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Single domain antibody against carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) inhibits proliferation, migration, invasion and angiogenesis of pancreatic cancer cells

Tsai-Mu Cheng [a,b,f,g], Yanal M. Murad [b,f,g], Chia-Ching Chang [a,c,*], Ming-Chi Yang [d], Toya Nath Baral [b], Aaron Cowan [b], Shin-Hua Tseng [a], Andrew Wong [b], Roger MacKenzie [b], Dar-Bin Shieh [e], Jianbing Zhang [b,*]

[a] Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 30050, Taiwan, ROC
[b] Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, ON, Canada
[c] Institute of Physics, Academia Sinica, Taipei 10529, Taiwan, ROC
[d] Life Science Group, Scientific Research Division, National Synchrotron Radiation Research Center, Hsinchu, Taiwan, ROC
[e] Institute of Oral Medicine, College of Medicine and Hospital, National Cheng Kung University, Taiwan 70101

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Abstract Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is over-expressed in pancreatic cancer cells, and it is associated with the progression of pancreatic cancer. We tested a single domain antibody (sdAb) targeting CEACAM6, 2A3, which was isolated previously from a llama immune library, and an Fc conjugated version of this sdAb, to determine how they affect the pancreatic cancer cell line BxPC3. We also compared the effects of the antibodies to gemcitabine. Gemcitabine and 2A3 slowed down cancer cell proliferation. However, only 2A3 retarded cancer cell invasion, angiogenesis within the cancer mass and BxPC3 cell MMP-9 activity, three features important for tumour growth and metas-

Abbreviations: MMP-2; Matrix metalloproteinase-2; MMP-9; Matrix metalloproteinase-9; Fc; fragment crystallizable; IC_{50}s; Half maximal inhibitory concentration; DMEM; Dulbecco's Modified Eagle Medium; PET; Positron emission tomography; FACS; Fluorescence-activated cell sorting; IgG; Immunoglobulin G; VTI1-Fc; Verotoxin1 sdAb Fc fusion; DAPI; 4′,6-diamidino-2-phenylindole

* *Corresponding authors:* Address: Department of Biological Science and Technology, National Chiao Tung University, 75 Bo-Ai Street, Hsinchu, Taiwan, ROC (C.-C. Chang). Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Dr., Ottawa, ON, Canada K1A 0R6. Tel.: +1 613 998 3373; fax: +1 613 952 9092 (J. Zhang).
E-mail addresses: ccchang01@faculty.nctu.edu.tw (C.-C. Chang), Jianbing.Zhang@nrc-cnrc.gc.ca (J. Zhang).
[f] T.M. Cheng and Y.M. Murad contributed equally to this work.
[g] Current address. at: Ph.D. Program for Translational Medicine, College of Medicine and Technology, Taipei Medical University, Taiwan, ROC (T.-M. Cheng), Advanced Medical Research Institute of Canada (Y.M. Murad).

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tasis. The IC_{50}s for 2A3, 2A3-Fc and gemcitabine were determined as 6.5 μM, 8 μM and 12 nM, respectively. While the 2A3 antibody inhibited MMP-9 activity by 33% compared to non-treated control cells, gemcitabine failed to inhibit MMP-9 activity. Moreover, 2A3 and 2A3-Fc inhibited invasion of BxPC3 by 73% compared to non-treated cells. When conditioned media that were produced using 2A3- or 2A3-Fc-treated BxPC3 cells were used in a capillary formation assay, the capillary length was reduced by 21% and 49%, respectively. Therefore 2A3 is an ideal candidate for treating tumours that over-express CEACAM6.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer death and its five-year survival rate is less than 5%.[1] The majority of pancreatic malignancies are pancreatic ductal adenocarcinomas (PDA).[2] Most pancreatic cancer patients present themselves with a locally advanced or metastatic disease that cannot be treated by employing curative resection due to highly aggressive local and regional invasion along with early metastasis. Gemcitabine and erlotinib are the only agents that are approved for treating pancreatic cancer. However, both drugs induce a poor response in patients and their use can result in patients developing multiple drug resistance.[2,3] Therefore, discovering new strategies for treating this disease is critically important.

