microbubble method to minimize the doses of naked DNA. Treatment with 25 μg of naked DNA suppressed tumor growth to a similar level as that observed by injection with 500 μg of naked DNA. Immunohistochemical examination of the tumor tissues confirmed that the intra-muscular injection of naked DNA encoding AMA also eliminated CD31-positive cells completely from the tumor tissues (Fig. 3d).

DISCUSSION

In this study, we first demonstrate that AM expression in cancer cell lines is additively, and very often synergistically, enhanced under hypoxic and glucose-deprived conditions. Several reports, including our previous study, affirm that AM expression increases under hypoxia. As tumor tissues are exposed to glucose-deprivation as well as hypoxia because of inadequate blood supply, we examined AM expression under hypoxic conditions, glucose-deprived conditions, as well as hypoxic plus glucose-deprived conditions. The results we obtained strongly suggest that AM is probably expressed at high levels in various tumor tissues, as these exist in low oxygen and low glucose microenvironments. We are currently examining possible mechanisms involved in the elevation of AM expression under hypoxic and glucose-deprived conditions. Our preliminary experiments suggest that ATF-4 might play a role in the enhancement of AM expression (data not shown), however this needs further confirmation.

In this paper, we also report that a single intra-tumoral or intra-muscular injection of naked DNA encoding AMA almost completely suppresses the growth of a pancreatic cancer cell line and a breast cancer cell line. Furthermore, we demonstrate that CD31-positive cells completely disappear in tumor tissues treated with naked DNA

encoding AMA. These results strongly imply that intra-muscular and intra-tumoral transfers of naked DNA encoding AMA might be effective for cancer therapy through the suppression of neo-vascularization, and that this strategy might be one of the alternative modalities for solid tumors. Previously we reported that daily intra-tumoral injections of adrenomedullin antagonist peptides for 10 days suppressed tumor formation through the suppression of neo-vascularization, which is consistent with this study. However, in the previous study only large blood vessel formation was suppressed, in contrast to the present study demonstrating that blood vessel formation was almost completely suppressed in the tumor tissues treated by naked DNA encoding AMA. The variation between the previous and the present study may be explained by the different effects of intermittent and continuous exposures of cancer cells to AMA. Moreover, Blezinger *et al*. also showed that intramuscular administration of naked DNA encoding the secreted form of endostatin suppressed the growth of primary tumors and metastatic tumors, through the sustained production and secretion of endostatin, which is in accordance with our study. Immunohistochemical staining with Flag–tag in the tumor tissues suggests that secreted AMA first reached the tumor tissues and then suppressed neo-vascularization.

The use of skeletal muscle as a target organ for gene transfer is a rapidly developing

area of gene therapy. Several factors make skeletal muscle an attractive route for gene therapy: 1) The tissue is abundant, making up about 40% of the body weight of an average adult; 2) Skeletal muscle can be used for most of the delivery systems currently used for gene transfer; 3) There is no significant replacement in skeletal muscle tissue, so that transgene expression can thus persist for relatively long periods [5]. While viral vectors are commonly used for gene transfer into skeletal muscle, naked DNA is now receiving considerable attention for gene transfer into muscle tissue, due to its safety, simplicity and reliability [4, 6, 7, 12]. However, as the transfection efficiency of naked DNA by intra-muscular injection is relatively low, this method would not suffice for the treatment of systemic diseases. In our study, ultrasound microbubbles-mediated gene transfer allowed us to reduce the amount of naked DNA from 500 μg/mouse to 25 μg/mouse, confirming recent reports, which state that ultrasound microbubbles-mediated gene transfer improves transfection efficiency and facilitates local gene expression in various tissues, such as cardiac muscle [13], skeletal muscle [14], carotid artery [15], and the fetus [16].

However, there are still several hurdles which must be overcome before we apply gene therapy using naked DNA to human clinical trials. The low transfection efficiency of naked DNA remains an unsolved issue. Following our murine model, we

would have to inject about 15-60 mg naked DNA into human muscle in order to obtain a significant anti-tumor effect, notwithstanding the use of ultrasound microbubbles-mediated gene transfer. Another very important fact to consider is the multifunctional characteristics of AM. AM was first found as a vasodilator [17], thus AMA might induce vascular contraction. Vasodilatation is caused by two mechanisms: by relaxation of vascular smooth muscle cells expressing CRLR and RAMP1 and by NO production by endothelial cells expressing CRLR and RAMP2 or RAMP3. To reduce this side effect, we are now designing appropriate peptides that can bind more efficiently to CRLR/RAMP2/3 rather than to CRLR/RAMP-1.

