Matrix Biology 30 (2011) 207–217

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

Syndecan-1 and -4 differentially regulate oncogenic K-ras dependent cell invasion into collagen through α2β1 integrin and MT1-MMP

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ARTICLE INFO

Article history:
Received 17 October 2010
Received in revised form 24 February 2011
Accepted 4 March 2011

Keywords:
K-ras
Integrin
MT1-MMP
Syndecan
Invasion
Contraction

ABSTRACT

Syndecans function as co-receptors for integrins on different matrixes. Recently, syndecan-1 has been shown to be important for α2β1 integrin-mediated adhesion to collagen in tumor cells by regulating cell adhesion and migration on two-dimensional collagen. However, the function of syndecans in supporting α2β1 integrin interactions with three-dimensional (3D) collagen is less well studied. Using loss-of-function and overexpression experiments we show that in 3D collagen syndecan-4 supports α2β1-mediated collagen matrix contraction. Cell invasion through type I collagen containing 3D extracellular matrix (ECM) is driven by α2β1 integrin and membrane type-1 matrix metalloproteinase (MT1-MMP). Here we show that mutational activation of K-ras correlates with increased expression of α2β1 integrin, MT1-MMP, syndecan-1, and syndecan-4. While K-ras-induced α2β1 integrin and MT1-MMP are positive regulators of invasion, silencing and overexpression of syndecans demonstrate that these proteins inhibit cell invasion into collagen. Taken together, these data demonstrate the existence of a complex interplay between integrin α2β1, MT1-MMP, and syndecans in the invasion of K-ras mutant cells in 3D collagen that may represent a mechanism by which tumor cells become more invasive and metastatic.

1. Introduction

In tissues cells form adhesions to three-dimensional (3D) flexible connective tissue rather than rigid two-dimensional (2D) matrix-coated surfaces. Therefore, it is not surprising that adhesion receptor induced signalling events between these two situations seem to be fundamentally different (Cukierman, et al., 2002). Two major families mediating contacts to the ECM are integrins and syndecans. These adhesion receptors recognize distinct classes of ligands:integrins bind to peptide sequences within the ECM proteins (Hynes, 2002) whereas the glycosaminoglycan chains (GAGs) of syndecans bind to heparin-binding sites in ECM molecules (Bernfield, et al., 1999). Integrin α2β1 is an important cellular collagen receptor (White, et al., 2004). However, this is cell type dependent and recent data demonstrate that in mouse embryonic fibroblasts α11β1 is the major collagen receptor (Carracedo, et al., 2010; Popova, et al., 2007). Integrin-mediated adhesion to type I collagen is known to trigger different cellular responses depending on the architecture of the matrix. Adhesion to 2D collagen-coated surfaces induces ERK activation and cell cycle progression in rat hepatocytes (Fassett, et al., 2003) while fibrillar 3D collagen blocks proliferation and attenuates Akt and IFNγ signalling in fibroblasts, human osteosarcoma Saos-2 cells, HEK293 cells, mouse embryonic fibroblasts, and human arterial smooth muscle cells (Fringer and Grinnell, 2001; Ivaska et al., 2002; Ivaska et al., 2003; Koyama, et al., 1996). In part this is due to the different mechanical tensions subjected to the cells by these two matrix conditions (Cukierman et al., 2002), but variation in usage of adhesion receptors could also be involved. Expression of syndecan-4 is induced in pathological scars (Chen et al., 2005) and engagement of syndecan-4 reduces cell motility and induces contractility via α5β1 integrin within a 3D fibrin–fibronectin matrix (Midwood et al., 2004). Therefore, syndecan family members could play a role in modulating integrin function in 3D collagen as well.

Syndecans have been shown to modulate integrin-mediated adhesion to different ECM proteins. Syndecan-4 has been shown in several studies to function as a co-receptor for α5β1 integrin. Cell spreading on fibronectin and the formation of stress fibers are also dependent on the ligation of integrin and syndecan. These joint signals are regulated in a RhoA-dependent manner (Bass and Humphries,

Abbreviations: 3D, three-dimensional; 2D, two-dimensional; ECM, extracellular matrix; MT1-MMP, membrane type-1 matrix metalloproteinase; MLC, myosin light chain; PKCα, protein kinase α.