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a member of the carcinoembryonic antigen (CEA) family of glycosylphosphatidylinositol anchored proteins. It is a tumour-associated antigen over-expressed in many malignant cells, such as pancreatic, lung and colon cancer cells. CEACAM6 over-expression in pancreatic cancer cells can be as high as 20- to 25-fold of the normal pancreatic cells.[4,5] Elevated CEACAM6 expression is associated with pancreatic cancer cell adhesion, migration, invasion, metastasis, angiogenesis and drug resistance.[6–9] Targeting CEACAM6 to inhibit these processes is logical since CEACAM6 over-expression is associated with many essential tumour cells functions.[6,10] CEACAM6-specific RNA interference (RNAi) decreases cancer cell proliferation, metastasis and angiogenesis in pancreatic cancer.[11] Monoclonal antibodies that target CEACAM6 block cell migration, adhesion to endothelial cells and colonic cancer invasion.[12]

In cancer, increasing extracellular proteolysis promotes cancer growth, tissue invasion and metastasis.[13] A characteristic of PDA is the increase Matrix metalloproteinases (MMPs) activity, particularly MMP-2 and MMP-9.[14] The MMP-2 and MMP-9 activity is correlated with the degradation of extacellular basement membrane, metastasis, invasion and growth in pancreatic cancer.[13,15] It was also reported that reducing the secretion and activity of MMP-2 and MMP-9 inhibited cell invasion ability in pancreatic cancer cells.[16]

Previously, we isolated and cloned an single domain antibody (sdAb) (2A3), from an immune llama library.[17] This sdAb binds to CEACAM6 on the cell surface. For eventual *in vivo* studies, we created a heavy chain antibody (HCAb) by fusing this sdAb to an fragment crystallisable (Fc) fragment (Fig. 1A). The HCAb version is preferred due to its long half life in the circulation, and its ability in inducing antibody dependent cellular cytotoxicity and complement dependent cytotoxicity. In this study, we report on using the 2A3 antibody to inhibit BxPC3 tumour cell proliferation and invasion, and BxPC3 tumour cell-induced angiogenesis. We also compare the effects of the 2A3 antibody with gemcitabine, which is the drug that is used for standard pancreatic cancer treatment.

2. Material and methods

2.1. Protein expression and purification

The His tagged 2A3 sdAb was purified by employing metal affinity chromatography as described.[18] The 2A3 HCAb was cloned into a mammalian expression vector pTT5 and the protein was expressed and purified as described.[19,20] Briefly, 293-6E cells were grown in 293-SFM (Invitrogen, Burlington, ON) and then transfected with the pTT5 constructs using PEI as a transfection agent. The cell culture medium was harvested after 5 d and 2A3-Fc was purified by using a Protein G column (GE Healthcare, Piscataway, NJ).

2.2. Cell culture

BxPC3 human PDA cells and Panc1 cells were obtained from ATCC (Manassas, VA). The BxPC3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) 1640 and Panc1 cells were cultured in DMEM, both were supplemented with 10% complement inactivated foetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs) were obtained from Invitrogen and were cultured in Medium 200 (Invitrogen) supplemented with 15% FBS and 2% low serum growth supplement (Invitrogen). All of the cell culture media contained 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified incubator with a 5% CO_2 supplemented atmosphere at 37 °C.