Collectively, our results suggest that the intra-tumoral or intra-muscular transfer of naked DNA encoding AMA might be a very promising tool for use in cancer treatment.

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FIGURE LEGENDS

Figure 1. AM mRNA expression in several pancreatic cancer cell lines under different culture conditions. A. Pancreatic cancer cell lines B. Cancer cell lines from other origins. Mean ± S.D. is shown.

According to the previous definition of tumor hypoxia (median pO_2 < 10 mmHg, approximately 1.25%), the cells were incubated at 1 % O_2 in a hypoxic chamber gassed with 1% O_2, 94 % N_2 and 5 % CO_2 (Wakenyaku Co. Ltd., Tokyo) and /or in glucose-free DMEM supplemented with 10% FBS (the final glucose concentration in glucose-free medium was determined to be 13 mg/dl). The cells were cultured for 16 hours under the following four conditions; N-N: normoxia and normal glucose concentration; N-L: normoxia and low glucose concentration; H-N: hypoxia and normal glucose concentration; H-L: hypoxia and low glucose concentration. Following mRNA extraction, cDNA was synthesised. Each cDNA (10 ng) was amplified in triplicates with the use of the SYBR-Green PCR assay kit and then detected on an ABI PRISM® 7900HT Sequence Detection System. β-actin mRNA was used to standardize the total amount of cDNA. The primers used were as follows: AMA, CCTTTTGAGGGCGACCTCCAAG and CTGGATGACGATGTCTGCGT; and β-actin, GCTCCTCCTGAGCGCAAGT and TCGTCATACTCCTGCTTGCTGAT. Relative

mRNA levels were determined by comparing the PCR cycle thresholds of the cDNA of the gene of interest to those of β-actin.

Figure 2. Tumor growth following injection of AMA expression vectors

A. Growth of PCI-43 (pancreatic cancer) injected with vectors intra-tumorally. B. Growth of PCI-43 injected with vectors intra-muscularly. C. Growth of MBA-MD231 (breast cancer) injected with vectors intra-muscularly. Mean tumor volume ± S.D. is shown for the 3 plots.

Full length human adrenomedullin precursor cDNA was isolated by RT-PCR using total RNA from hypoxia-treated K562 cells. Adrenomedullin antagonist (AMA) expression vectors were generated by PCR combined with site-directed mutagenesis. AMA214 lacked 27-115 amino acids, and the threonine at the 116 amino acid position was changed to serine. AMA224 lacked 95-115 amino acids and the threonine at the 116 amino acid position was changed to serine, which retained the N-20 terminal peptide. AMA214 and AMA224 cDNAs were cloned respectively into p3xFLAG-CMV-14 (SIGMA), both of which carried three repeats of FLAG-tag at the C-terminus of the AMA. The plasmids used for transfection were p3Xflag AMA214, p3Xflag AMA 224 and p3Xflag control vector.

Figure 3. CD31-positive cells in the tumor tissues.

A. Immunohistochemical staining of pancreatic cancer tissues treated with an intra-tumoral injection of AMA expression vector using an anti-CD31 antibody. B. Immunohistochemical staining of pancreatic cancer tissues treated with an intra-muscular injection of AMA expression vector using an anti-CD31 antibody. C. Immunohistochemical staining of pancreatic cancer tissues using an anti-Flag antibody to stain the adrenomedullin antagonist. D. Immunohistochemical staining of breast cancer tissues treated with an intra-muscular injection of AMA expression vector using an anti-CD31 antibody. Expressions of CD31 and adrenomedullin antagonist were analyzed by immunohistochemical staining using the streptavidin-biotin technique (Histofine SAB-PO kit, Nichirei, Tokyo). Snap-frozen tissue specimens were used for the analysis. The tissue sections were pre-incubated for 30 minutes with PBS containing 1% bovine serum albumin and 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-adrenomedullin 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 with the biotin-conjugated anti-rat secondary 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. Immunocytochemical staining for AMA was performed, with the use of an anti-Flag antibody (Peninsula Laboratories INC., San Carlos, CA) and a Histofine simple staining PO kit (Nichirei, Tokyo), according to the manufacturer's instructions.

