Activated leukocyte cell adhesion molecule regulates the interaction between pancreatic cancer cells and stellate cells

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Abstract. Activated leukocyte cell adhesion molecule (ALCAM/CD166) is a transmembrane glycoprotein that is involved in tumor progression and metastasis. In the present study, the expression and functional role of ALCAM in pancreatic cancer cells and pancreatic stellate cells (PSCs) was investigated. Tissue specimens were obtained from patients with pancreatic ductal adenocarcinoma (n=56) or chronic pancreatitis (CP; n=10), who underwent pancreatic resection, and from normal pancreatic tissue samples (n=10). Immunohistochemistry was used to analyze the localization and expression of ALCAM in pancreatic tissues. Subsequently, reverse transcription-quantitative polymerase chain reaction and immunoblotting were applied to assess the expression of ALCAM in pancreatic cancer Panc-1 and T3M4 cells, as well as in PSCs. An enzyme-linked immunosorbent assay was used to measure ALCAM levels in cell culture medium stimulated by hypoxia, tumor necrosis factor (TNF)-α and transforming growth factor-β. Silencing of ALCAM was performed using ALCAM small interfering (si)RNA and immunocytochemistry was used to analyze the expression and functional role of ALCAM during the tumorigenesis of different types of cancer. It was proposed that ALCAM may promote the survival of breast cancer cells by inhibiting apoptosis and autophagy (14). Evidence has also suggested that ALCAM may suppress the migration and invasion of cancer cells by controlling the activity of matrix metalloproteinases (15). For example, the expression of ALCAM is associated with the development and malignant progression of various types of cancer, including colon, gastric, liver, lung, prostate, pancreatic cancer, breast carcinomas and melanoma (3-11). Furthermore, it is a marker for cancer stem cells in colon and prostate cancer (12,13). However, the exact role of ALCAM during the tumorigenesis of different types of malignant tumor remains to be elucidated. It was proposed that ALCAM may promote the survival of breast cancer cells by inhibiting apoptosis and autophagy (14). Evidence has also suggested that ALCAM may suppress the migration and invasion of cancer cells by controlling the activity of matrix metalloproteinases (15). For example, the expression of ALCAM is associated with the suppression of breast cancer cell invasion (16). Furthermore, ALCAM-negative pancreatic cancer cells demonstrated stronger invasive and migratory activities compared with ALCAM-positive cancer cells (17). Our previous study demonstrated that silencing ALCAM caused no affect on cell growth or invasion in Su86.86 pancreatic cancer cells, but significantly reduced cell adhesion and increased pancreatic cancer cell resistance towards chemotherapeutic agents (4). However, the underlying mechanism remains to be elucidated.
In the present study, the expression of ALCAM in pancreatic cancer cells and pancreatic stellate cells (PSCs) was analyzed, and the role of ALCAM in the growth, proliferation, invasion and cell-cell interaction of pancreatic cancer cells and PSCs was further investigated.

Materials and methods

Tissue specimens and cell cultures. Tissue specimens were obtained from patients who underwent pancreatic resection. The patients underwent surgery for a range of pancreatic diseases, including pancreatic ductal adenocarcinoma (PDAC; n=56) and chronic pancreatitis (CP; n=10). Normal pancreatic tissue samples (n=10) were obtained during resection for tumor infiltration of the peripancreatic area by another malignancy (i.e. colon cancer) or metastasis to the pancreas by kidney tumors that required resection of healthy pancreatic tissue. Tissue collection was approved by the Ethics Committees of the Technical University of Munich (Munich, Germany) and the University of Heidelberg (Heidelberg, Germany). Written informed consent was obtained from all patients.