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doi:10.1016/j.matbio.2011.03.003

2002; Bass et al., 2008; Saoncella et al., 1999; Woods et al., 1986). In addition, protein kinase Cα (PKCα) has been shown to be activated by syndecan-4 and α5β1 to induce stress fibers and focal adhesions (Mostafavi-Pour et al., 2003; Oh et al., 1997).

On vitronectin, the ectodomain of syndecan-1 is able to activate both αvβ3 and αvβ5 integrins but via distinct mechanisms. Activation of αvβ3 requires the HS chains of the ectodomain whereas in the case of αvβ5 these are dispensable (Beauvais et al., 2004; McQuade et al., 2006). Cell adhesion to 2D collagen has been recently shown by us and others to require co-operation between α2β1 integrin and syndecan-1. Syndecan-1 expression enhances binding to 2D collagen as well as activation of RhoA and suppression of Rac downstream of α2β1 integrin (Ishikawa and Kramer, 2010; Vuoriluoto et al., 2008). However, it remains unclear whether syndecans act as α2β1 co-receptors also in 3D collagen.

Mutations activating K-ras are found in many cancer types (Karnoub and Weinberg, 2008). However, the pathways regulating cell invasion of K-ras transformed cells are not fully understood. Here we show that expression of mutant K-ras regulates expression of α2 integrin, syndecan-1, syndecan-4, and MT1-MMP in an invasive breast cancer cell line. Furthermore, we demonstrate that mutant K-ras is important for the α2β1 integrin- and MT1-MMP-dependent invasion of these cells into collagen. Interestingly, silencing of syndecan-1 and -4 both induce cell invasion but with distinct characteristics. Silencing of syndecan-1 induces single-cell invasive migration whereas silencing of syndecan-4 induces collective invasion of cell strands.

2. Results

2.1. Oncogenic K-ras regulates invasion and gene expression of α2β1 integrin, MT1-MMP, and syndecan-1 and -4

Several gene expression signatures reflecting the activation state of an oncogenic pathway have been identified by analyzing gene expression profiles using cDNA microarrays. The oncogenic signature of mutant K-ras in human primary mammary epithelial cells contains altered expression of hundreds of genes, among them a putative upregulation of *ITGA2* (α2 integrin), *MMP14* (MT1-MMP), *SDC1* (syndecan-1), and *SDC4* (syndecan-4) (Bild et al., 2006). Integrin α2β1 is a receptor for type I collagen (White et al., 2004) and MT1-MMP is a transmembrane collagenase important for cell invasion through collagen (Sabeh et al., 2004). Furthermore, we have recently shown that lack of glycosaminoglycans renders α2β1 expressing cells unable to bind to collagen and that cell surface proteoglycan syndecan-1 supports α2β1-mediated cell adhesion to 2D collagen (Vuoriluoto et al., 2008). Thus, we became interested to investigate whether mutant K-ras would regulate cell invasion to collagen via regulation of α2β1-integrin, MT1-MMP, and syndecans. MDA-MB-231 human breast adenocarcinoma cells were chosen as a model since these cells carry an activating mutation of K-ras (G13D) and express all these proteins at relatively high levels (Beauvais and Rapraeger, 2003; Jones, et al., 1997; Kozma, et al., 1987; Munoz-Najar, et al., 2006; Vuoriluoto, et al., 2008). In line with the published K-ras signature (Bild et al., 2006) RNAi-mediated silencing of *KRAS* (siRNA1) in these cells resulted in a significant downregulation of also *ITGA2*, *MMP14* (gene for MT1-MMP), *SDC1*, and *SDC4* expression (Fig. 1A). This indicates that mutant K-ras is indeed associated with the expression of these genes and that loss of oncogenic K-ras in breast adenocarcinoma cells results in down-regulation of their expression.