Fig. 1a

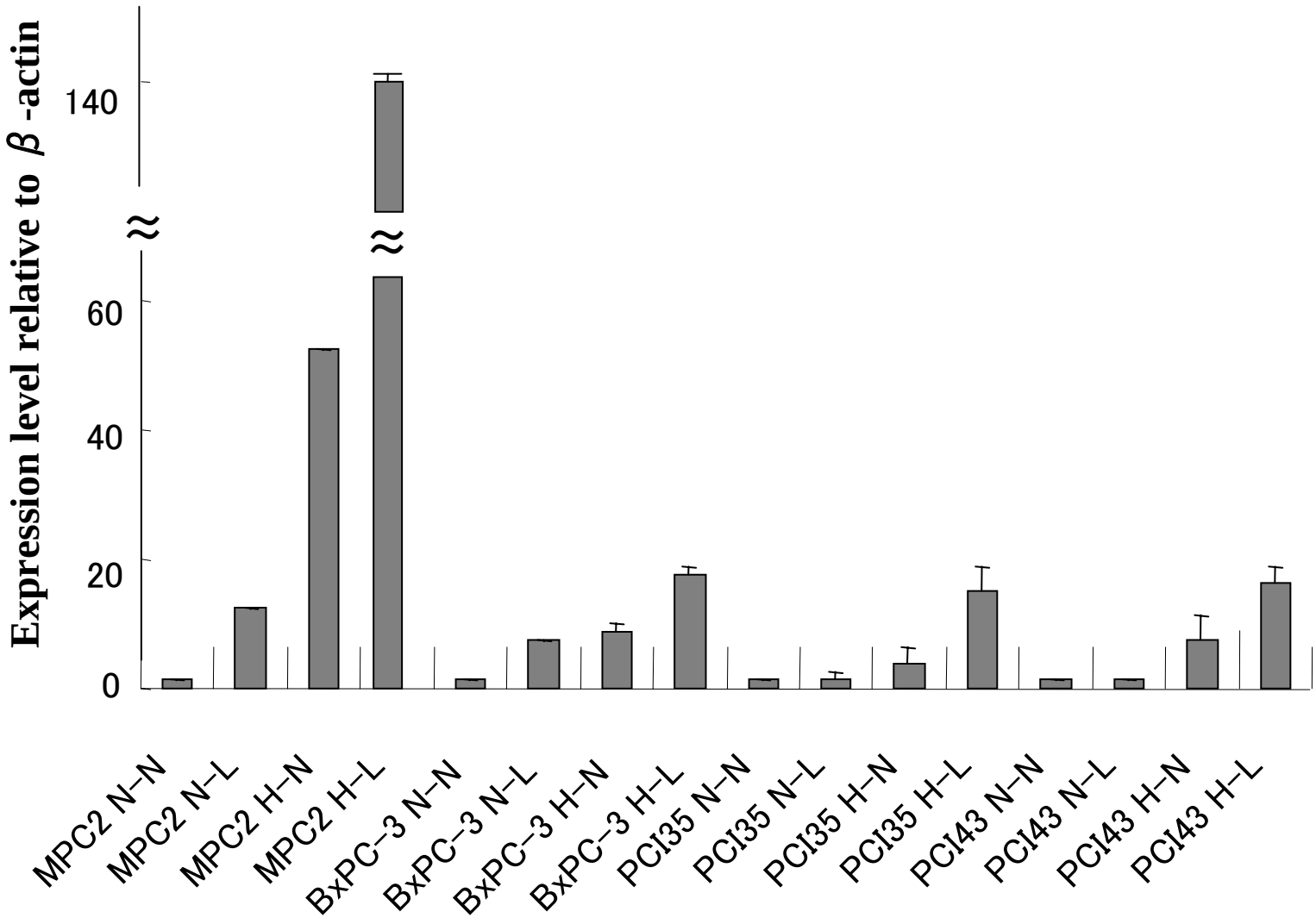
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Fig. 1b