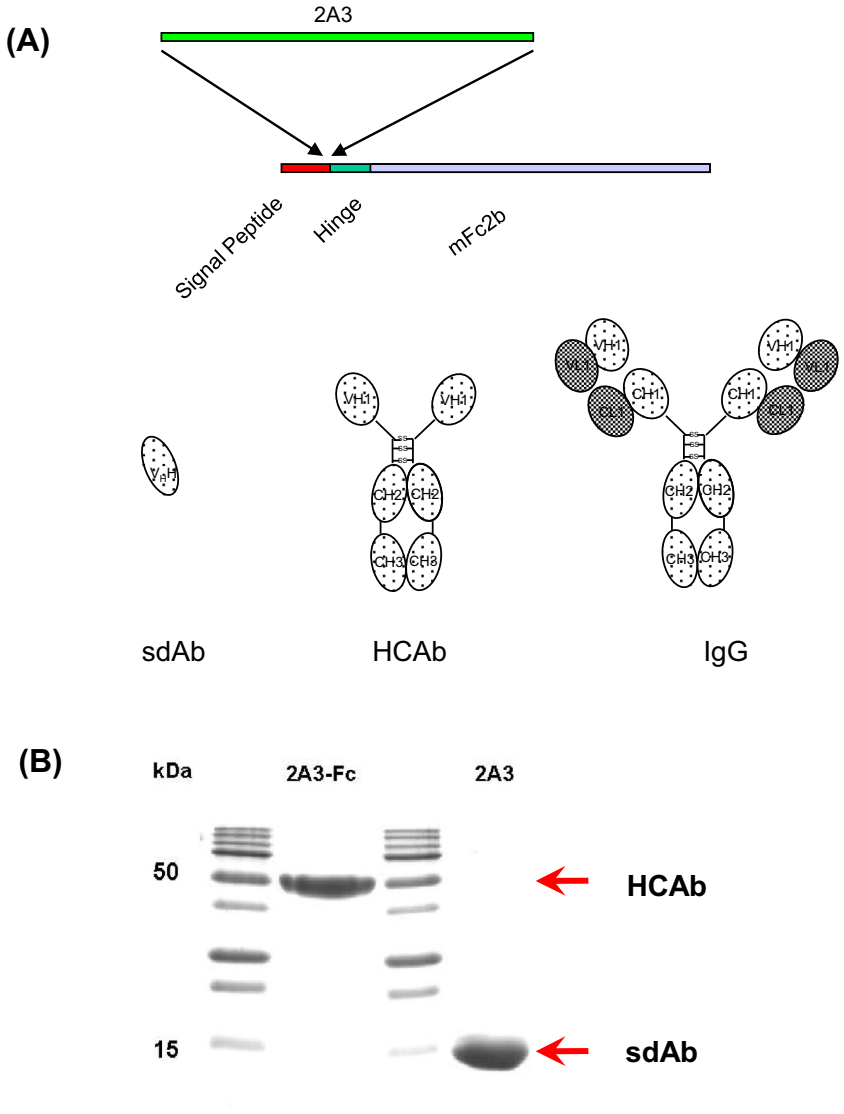

Fig. 1. Expression and evaluation of 2A3 and 2A3-Fc. (A) Primary structure (top panel) and schematic drawing (bottom panel) of the single domain antibody (sdAb) 2A3 and the heavy chain antibody (HCAb). sdAbs are the variable regions of camelid (e.g. llama) heavy chain antibodies. They can be cloned and expressed either alone (in bacterial systems), or they can be cloned and expressed in a mammalian system in fusion with an Fc fragment of an antibody (e.g. mouse Fc fragment). The sdAb 2A3 was subcloned into a mammalian expression vector pTT5 with the mouse Fc2b fragment as indicated. (B) The recombinant HCAb is approximately half the size of a conventional immunoglobulin G (IgG). The sdAb 2A3 and HCAb 2A3-Fc were expressed and purified to >95% purity (by SDS–PAGE) as described in methods.

2.3. Proliferation assay

Proliferation assays were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Oakeville, ON). Cells at 5000 cells/well were plated in 96-well plates (Falcon, Becton Dickinson, NJ) and the antibodies were added to the wells 24 h later. The cells were assayed for viability after 72 h. Briefly, 20 μl of MTT reagent was added to each well to give a final concentration of 300 μg/ml. Four hours later, the adherent cells were lysed by using 100 μl dimethyl sulphoxide per well. The absorbance of the formazan product was measured at 570 nm.

2.4. Gelatin zymography, MMP-2 and MMP-9 activity assays

Gelatin zymography is used to detect the activity of gelatinases, namely, matrix metalloproteinases MMP-2 and MMP-9.[21] Briefly, conditioned media were obtained from BxPC3 cell cultures that had been treated with 2A3 or gemcitabine. The media were applied to a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) that was supplemented with 1.5 mg/ml gelatin. SDS was removed from the gel by washing four times with 2.5% Triton X-100 for 30 min at room temperature while gently shaking.[21]

The gel was then incubated in development buffer which comprised 50 mM Tris–HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 5 μM $ZnCl_2$, 0.02% Brij-35 and 0.05% NaN_3 at pH 7.4, overnight at 37 °C and was stained with Coomassie blue. Clean bands that were produced by MMP-2 and MMP-9 activity were visible against the blue background after de-staining. The relative activities of the gelatinases were determined by using a densitometer (AlphaView, FluorChem SP, Alpha Innotech Corporation, San Leandro, CA, United States America (USA)).

2.5. Matrigel invasion assay

The assay was carried out by using bio-coated Matrigel invasion chambers (BD Biosciences, Bedford, MA). Each chamber consisted of an 8 μm pore size PET membrane which had a thin layer of Matrigel basement. BxPC3 cells were detached with cell dissociation buffer (Invitrogen) and then they were centrifuged at 300*g* for 5 min. The cells were re-suspended in RPMI medium and seeded on the tops of insert wells at 1×10^5 cells/well density and cultured at 37 °C in a humidified CO_2 chamber. After 20 h, the non-invasive cells were scrubbed from the top of the insert well using a cotton-swap. The invasive cells that were on the bottom surface were stained with Calcein AM (Invitrogen) and were photographed with an Olympus BX51 microscope. All of the experiments were performed in triplicate.