Freshly removed tissues were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin for histological analysis. A portion of the tissue samples was preserved in RNAlater (Ambion Europe Ltd., Huntingdon, Cambridgeshire, UK) or snap-frozen in liquid nitrogen immediately upon surgical removal, and maintained at -80˚C until use. Pancreatic cancer cell lines (Panc-1 and T3M4) were obtained from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS; Invitrogen; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and streptomycin (complete medium) at 37˚C in a 5% CO₂ humidified atmosphere. PSCs between passages 3 and 6 were cultured in a 1:1 (vol/vol) mixture of Ham's F12 medium (Invitrogen; Carlsberg, Denmark) and Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) to 70% confluence and used at passages 3 and 6. PSCs were cultured in Ham's F12 medium containing 3% hydrogen peroxide at room temperature for 10 min. Following blocking of non-specific reactivity with diluted normal goat serum, the tissue sections were incubated with rabbit anti-human ALCAM polyclonal antibodies (4 µg/ml; 1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. The tissue sections were subsequently incubated with horseradish peroxidase (HRP)-linked goat anti-rabbit antibodies (1:5,000; Dako GmbH, Hamburg, Germany), followed by reaction with diaminobenzidine and counterstaining with Mayer's hematoxylin. In addition, to confirm the specificity of the primary antibodies, tissue sections were incubated in the absence of the primary antibodies and with negative control rabbit immunoglobulin G (1:50; Santa Cruz Biotechnology, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). All reagents and equipment for mRNA and cDNA preparation were purchased from Roche (Mannheim, Germany). The forward and reverse primer sequences were as follows: ALCAM sense, 5'-TAG CAG GAA TGC AAC TGT CAG-3'; ALCAM anti-sense, 5'-CGC AGA CAT AGT TTC CAG-3'. mRNA was prepared by automated isolation using the MagNA Pure LC instrument and isolation kit I (for cells) and kit II (for tissues). cDNA was reverse-transcribed into cDNA using the cDNA synthesis kit for RT-PCR (AMV), according to the manufacturer's protocol. qPCR was performed on a LightCycler 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland) with the Light Cycler Fast Start DNA SYBR Green kit. The PCR program consisted of an initial denaturation cycle (10 min at 95˚C) followed by 40 cycles of denaturation (15 sec at 95˚C) and annealing and elongation (60 sec at 60˚C). The relative number of specific transcripts was normalized against the levels of cyclophilin B and hypoxanthine guanine phosphoribosyltransferase, and the data were analyzed using the 2^(-ΔΔCt) method (22).

Immunohistochemistry. Immunohistochemistry was performed using the Dako Envision System (Dako Cytomation GmbH, Hamburg, Germany). Consecutive paraffin-embedded tissue sections (3-5 µm thick) were deparaffinized and rehydrated using routine methods (23). Antigen retrieval was performed by pretreatment of the slides in citrate buffer (pH 6.0) in a microwave oven for 10 min. Endogenous peroxidase activity was quenched by incubation in deionized water containing 3% hydrogen peroxide at room temperature for 10 min. Following blocking of non-specific reactivity with diluted normal goat serum, the tissue sections were incubated with rabbit anti-human ALCAM polyclonal antibodies (4 µg/ml; 1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. The tissue sections were subsequently incubated with horseradish peroxidase (HRP)-linked goat anti-rabbit antibodies (1:5,000; Dako GmbH, Hamburg, Germany), followed by incubation with diaminobenzidine and counterstaining with Mayer's hematoxylin. In addition, to confirm the specificity of the primary antibodies, tissue sections were incubated in the absence of the primary antibodies and with negative control rabbit immunoglobulin G (1:50; Santa Cruz Biotechnology, Inc.).

Immunocytochemistry. The cells (Panc-1, T3M4 and PSCs) were transfected with control or ALCAM small interfering (si)RNA for 48 h, trypsinized and seeded onto slides for 12 h. Following washing three times with phosphate-buffered saline (PBS), the cells were incubated with 4% paraformaldehyde for 10 min, 30 mM glycine/PBS for 5 min and 0.1% Triton X-100 for 5 min. After washing three times with PBS, the cells were incubated with 3% H₂O₂ for 10 min, followed by incubation with the primary antibody diluted in universal block DAKO (ALCAM, 1:50; keratin, 1:500, Santa Cruz Biotechnology, Inc.) for 1 h. Following washing with Tris-buffered saline (TBS)/bovine serum albumin (BSA; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and Tween 20 (0.05%), the secondary antibody labeled with HRP (1:1,000, Chemicon, Hofheim, Germany) was added, followed by color reaction and counterstaining as in immunohistochemistry.