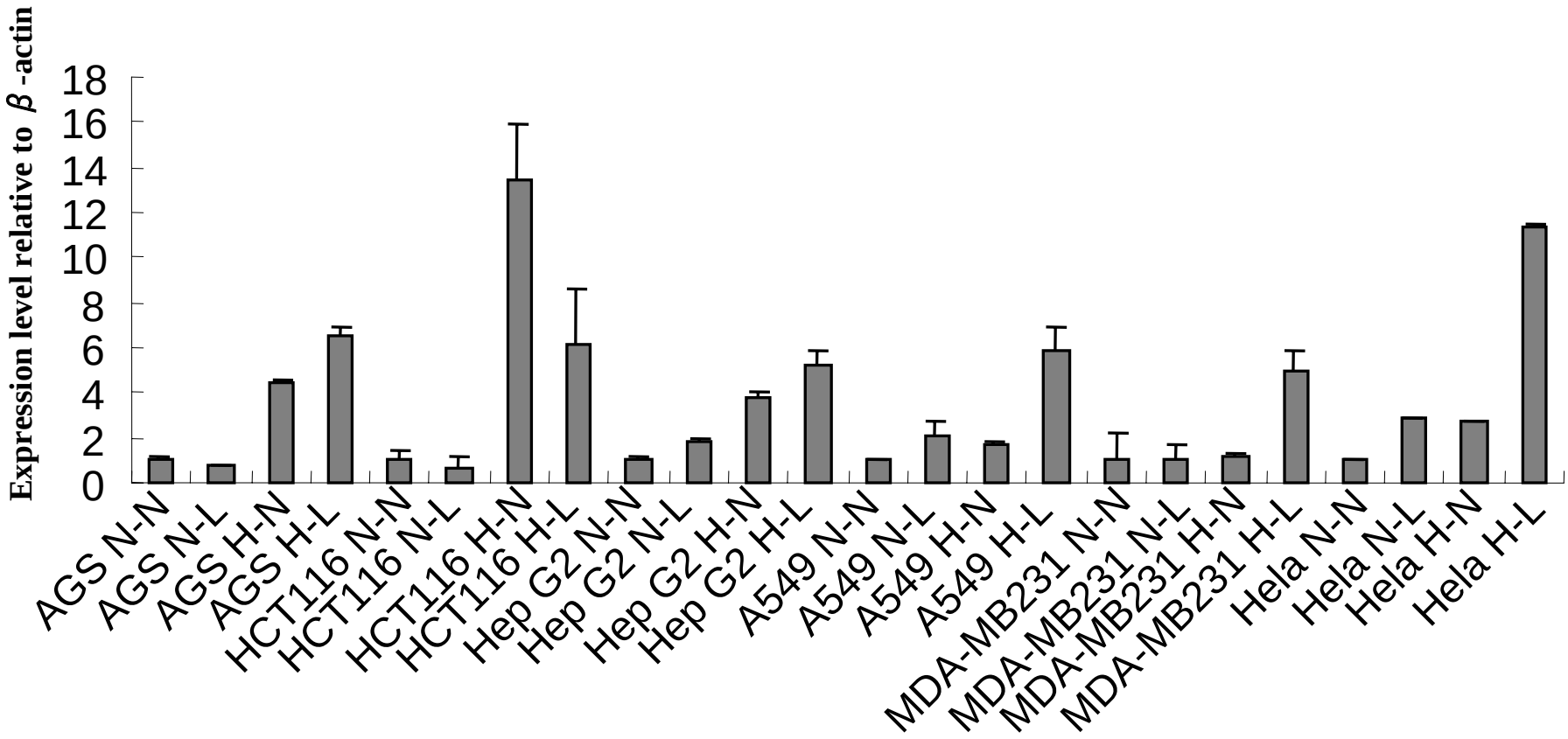
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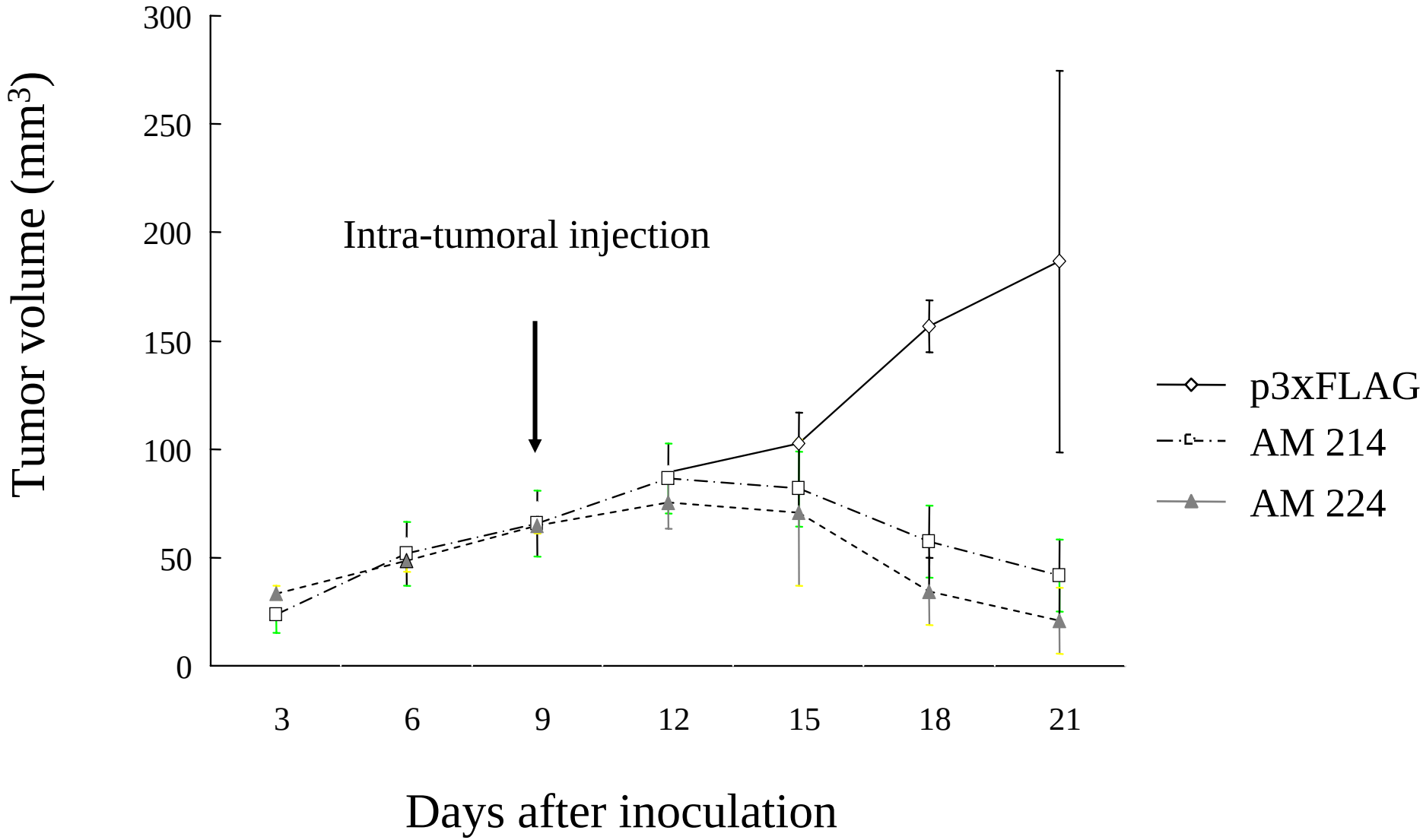
Fig. 2a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200
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18
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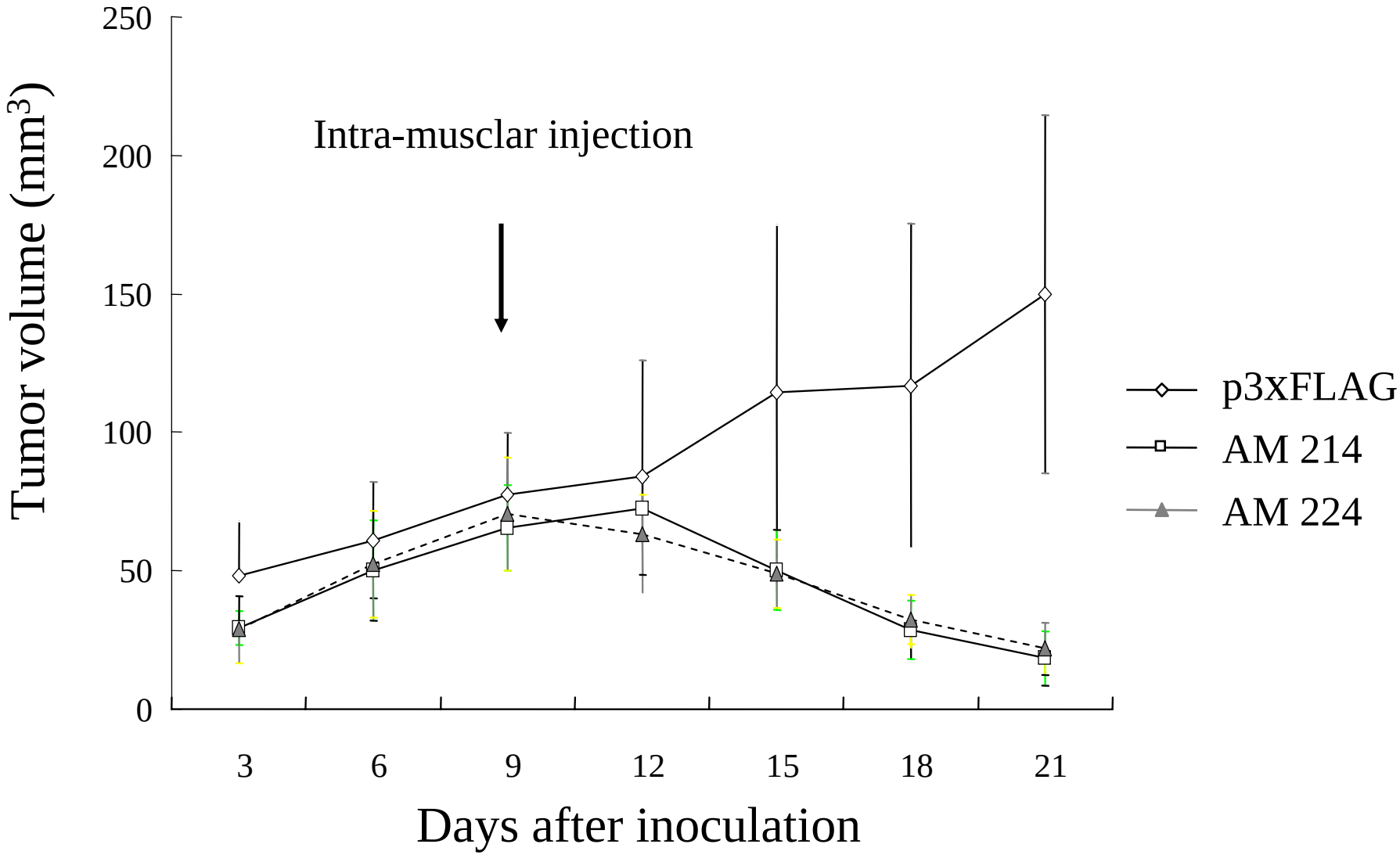
Days after inoculation
p3xFLAG
AM 214
AM 224