2.6. Angiogenesis test

Approximately 200 μl of Geltrex reduced growth factor basement membrane matrix (Invitrogen) was plated onto a 9 mm diameter cell culture insert well which had a 0.45 μl pore size (Millipore, Billerica, MA). The matrix was then polymerised at 37 °C for 30 min. Conditioned media from BxPC3 cells treated with 2A3 antibody for 24 h were used to re-suspend HUVECs at 1×10^5 cells/ml. HUVEC cells were then seeded onto the polymerised matrix at 5×10^4 cells per well. The cells were cultured at 37 °C for 16 h and then stained with Calcein

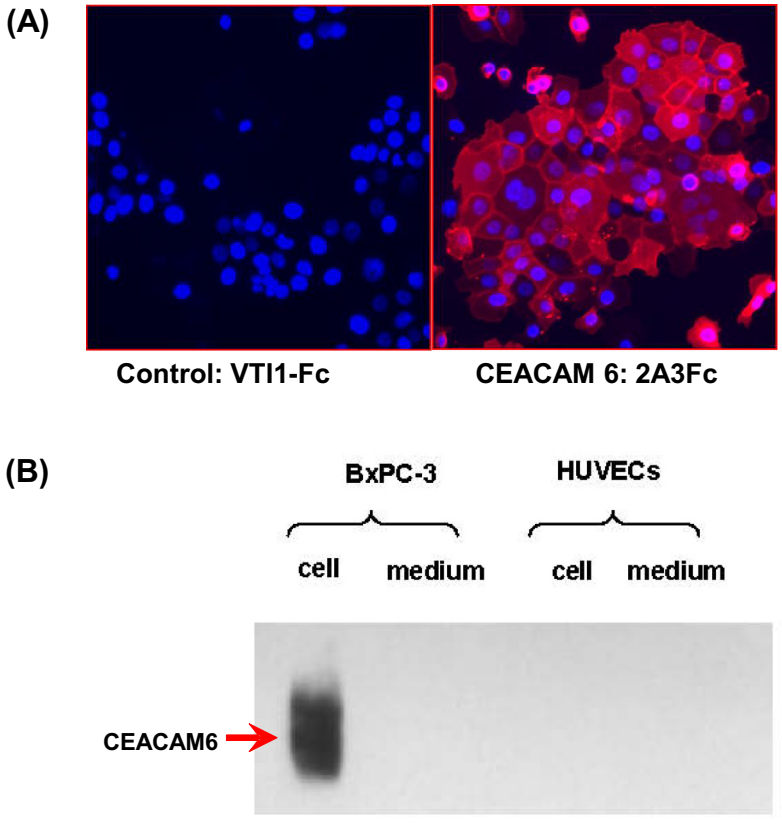

Fig. 2. Expression level of carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) by BxPC3 and human umbilical vein endothelial cells (HUVECs) and in their conditioned media. (A) We used 2A3-Fc antibody conjugated with Alexa Fluor 594 for immunocytochemistry to visualise CEACAM6 expression on BxPC3 cell surface (red, right). An isotype control VTI1-Fc was used as a negative control (left). 4′,6-diamidino-2-phenylindole (DAPI) was used as nuclear stain (Blue). (B) While BxPC3 cells showed very high levels of CEACAM6 expression, we were not able to detect CEACAM6 in the culture medium. We could not detect expression of CEACAM6 on HUVEC cells by Western blotting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AM (Invitrogen) for 30 min. Photographs were taken by employing fluorescence microscopy (Olympus BX51). Three fields were randomly chosen and the lengths of the capillaries in each field were measured.

3. Results

3.1. Antibody cloning, expression and purification

The sdAb 2A3 was expressed by using an *Escherichia coli* (*E. coli*) expression system. The protein was purified by immobilised metal affinity chromatography. More than 25 mg of >95% pure proteins were obtained per litre of culture.

The HCAb 2A3-Fc was cloned into pTT5 expression vector and later transfected into 293–6E cells (Fig. 1). The expressed protein was then purified from the culture medium using a Protein G column. More than 30 mg of >95% pure protein were obtained per litre of culture (Fig. 2, left).

3.2. CEACAM-6 expression in BxPC3 and HUVECs cells

We examined CEACAM6 expression in BxPC3 cells and HUVECs and in conditioned culture media. BxPC3 cells had very high expression levels that were detected by Western blotting of cell lysates, fluorescence-activated cell sorting (FACS) analysis, and immuno-histochemical staining (Fig. 2). We could not detect CEACAM6 expression in HUVECs, neither could we detect any CEACAM6 in the culture media of either type cells (Fig. 2B). Moreover, CEACAM6 expression was absent from the control tumour cell line Panc1. The absence of CEACAM6 in the conditioned media made the following evaluation more straightforward because soluble CEACAM6 antigen in the medium might neutralise and block the antibody from targeting the antigen on the cell surface.