Enzyme-linked immunosorbent assay (ELISA). The ALCAM ELISA kit was used (R&D Systems, Wiesbaden-Nordenstadt, Germany) to detect ALCAM levels in the serum and tissue culture medium at room temperature. Briefly, 96-well Nunc Immuno plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µl (2 µg/ml) of ALCAM capture antibodies (1:500; R&D Systems, Inc., Wiesbaden-Nordenstadt, Germany) in PBS (pH 7.0). PBS with 0.05% Tween 20 was used as the washing solution. Non-specific binding sites were blocked with 300 µl blocking buffer (1% BSA in PBS) for 1 h at 37˚C. Either recombinant human ALCAM or serum/cell culture supernatant (100 µl/well) were added and incubated for 2 h at 37˚C. Following washing, 100 µl of biotin-conjugated goat anti-human ALCAM detection antibodies (50 ng/ml) were added into each well and incubated for 2 h at room temperature. HRP-conjugated streptavidin (100 µl) 1:200 diluted
in PBS was added to each well and incubated for 20 min at 37°C. Following washing with PBS with Tween 20 three times, 100 µl of 1:1 mixed TMB substrate reagent A and reagent B (BD Biosciences, San Diego, CA, USA) were added for 20 min at 37°C. Colorimetric reactions were stopped by adding 50 µl of 2 N H₂SO₄ and analyzed using a microplate reader at 450 and 570 nm for correction.

**Immunoblotting.** Cultured pancreatic cancer cells and PSCs were lysed in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate and one tablet EDTA-free protease inhibitor cocktail (Roche) for 30 min. Cell lysates were collected following centrifugation at 15,000 g for 10 min at 4°C. The total protein (20 µg) was loaded onto 10% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 20 ml TBS, 5% skim milk and 0.05% Tween-20 for 1 h, and then incubated with rabbit anti-human ALCAM polyclonal antibodies (1:200; Santa Cruz Biotechnology, Inc.) overnight at 4°C, and then incubated with HRP-conjugated anti-rabbit antibody (1:2,500; Santa Cruz Biotechnology, Inc.) or anti-Erk2 (1:2,500; Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membranes were blocked with 20 ml TBS, 5% skim milk and 0.05% Tween-20 for 1 h, and then incubated with HRP-conjugated anti-rabbit antibody (1:2,500; Chemicon) for 1 h at room temperature. Signals were detected using the enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

**Hypoxia.** Panc-1, T3M4 and PSC cells were exposed to hypoxic conditions of 0.75% O₂, 10% CO₂ and 89.25% N₂, as described previously (24). The medium was switched to serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) prior to subjecting the cells to hypoxia. Control cultures were grown in serum-free medium under normoxia in a 5% CO₂ incubator (Forma Scientific Co., Marietta, OH, USA). After incubation for 48 h, supernatants were collected for ALCAM assay using ELISA.

**siRNA transfection.** Human ALCAM specific siRNA (sense, GCC CGA UGG CUC CCC AGU A; antisense, UAC UGG GGA GCC AUC GGG C) (14) was purchased from Qiagen (Hilden, Germany). The cells were grown to 50-70% confluence. siRNA transfection was performed with HiPerFect transfection reagent (Qiagen), according to the manufacturer's instructions. The final concentration of the control and specific siRNA was 5 nM. The efficacy of the siRNA transfection was ascertained by immunoblot analysis and ELISA after 72 h of transfection.

**Invasion assay.** To assess cell migration in vitro, Transwell migration chambers with an 8 µm pore size (BD Biosciences) were used and reconstituted with 600 µl serum-free RPMI-1640 medium in the top and bottom chambers for 24 h. The Panc-1, T3M4 and pancreatic stellate cells were trypsinized and seeded into the top chamber at a density of 2.5x10⁴ cells/well in 600 µl RPMI-1640 containing 10% FCS. Following incubation at 37°C for 20 h, the cells remaining attached to the upper surface of the filters were carefully removed with cotton swabs, while cells that reached the underside of the chamber were stained with hematoxylin and eosin and counted under a microscope in five random fields at a magnification of x200.