Fig. 2b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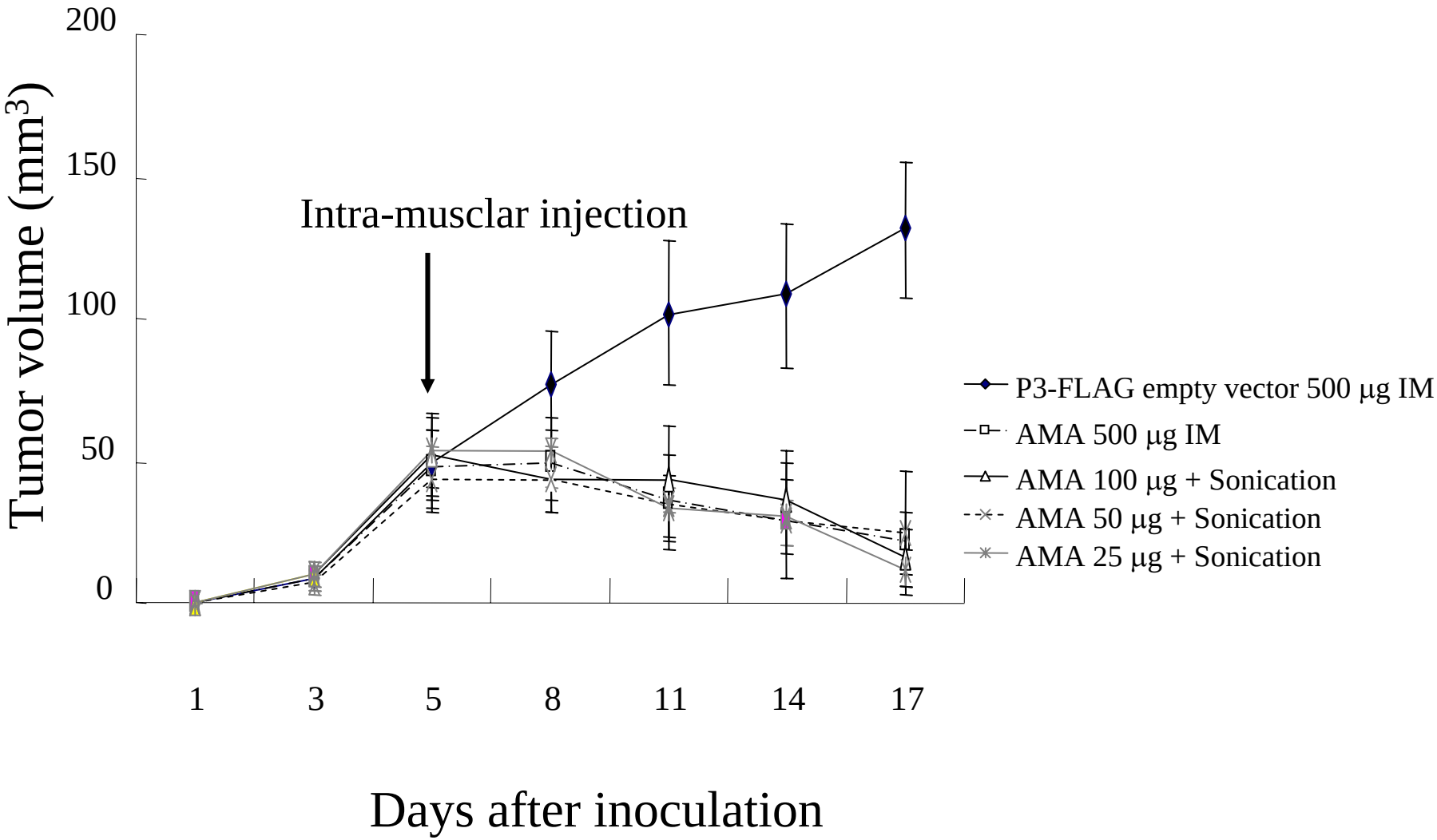
AM 214
AM 224