3.3. Inhibition of BxPC3 cell proliferation and MMP-9 activity by anti-CEACAM6 antibodies

Inhibition of cell proliferation of BxPC3 by 2A3 and 2A3-Fc was tested by MTT assay. verotoxin1 sdAb Fc fusion (VTI1-Fc), an isotype control HCAb that does not bind to CEACAM6 was used as a negative control. The 2A3 antibody at 50 μg/ml inhibited approximately 27% of cell proliferation in contrast to starvation medium control (Fig. 3A). The inhibition effect of 2A3-Fc was also tested at a concentration of 360 μg/ml. This concentration was chosen because it has the same molar

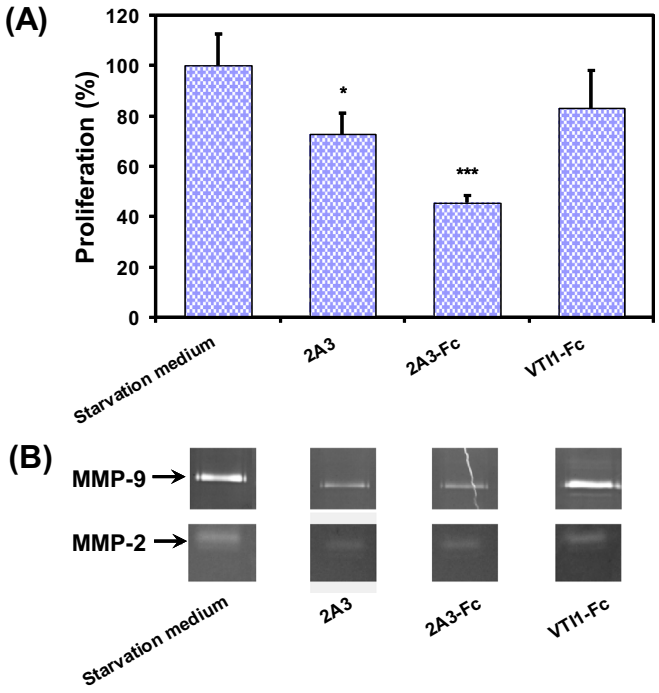

Fig. 3. Inhibition of BxPC3 cell proliferation and MMP-9 and MMP-2 activities by single domain antibodies (sdAbs). (A) Anti-carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) antibodies were tested in MTT assays for their effect on BxPC3 cells proliferation. About 50 μg/ml of sdAb or 360 μg/ml of heavy chain antibody (HCAb) were used to treat the tumour cells. VTI1-Fc, an isotype control HCAb that does not bind to CEACAM6 was used as a negative control. The cells were allowed to grow for 72 h, after which the MTT assays were performed. (B) MMP-9 and MMP-2 activities were examined by gelatin zymography. The 2A3 and 2A3-Fc antibodies showed significant reduction in MMP-9 activity in the media from BxPC3 cells. The zymography assays were performed on medium aliquots taken from BxPC3 cultures at the time the proliferation assays were started.

concentration of 50 μg/ml of 2A3. 2A3-Fc had obviously better inhibition effect, inhibiting 55% of BxPC3 cell proliferation. On the other hand, 2A3 antibody treatment had no effect on the proliferation of Panc1 control cells. The 2A3/2A3Fc antibodies were used to test their influence on MMP-2 and MMP-9 activity. Results indicate that both 2A3 and 2A3-Fc were able to reduce MMP-9 level, but not MMP-2 activity (Fig. 3B).

3.4. Anti-CEACAM6 sdAb decreased proliferation and MMP-9 activity in BxPC3 cells in a dosage-dependent manner

We further determined whether the inhibition of BxPC3 cell proliferation and MMP-2 or MMP-9 activity is dose dependent. The effect of gemcitabine was also tested. The 2A3 and 2A3-Fc antibodies and gemcitabine decreased BxPC3 cell proliferation to less than 10% at dosages of 25 μM and 50 nM, respectively. The half maximal inhibitory concentration (IC_{50}s) for 2A3, 2A3-Fc and gemcitabine were determined as 6.5 μM, 8 μM and 12 nM, respectively (Fig. 4A).

Anti-CEACAM6 antibodies down-regulated MMP-9 activity, but not MMP-2 activity in the BxPC3 cells (Fig. 4B). The culture medium MMP-9 activity of 2A3 treated BxPC3 was down-regulated by 33% compared to non-treated cells (Fig. 4B). Gemcitabine decreased BxPC3 cell proliferation more effectively than did the sdAb. However, this effect was only a result of cell toxicity. We did not detect an effect on MMP-9 activity. On the contrary, a slightly increased MMP-9 activity was detected for the BxPC3 cells that were treated with gemcitabine.

3.5. Anti-CEACAM6 sdAb inhibited BxPC3 invasion

BxPC3 cells that were cultured in starvation medium (RPMI 1640 with 0.1% FBS) were able to degrade the matrigel and migrate through the basement membrane. The invasiveness was reduced for BxPC3 cells that were treated with anti-CEACAM6 antibody 2A3, compared to the control (non-treated) cells (Fig. 5, top). The invasiveness of the cells that were treated with 40 μM of 2A3 or 2A3-Fc decreased to 27.4% (27.4 ± 8.4% and 27.4 ± 4.8%, respectively) (Fig. 5, bottom). Thus, BxPC3 cell invasiveness can be modulated by targeting CEACAM6 using 2A3 antibody.