**Cell interaction assay.** Panc-1 or T3M4 cells in RPMI-1640 supplemented with 10% FCS and PSCs in a 1:1 (vol/vol) mixture of low glucose (1,000 mg/l) Dulbecco’s modified Eagle’s medium with Ham’s F12 medium containing with 10% FCS were transfected with ALCAM siRNA or control siRNA for 2 days. The tumor cells and PSCs were trypsinized, counted, mixed and seeded directly for co-culture at a density of 4,000 cells/well in 24-well plates. After 2 days of culture, immunocytochemistry using keratin as a specific marker for cancer cells was performed to analyze the interaction between tumor cells (Panc-1 and T3M4) and PSCs. Cell-cell interactions were counted randomly in five areas under the microscope and calculated as a percentage (interaction number/total number of cells). All assays were performed in triplicate and repeated three times.

**Statistical analysis.** The data are presented as the mean ± standard error of the mean for in vitro assays, and median and individual data for the RT-qPCR and ELISA results, unless indicated otherwise. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U test and the Kruskal-Wallis test were
utilized, and groups were compared using Dunn's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. The mean difference between groups was estimated with a 95% confidence interval.

Results

ALCAM expression and localization in pancreatic tissues. Our previous study (4) demonstrated that ALCAM was expressed on the membrane of islet cells in the normal pancreas whereas normal pancreatic ducts were negative for ALCAM. ALCAM was expressed in ductal and acinar cells in CP tissues. Furthermore, ALCAM expression was generally low in PDAC, while membranous or cytoplasmic ALCAM expression was found in certain types of tumor (9). The present study demonstrated strong ALCAM expression in PSCs of CP tissues (Fig. 1A), and PSCs surrounding pancreatic intraepithelial neoplasias (Fig. 1B), as well as in pancreatic cancer cells (Fig. 1C).

ALCAM expression in pancreatic cancer cells and PSCs. A previous study demonstrated that ALCAM was expressed in pancreatic cancer cell lines (9). The present study compared the expression of ALCAM in pancreatic cancer Panc-1 and T3M4 cells with its expression in PSCs. As shown in Fig. 2A, ALCAM mRNA was highly expressed in PSCs, while it was low to moderately expressed in T3M4 and Panc-1 cells. Similar to mRNA expression, western blot analysis demonstrated that ALCAM protein levels were high in PSCs and T3M4 cells, but low in Panc-1 cells (Fig. 2B).

Soluble levels of ALCAM are regulated by tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and hypoxia. To assess the secretion of ALCAM following stimulation in pancreatic cells, Panc-1 and T3M4 cells, and PSCs were treated with TNF-α, TGF-β and hypoxia for 48 h, and ALCAM protein levels were detected in cell culture supernatant by ELISA. The results demonstrated that ALCAM levels...
were significantly increased by TNF-α in Panc-1 (P<0.001), T3M4 (P=0.003) and PSCs (P<0.001), while ALCAM levels were significantly decreased by hypoxia in PSCs (P<0.001).

Following treatment with TGF-β, ALCAM levels did not change in Panc-1 cells, increased in T3M4 cells (P=0.043) and decreased in PSCs (P=0.01; Fig. 3).

**ALCAM promotes PSC invasion.** As previously demonstrated by our group, ALCAM silencing did not affect pancreatic cancer cell growth or invasion but significantly reduced cell adhesion in Su86.86 pancreatic cancer cells (4). To assess the role of ALCAM in the regulation of invasion of pancreatic cells and PSCs, ALCAM was knocked down using siRNA in Panc-1 and
T3M4 cells, as well as in PSCs. Immunocytochemistry demonstrated that ALCAM was predominantly expressed in the cytoplasm in Panc-1 and PSC cells, and in the membrane of T3M4 cells. Transfection of these cells with ALCAM siRNA for 48 h resulted in a decrease in ALCAM expression (Fig. 4A). ALCAM silencing by siRNA did not affect the invasion of Panc-1 and T3M4 cells, but resulted in 50% inhibition of invasion of PSCs (P=0.047; Fig. 4B).