Fig. 2c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3
5
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11
14
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Days after inoculation
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AMA 500 μg IM
AMA 100 μg + Sonication
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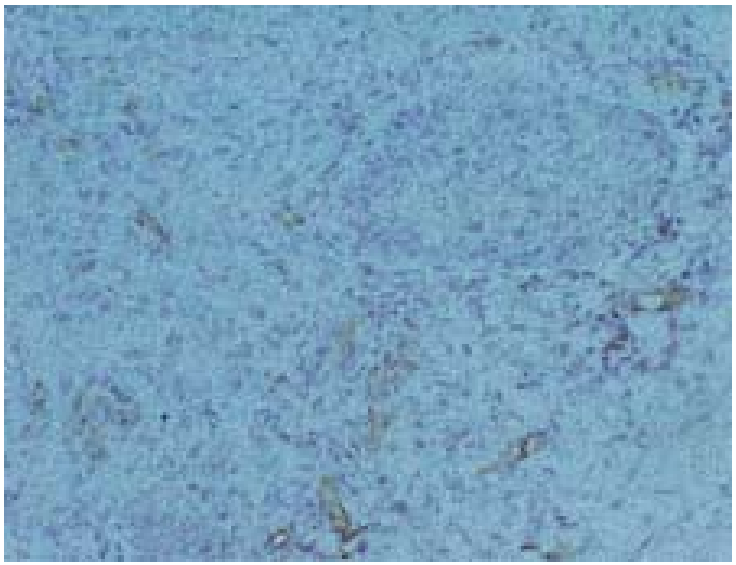
AMA 25 μg + Sonication