3.6. Anti-CEACAM6 sdAb decreased BxPC3 induced HUVECs angiogenesis

The *in vitro* capillary formation assay was used to test the ability of media of sdAb-treated BxPC3 cells to inhibit HUVECs angiogenesis. For the anti-angiogenesis assay, tumour-induced angiogenesis was suppressed significantly when 2A3- and 2A3-Fc-treated BxPC3 culture medium inhibited endothelial cell capillary-like structure formation (Fig. 6). After 24 h, media from BxPC3 cells that were treated with 2A3 or 2A3-Fc significantly inhibited HUVECs from forming capillary-like structures (Fig. 6A, top). The 2A3 inhibited 21% of endothelial cell angiogenesis. In addition, 49% of angiogenesis was inhibited by 2A3-Fc. In contrast, the inhibitory effects on angiogenesis were not observed when culture medium from gemcitabine-treated BxPC3 cells was used (Fig. 6, bottom). Therefore sdAb 2A3 and the corresponding HCAb (2A3-Fc) likely can promote anti-angiogenic effects which are mediated by BxPC3 tumour cells.

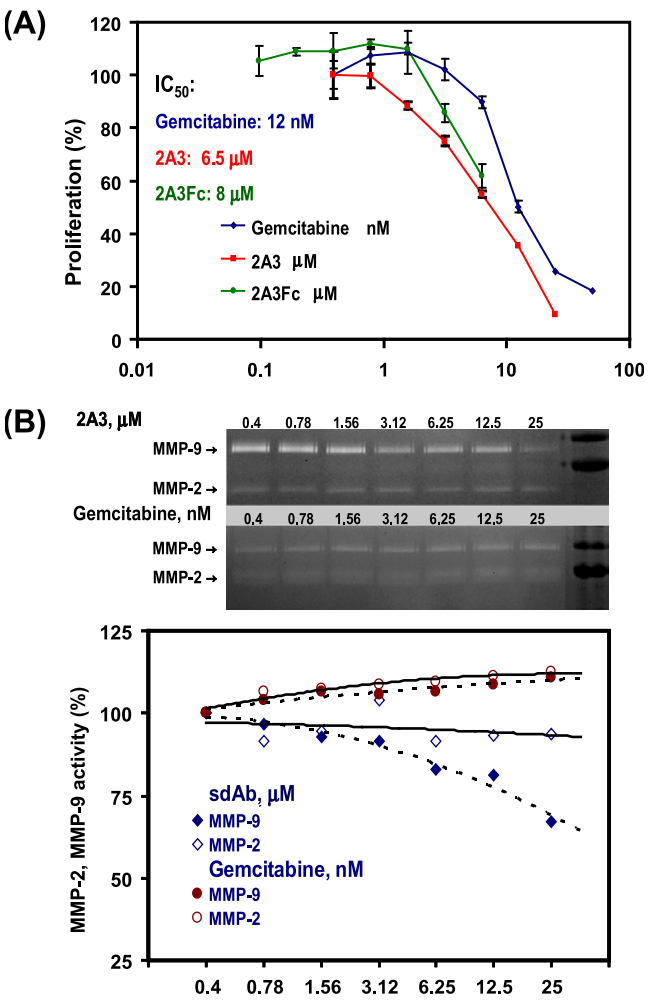

Fig. 4. *In vitro* proliferation and gelatinase activity assays. (A) Percentage of proliferating BxPC3 cells after being treated with various amounts of 2A3 or 2A3-Fc and gemcitabine for a 72 h. Cell proliferation in starvation medium is used as control. Data were collected from five repeated experiments and normalised. The IC_{50}s for 2A3, 2A3-Fc and gemcitabine were calculated as 6.5 μM, 8 μM and 12 nM, respectively. (B) Top panel, MMP-2 and MMP-9 activities were examined by gelatin zymography. 2A3/2A3Fc, but not gemcitabine, inhibited MMP-9 activity. MMP-2 activity was not affected by the treatment. Bottom panel, The scale of MMP-9 activity reduction caused by 2A3/2A3-Fc treatment.