ALCAM silencing results in decreased interaction between Panc-1 cells and PSCs. A previous study demonstrated that ALCAM is important in the regulation of tumor cell-stromal cell interactions (25). To assess whether silencing of ALCAM alters the interaction between tumor and stromal cells, Panc-1 and PSCs were co-cultured. Silencing of ALCAM by siRNA led to a decreased interaction between Panc-1 cells and PSCs (Fig. 5).

Discussion

The molecular functions of ALCAM in the tumorigenesis of different types of cancer are largely unknown. In the present study, immunohistochemistry demonstrated that ALCAM was only partially expressed in the membrane and cytoplasm of pancreatic cancer cells, which was consistent with the results of a previous study by Hong et al (4). ALCAM expression is generally variable in pancreatic cancer cells (17). In the present study, RT-qPCR and immunoblot analyses revealed that the expression of ALCAM was higher in PSCs compared with that in Panc-1 and T3M4 pancreatic cancer cells, suggesting that ALCAM may be more important in PSCs compared with in pancreatic cancer cells.

It was reported that ALCAM protected breast cancer cells against apoptosis and autophagy, suggesting that ALCAM may promote tumorigenesis (14). However, another study demonstrated that ALCAM suppressed breast cancer cell invasion, suggesting inhibition of tumorigenesis at the late stages (16). In addition, CD166+ pancreatic cancer cells are strongly tumorigenic, while CD166- pancreatic cancer cells exhibit comparatively stronger invasive and migratory activities (16). These data suggested that ALCAM may either promote or suppress tumorigenesis. However, the effect of ALCAM on PSCs is unknown. Notably, the results of the present study demonstrated that ALCAM silencing by siRNA decreased the invasive ability of PSCs, which are a component of the pancreatic tumor microenvironment. ALCAM shedding would release ALCAM into the tumor environment and circulation to exert its function. When the malignant environment was mimicked in vitro using TNF-α, TGF-β and hypoxia, ALCAM shedding was significantly induced by TNF-α from pancreatic cancer cells and PSCs, while it was decreased by hypoxia and TGF-β, particularly in PSCs. The results are consistent with the observation that ALCAM is a cytokine-regulated cell adhesion molecule (26). This indicated that the expression of ALCAM is regulated by an altered tumor microenvironment.

The tumor microenvironment is important in the progression, invasion and metastasis of cancer cells (27,28). ALCAM is variably expressed in stromal and tumor cells, and its expression is affected by the tumor microenvironment. Hong et al reported an association between CD166 and adhesiveness (4). Adhesiveness may cause the functional differences between CD166+ and CD166- cells. Thus, it may be hypothesized that ALCAM-ALCAM interactions facilitate stromal-cancer cell adhesiveness. In support of this hypothesis, in the present study, ALCAM silencing by siRNA did not significantly alter the proliferation of pancreatic cancer cells, but decreased the invasive ability of PSCs. Furthermore, co-culture experiments of PSCs with pancreatic cancer cells demonstrated that silencing of ALCAM in PSCs and Panc-1 cells resulted in decreased tumor cell-PSC adhesiveness. It is well known that once tumor growth has reached a critical mass, the metastatic spread of tumor cells is dependent on their dissociation from the primary tumor and migration towards the systemic circulation. Primary tumors with invasive properties usually exhibit reduced intercellular adhesion, which allows cells to break away from the parental cell mass. Thus, it is possible that ALCAM may indirectly regulate the metastatic potential of pancreatic cancer cells by modulating tumor-stroma interactions.

In conclusion, ALCAM is upregulated in PSCs of pancreatic cancer tissues, promotes PSC invasion and increases the interaction between Panc-1 cells and PSCs, suggesting a potential role of ALCAM in regulating pancreatic cancer cell-PSC interactions.

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