Fig. 3a

p3xFLAG-CMV-vector



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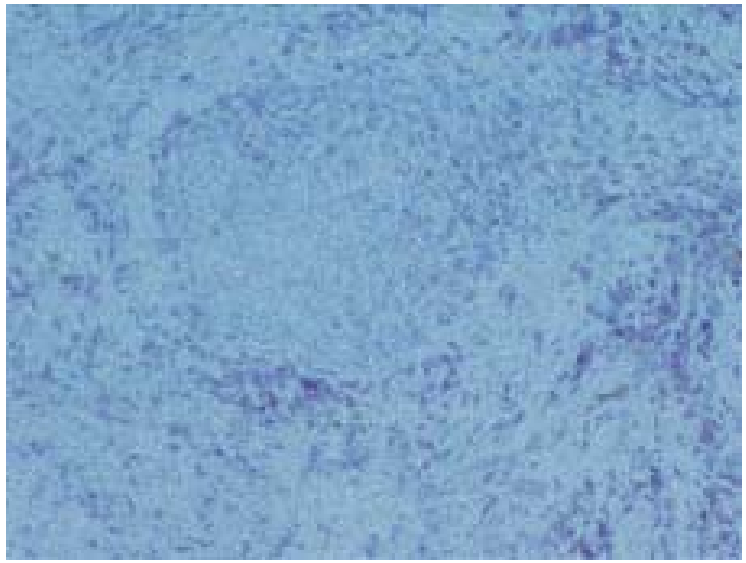

Anti-CD31 ab

Fig. 3b

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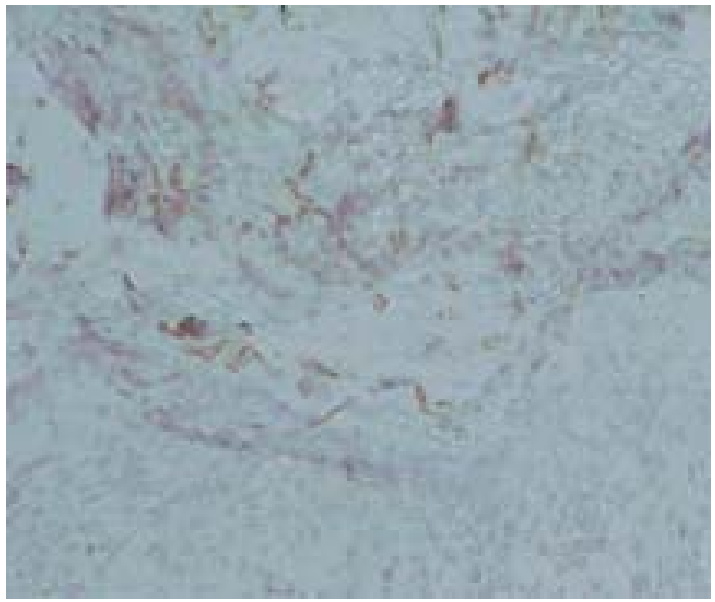