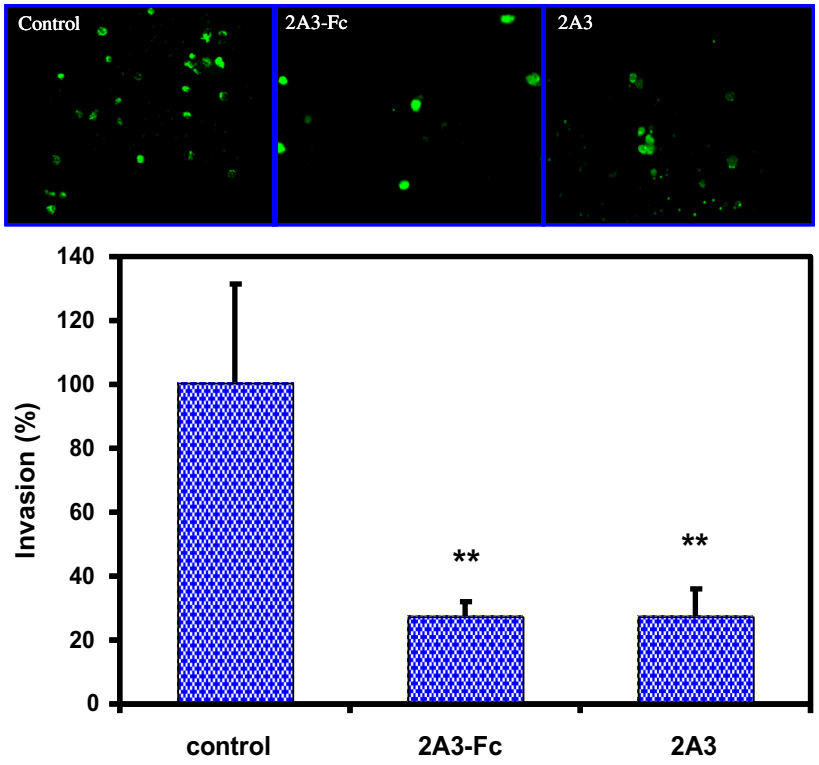

Fig. 5. Inhibition of BxPC3 invasion by single domain antibodies (sdAbs). Top panel, BxPC3 invasion activity was inhibited by 2A3/2A3-Fc-treatment. Cells from three random selected fields were counted and normalised against a starvation medium control. The invasive cells on the bottom surface were stained with Calcein AM and photos were taken at 100× magnification. Bottom panel, scale of the reduction of BxPC3 cells by 2A3 and 2A3-Fc. The invasive cells on the bottom surface were stained with Calcein AM and counted by fluorescence microscopy. All experiments were performed in triplicate. ** $P < 0.01$.

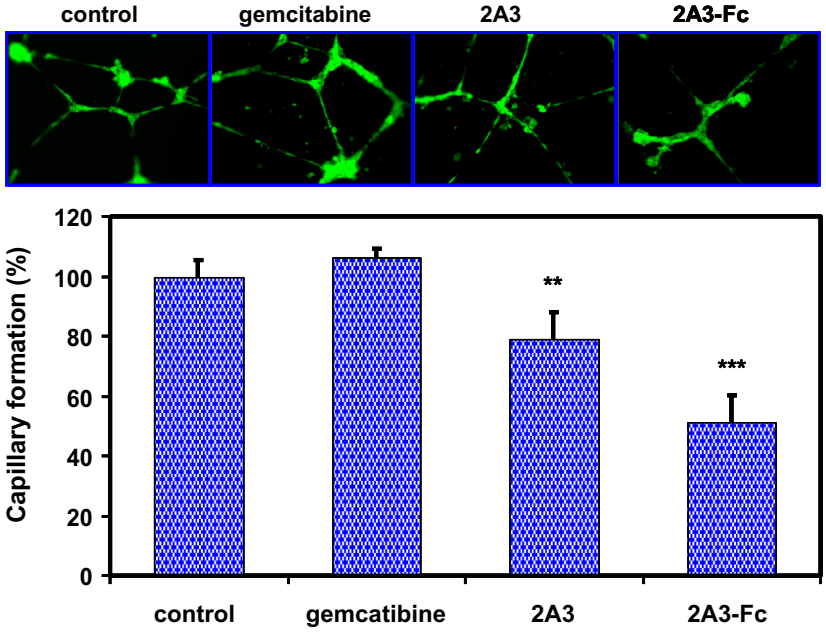

Fig. 6. Inhibition of angiogenesis by 2A3 and 2A3-Fc. Top panel, conditioned media from BxPC3 cells that were treated with 2A3 or 2A3-Fc inhibited tubule formation by human umbilical vein endothelial cells (HUVECS) during a tubule formation assay. Bottom panel, capillary-like tube formation (angiogenesis) was inhibited by 2A3 or 2A3-Fc. Single domain antibody (sdAb) 2A3 inhibited tubule formation by 21% and 2A3-Fc inhibited tubule formation by 49%. Three fields that were randomly chosen were captured and the length of the capillary in each field was measured. ** $P < 0.01$ in 2A3 and *** $P < 0.001$ in 2A3-Fc.