Fibrillar collagen gels prepared from pepsin-extracted collagen are devoid of the naturally occurring intermolecular crosslinks and cells are able to invade into this type of collagen independently of collagenases (Sabeh et al., 2004, 2009). In contrast, membrane-anchored MT1-MMP pericellular collagenolytic activity is a prerequisite for tumor cell invasion through matrix prepared from acid-extracted collagen, where the sites for intercellular crosslinks have been preserved during isolation (Sabeh et al., 2004, 2009). Due to the different biological requirements for cell invasion into these structurally distinct matrixes, we chose to investigate cell invasion using both systems. We found that MDA-MB-231 cells invaded efficiently into both pepsin-extracted as well as cross-linked 3D collagen matrixes. Importantly, transfection with K-ras siRNA2 (Supplementary Fig. 1) inhibited cell invasion into both types of collagen (Fig. 1B, C). These data are in line with the gene expression changes obtained with K-ras siRNA1 (Fig. 1A). Furthermore, the invasion was absolutely dependent on α2β1 integrin since α2 integrin function blocking antibody completely inhibited invasion (Fig. 1D). These data demonstrate that mutant K-ras functionally contributes to the high invasive capacity of MDA-MB-231 cells in collagen. This would correlate with mutant K-ras inducing gene expression of α2β1 integrin in these cells.

2.2. GAGs are required for α2β1 integrin-mediated collagen contraction

Recently, matrix contraction has been shown to correlate with the ability of cancer-associated fibroblasts to enable cancer cell invasion into collagen (Gaggioli et al., 2007). However, whether this applies to invasive carcinoma cells is not clear. In a free floating 3D collagen lattice, cells have been shown to remodel the matrix by contracting it in an α2β1-dependent manner (Langholz et al., 1995). However, more recently collagen-binding integrin α11β1 has also been implicated in collagen gel contraction by fibroblasts (Carracedo et al., 2010; Popova et al., 2007). We found that integrin α2β1 was important for collagen matrix contraction by MDA-MB-231 human breast adenocarcinoma cells stably transfected to express the control GFP vector. GFP expressing cells were capable of contracting the matrix and this was α2β1 integrin-dependent since α2 function blocking antibody impaired collagen contraction by MDA-MB-231 cells (Fig. 2A). Thus, both collagen invasion and matrix contraction are α2β1-dependent in these cells.

To investigate the possible contribution of syndecan-1 and -4 in cell–collagen interactions in 3D we first analyzed the requirement for glycosaminoglycans (GAGs) in collagen matrix contraction. To this end we exploited wild-type and mutant CHO cells. The CHO745 mutant cell line is defective in the biosynthesis of both HS and CS GAG chains (Esko et al., 1985) and CHO cells have no endogenous collagen-binding integrins. Stable CHO wild-type and CHO745 cell lines expressing α2β1 integrin at equal levels (Vuoriluoto et al., 2008) were embedded in a collagen gel. CHOwtα2 contracted the matrix efficiently, whereas contraction by CHO745α2 cells was severely impaired (Fig. 2B). Taken

Fig. 1. Oncogenic K-ras regulates invasion and expression of invasive genes. (A) TaqMan qRT-PCR analysis shows the expression of *ITGA2* (α2 integrin), *MMP14* (MT1-MMP), *SDC1* (syndecan-1), and *SDC4* (syndecan-4) in MDA-MB-231 cells after silencing of K-ras (K-ras siRNA1) relative to scrambled siRNA transfected cells. Cellular RNA was collected 48–72 h after transfection. Results were normalized using GAPDH as an endogenous control and shown are the remaining mRNA levels relative to Scr siRNA transfected cells (mean ± SEM of 4–8 experiments with 2–3 parallels). (B–C) MDA-MB-231 GFP cells were transfected with a scrambled (siScr) or an siRNA targeting K-ras (siKras siRNA2) and allowed to invade into (B) 3D pepsin-extracted collagen for 4 days or (C) cross-linked, acid-extracted collagen for 7 days, fixed, and imaged. Shown are the quantifications of two experiments in both matrixes (mean ± SEM; 2–4 parallels imaged at 3–4 different positions; $^{***}p<0.001$; $^{*}p<0.02$) and representative images. The z axis maximum projections are 380 μm thick and the dashed lines indicate the top of the collagen gel. (D) MDA-MB-231 GFP cells were allowed to invade with or without an α2 function blocking antibody (anti-α2) into 3D pepsin-extracted collagen for 4 days and into cross-linked, acid-extracted collagen for 7 days, fixed, and imaged. Shown are examples of z axis maximum projections of 240 μm thick images and individual 5 μm stack images starting from the top of the collagen matrix (indicated with a dashed line). The experiment was performed twice in pepsin-extracted collagen and four times in cross-linked, acid-extracted collagen.