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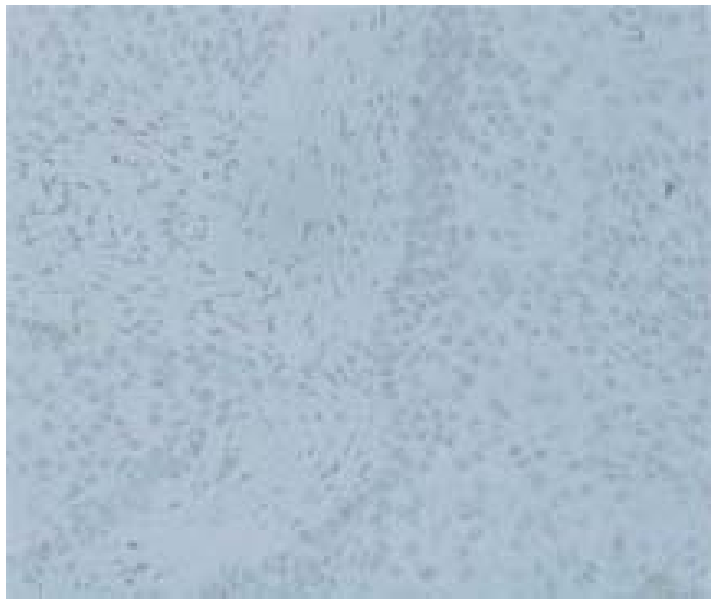
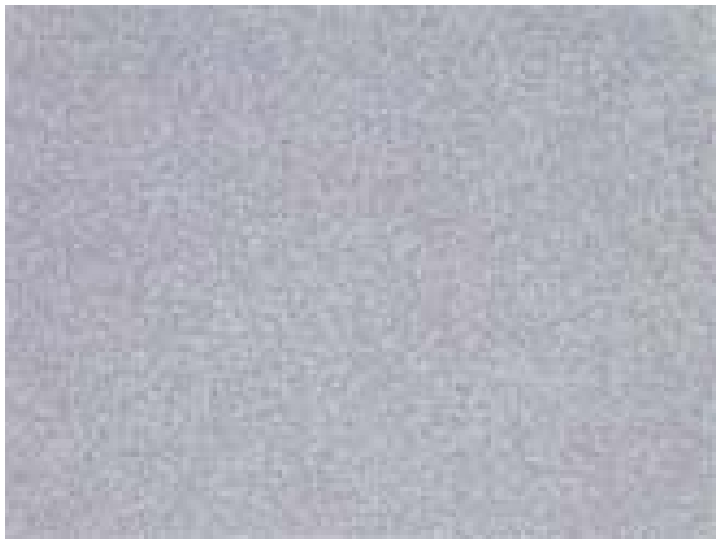


Anti-CD31 ab

Fig. 3c

p3xFLAG-CMV-vector

p3xFLAG-CMV-AM 214

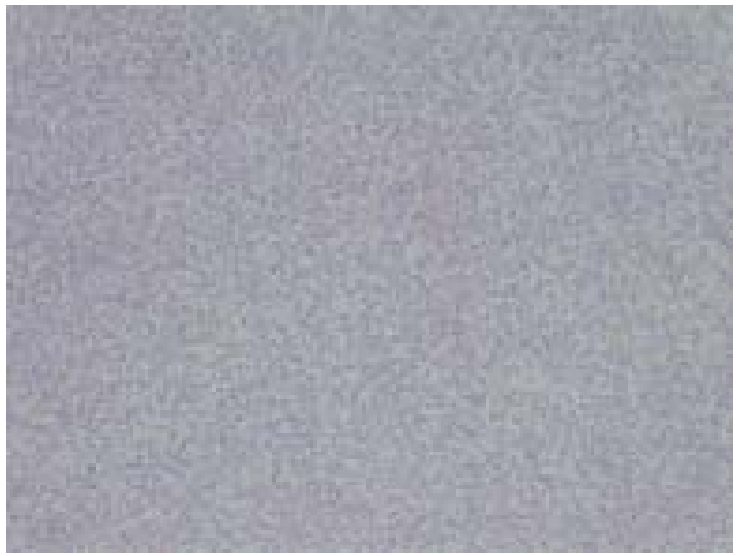


Anti-Flag Ab

Fig. 3d

p3xFLAG-CMV-vector

p3xFLAG-CMV-AM 214



Anti-CD31 ab