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Alternative splicing of KAI1 abrogates its tumor-suppressive effects on integrin αvβ3-mediated ovarian cancer biology

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ABSTRACT

Loss or downregulation of the tumor-suppressor KAI1 correlates with poor cancer patient prognosis. KAI1 functions by interacting with other proteins, including integrin cell adhesion and signaling receptors. We previously showed that KAI1 physically and functionally crosstalks with the tumor-biologically relevant integrin αvβ3, thereby suppressing ovarian cancer cell migration and proliferation. Interestingly, in metastases, a KAI1 splice variant had been identified, indicating poor patient prognosis. Thus, we here characterized differential effects of the two KAI1 proteins upon their cellular restoration. Opposite to KAI1, KAI1-splice reduced αvβ3-mediated cell adhesion, thereby inducing cell migration. This was accompanied by elevated αvβ3 levels and drastically elevated focal adhesion kinase activation, however, without any obvious colocalization with αvβ3, as observed for KAI1. Moreover, codistribution of KAI1 with the cell/cell-adhesion molecule E-cadherin was abrogated in KAI1-splice. Whereas KAI1 diminished cell proliferative activity, KAI1-splice prominently enhanced cell proliferation concomitant with elevated transcription and cell-surface expression of the epidermal growth factor receptor. Thus KAI1-splice does not only counteract the tumor-suppressive actions of KAI1, but – beyond that – promotes αvβ3-mediated biological functions in favor of tumor progression and metastasis.

1. Introduction

KAI1 (CD82, kangai, or C33), a member of the tetraspanin family, is a type III transmembrane protein traversing the cell membrane four times thereby forming one small (ECL1) and one large extracellular loop (ECL2) as well as a very short intracellular loop. So far, tetraspanins have not been shown to exert intrinsic enzymatic activities but rather are known to organize large multimeric complexes. The ECL2 encompasses a constant region which is instrumental for oligomerization with other tetraspanins, and a variable portion which is implicated in membrane organization of non-tetraspanin proteins, including receptor tyrosine kinases, adaptor proteins that are integral to signaling cascades, and cell adhesion and signaling receptors of the integrin superfamily [1–3]. These interaction partners are recruited into specialized "tetraspanin-enriched microdomains" (TEM) where signal transduction takes place [4–7]. Through these interactions, tetraspanins regulate a variety of cellular events, including transcription, cell signaling, adhesion, migration, survival, endo- and exocytosis, differentiation, and fusion, by yet not fully resolved mechanisms [8–10].

KAI1 was originally identified as a suppressor of metastatic spread in a rat prostate cancer model [8,9,11,12]. By now, accumulating evidence underlines the role of KAI1 as a broad-spectrum suppressor of invasion and metastasis. In malignant solid tumors, detection of KAI1 indicates a better prognosis for cancer patients, consequently, its loss or low expression directly correlates with poor prognosis in many other cancer types, including that of the prostate, lung, pancreas, breast, bladder, colon, skin, cervix, endometrium, and ovary [12–24]. Decrease or loss of KAI1 has also been observed in many metastatic tumor cell lines. Its reintroduction into tumor cells inhibited cancer cell migration and invasion in vitro as well as suppression of cancer metastasis in animal models [10,22,25–32]. Moreover, a KAI1 splice variant (KAI1-SP) lacking exon 7 has been detected in metastatic tissues of gastric cancer patients correlating with poor prognosis. Thus, the splice variant of KAI1 seems no longer to efficiently suppress gastric cancer metastasis. Also, mouse colon adenocarcinoma cells, stably expressing the KAI1 splice variant, displayed increased in vivo tumorigenicity, enhanced in vitro invasive potential, and cell/ECM adhesion [33]. These findings indicate prominent functional differences between the two KAI1 molecules regarding cell biological events, such as cell adhesion, spreading, and migration. These cellular activities seem to be majorly accomplished by its interactions with integrins, their compartmentalization, internalization, recycling, and signaling [34,35].

Integrins are transmembrane glycoproteins composed of non-covalently associated α- and β-chains which recognize proteins of the

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extracellular matrix (ECM). In addition to their cell anchoring function, by binding to ECM ligands, integrins propagate bidirectional signaling across cell membranes, thereby affecting a variety of cellular functions, such as cell growth, migration, and differentiation in physio- and pathophysiological settings, including cancer. The well characterized integrin αvβ3 has prominent functions in cancer biology, due to its role as a marker of angiogenesis and tumor progression in several different cancer types, including ovarian cancer [36,37]. Epithelial ovarian cancer metastasis is characterized by spread of tumor cells within the peritoneal cavity and their subsequent invasion and proliferation within the organs of the small pelvis [38,39]. Regarding these adhesion-dependent events, αvβ3 in concert with its major ECM ligand vitronectin (VN) is crucially involved [40–43]. In a previous study in human ovarian cancer cells, we proved that αvβ3, upon engagement by VN, significantly enhanced cell adhesion, motility, and proliferation [44].

KAI1 has already been shown to interact with β1 integrins, such as α3β1, α4β1, α5β1, α6β1, and αLβ2, respectively [1,35,45]. We previously documented that KAI1 also crosstalks with the tumor biologically relevant integrin αvβ3 [46]. Moreover, other interaction partners include E-cadherin [33], EWI-2 [29], epidermal growth factor receptor (EGF-R) [47,48], tetraspanins CD9 and CD81, and protein kinase C [49]. Herewith, KAI1 inhibits important steps related to tumor metastasis, including cell motility and invasion, proliferation, induction of apoptosis and senescence in response to extracellular stimuli.