4. Discussion

The majority of cancers deaths are due to metastatic cancer cells and outgrowths of tumours at distant sites. Cancer cell invasion into peripheral tissues allows the cells to enter the circulation. They then anchor onto secondary tissues and organs: this step is essential for tumour metastasis. Inhibiting invasion is a key step for decreasing cancer cell metastasis. CEACAM6 over-expression in pancreatic cancer tissues promotes pancreatic cancer cell invasion, metastasis and angiogenesis.[4–6,8,22,23] Recently, CEACAM6 has become a target for pancreatic cancer therapy.[10,12,24,25] The sdAb 2A3 and its HCAb version 2A3-Fc can block the CEACAM6 antigen, and decrease its invasiveness. An important advantage of antibody therapy compared to chemotherapeutic agents is that antibodies are more specific for tumours that over-express tumour antigens. Therefore this might result in reduced general cell toxicity and cancer cell chemo-resistance.

Proteolytic degradation of extracellular matrix (ECM) is critical for cancer cell migration and for cancer cells to enter the circulation. MMP-2 and MMP-9 are expressed highly in human pancreatic cancer tissues.[26] MMP-2/MMP-9 mediated extra-cellular matrix degradation leads to cancer cell invasion and metastasis. Its association with cancer progression has also been an important principle of cancer research.[13] MMP-9 is associated with ECM turnover and cell migration through the ECM. It is a key enzyme that regulates cancer cell invasion and metastasis.[27,28] MMP inhibitors have been used in combination with gemcitabine to treat pancreatic cancer patients.[26] In animal models, gemcitabine and MMP inhibitor combinational therapy can be used to reduce cancer implantation and improve survival compared with using gemcitabine or the inhibitor alone. However, the results of clinical trails which involved using MMP inhibitors to treat patients were not significant.[29,30] The poor clinical data may be attributable to the lack of MMP species specificity of the inhibitors that were used. In this study, the 2A3 antibody inhibited MMP-9 activity in culture medium that was derived from BxPC3 cells but it had no effect on MMP-2 activity. Although gemcitabine profoundly affects cell survival, it does not affect MMP-2 and MMP-9 activity in BxPC3 cells. The effect of gemcitabine on MMP-2 and MMP-9 was similar to that which was noted in previous reports, where MMP-9 expression did not change in pancreatic tumour tissues of gemcitabine-treated mice.[31] There is no significant difference in serum MMP-2 activity between gemcitabine-treated and control mice.[26] In this study, in addition to inhibiting cell proliferation, MMP-9 activity in the culture medium was down-regulated by 2A3 and 2A3-Fc. A 5 μM dosage of sdAb 2A3 reduced MMP-9 activity by ~25%. On the other hand, 2A3 reduced BxPC3 invasiveness by ~73%. Except for its cell toxicity effect, gemcitabine did not affect MMP activity or invasion. It even increased MMP-2/MMP-9 activity slightly (~10%) at high dosages.

Angiogenesis is another critical step in the progression of pancreatic cancer. Relatively complex and dense micro-vessels are localised in pancreatic cancer tissue. Highly vascular tumours are associated with an increased risk of hepatic metastasis and a poor survival rate.[32] We tested the ability of conditioned media from BxPC3 cells to inhibit capillary-like structures from being formed by endothelial cells (angiogenesis) *in vitro*. From our data for a matrix tubule formation assay we found that conditioned media from 2A3- and 2A3-Fc-treated BxPC3 cells decreased the total tube length significantly (the tubing formation activity was reduced by ~21%). In contrast, gemcitabine was not found to have such effect, which is consistent with report from others.[33] Based on report that CEACAM6 plays a role in invasion and angiogenesis in pancreatic cancer, our results suggest that this function can be blocked by 2A3 or 2A3-Fc antibodies. Furthermore, anti-angiogenic agents permit better delivery of gemcitabine into tumour vascular and interstitial spaces.[33] Our results suggest that anti-CEACAM6 sdAbs have such functions. We also would like to note that the effects of the 2A3 antibody described in this paper are not limited to BxPC3 cells. Testing other cell line that over express CEACAM6 (e.g. MCF7 and ZR-75-30) have shown similar effects to those seen in BxPC3 (non-published data).

In conclusion, treating BxPC3 tumour cells with 2A3/2A3-Fc reduces cell proliferation, invasion and MMP-9 activity. Such treatment also reduces the ability of the conditioned media of pancreatic tumour cells in promoting HUVEC cell angiogenesis. In contrast, gemcitabine only affects BxPC3 cells proliferation but does not affect MMP activity and does not reduce HUVEC capillary-like structure formation. Therefore the 2A3 antibody can be a useful addition to gemcitabine in treating pancreatic cancer.

Conflict of interest statement

None declared.

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