In recent year, many experimental in vitro and in vivo approaches have been undertaken to further the understanding of mechanisms by which KAI1 might exert its tumor-suppressive properties. To this end, in the present study, we were interested whether reintroduction of KAI1 and its splice variant, respectively, by stable transfection of human ovarian cancer cells affects αvβ3-induced tumor biological effects.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle medium (DMEM), fetal calf serum (FCS), Lipofectin®, the plasmid pcDNA3.1/Hygro, geneticin, hygromycin, Alexa-488- and Alexa 568-labeled goat-anti-mouse, and goat-anti-rabbit IgG were from Invitrogen, Carlsbad, CA, USA. Vitronectin, monoclonal antibodies (mAb) raised against the EGF-R, mAb directed to p-FAK (Y397), the dual luciferase reporter gene assay kit, and the Renilla luciferase reporter gene vector pRL-SV40 plasmid were obtained from Beckton-Dickinson Biosciences, Franklin Lakes, NJ, USA. Collagen type I, p-nitrophenyl-N-acetyl-β-D-glucosaminide, and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide thiazolyl blue (MTT) were from Sigma-Aldrich, St. Louis, MO, USA. DNase I and the reverse transcriptase were from Roche Diagnostics GmbH, Mannheim, Germany. Monoclonal Ab directed to αvβ3 (# 23C6) and polyclonal Ab (pAb) directed to αv (# AB1930) were purchased from Millipore, Schwalbach, Germany. Monoclonal Ab directed to KAI1 (# TS82b) was from Diaclone, Stamford, CT, USA. Rabbit pAb raised against E-cadherin (# 24E10) were from Cell Signaling, Leiden, The Netherlands; Monoclonal Ab to EGF-R for FACS analysis were purchased from Stressgen, Victoria, Canada. The RNA isolation kit RNA-Clean® was ordered from TM System, AGS, Heidelberg, Germany. The plasmid purification kit was from Macherey-Nagel, Easton, PA, USA. The RNeasy Mini Kit was from Qiagen, Hilden, Germany. The First Strand cDNA Synthesis Kit was obtained from Roche, Germany. Microchamber cell culture slides for CLSM studies were purchased from Nunc Lab-Tek, Naperville, IL, USA. Specialized cell culture glass bottom dishes (P45G-0-14-C-gm) for cell migration assays were ordered from MatTek Corporation, Ashland, USA. The EGF-R promoter luciferase reporter gene plasmid was a kind gift by Prof. Dr. Ester Gonzalez, Saint Louis University, Division of Nephrology, Saint Louis, USA.

2.2. Cell culture

Origin and cultivation of the human ovarian cancer cell line OV-MZ-6 as well as its integrin expression and adhesion profiles had been reported earlier. Stable OV-MZ-6 cell transfectants overexpressing αvβ3 were generated as previously described [44,50].

2.3. Reverse transcriptase PCR

RNA of ovarian cancer tissues was prepared using standard procedures and transcribed into cDNA. The whole coding region of KAI1 was amplified using flanking primers as previously described [33] which yielded an 801 bp fragment for KAI1-WT and a 717 bp fragment for KAI1-SP which lacks exon 7. Nested primer pairs generating 255 bp and 171 bp fragments [33] were further used to screen ovarian cancer tissues for endogenous KAI1-WT and/or KAI1-SP expression. Hypoxanthine–guanine phosphoribosyltransferase (HPRT) served as housekeeping gene by applying the primers 'HPRT-forward': 5′-TATT GTAATGACCAGTCAACAG-3′ and 'HPRT reverse': 5′-GGTCCTTTTCACCA GCAAG-3′.

2.4. Cloning of KAI1-SP cDNA

A cDNA preparation of an ovarian cancer tissue exhibiting high endogenous KAI1-SP levels was used to generate the KAI1-SP transcript as described for KAI1-WT cDNA cloning [46]. The insert was finally checked for correct sequence using an ABI 3100 capillary sequencer (Applied Biosystems, Darmstadt, Germany).

2.5. Stable transfection of human ovarian cancer cells with KAI1 cDNA

Stable transfection of human OV-MZ-6 ovarian cancer cells, selection and isolation of transfected individual cell clones were performed upon hygromycin selection as described earlier [46,50].

2.6. Detection of KAI1 proteins by immunocytochemical staining

Human OV-MZ-6 cells transfected with expression vectors either encoding KAI1-WT or KAI1-SP were grown on microchamber cell culture slides and immunodetection of the two KAI1 proteins conducted by confocal laser scanning microscopy (CLSM) as described earlier [46].

2.7. Flow cytofluorometry (FACS)

2.7.1. KAI1 proteins

Human OV-MZ-6 cells, either transfected with KAI1-WT or KAI1-SP, were harvested, fixed in 2% (w/v) PFA, blocked, and incubated with mAb directed to KAI1 (# TS82b). Signals were detected upon addition of Alexa-488-conjugated goat-anti-mouse IgG.

2.7.2. Integrin αvβ3

Cellular expression of αvβ3 was analyzed as described earlier [51,52].

2.8. Immunocytochemical double staining of colocalization of KAI1 proteins with αvβ3 or E-cadherin

Human OV-MZ-6 cells overexpressing αvβ3 and either KAI1-WT or KAI1-SP were passed to VN-coated microchamber cell culture slides. Staining procedures were performed and evaluated by CLSM as described earlier [46]. Monoclonal Ab directed to KAI1 as well as pAb to αv or pAb to E-cadherin were simultaneously incubated with the cells in PBS, 1% (w/v) BSA, for 2 h at RT, and detected by the addition of secondary Ab Alexa-488-labeled goat-anti-mouse and Alexa-568-labeled goat-anti-rabbit IgG for 45 min. Colocalization was documented by

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