European Journal of Cell Biology 89 (2010) 843–852

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.de/ejcb

Native type IV collagen induces cell migration through a CD9 and DDR1-dependent pathway in MDA-MB-231 breast cancer cells

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ARTICLE INFO

Article history:
Received 21 November 2009
Received in revised form 2 July 2010
Accepted 2 July 2010

Keywords:
Breast cancer
CD9
DDR1
type IV Collagen

ABSTRACT

CD9 is a member of the tetraspanin family and is widely expressed in the plasma membrane of several cell types as well as malignant cells. CD9 associates with a number of transmembrane proteins, which facilitates biological processes, including cell signaling, adhesion, migration and proliferation. DDR1 is activated by native type IV collagen and overexpressed in human breast cancer. Type IV collagen is the main component of basement membranes, and may interact with cell surface biomolecules, promoting adhesion and motility. However, the role of DDR1 and type IV collagen in the regulation of CD9-cell surface levels and migration in breast cancer cells has not been studied in detail. We demonstrate here that native type IV collagen induces a transient increase of CD9-cell surface levels through a DDR1-dependent pathway in MDA-MB-231 breast cancer cells, as revealed by flow cytometry and Western blotting using specific antibodies that recognize CD9. In contrast, type IV collagen does not induce any increase of CD9-cell surface levels in the mammary non-tumorigenic epithelial cells MCF10A and MCF12A. Transient increase of CD9-cell surface levels is coupled with clathrin-mediated endocytosis and it is dependent of DDR1 expression. In addition, type IV collagen induces cell migration through a DDR1 and CD9-dependent pathway. In summary, our data demonstrate, for the first time, that native type IV collagen induces a transient increase of CD9-cell surface levels and cell migration through a DDR1 and CD9-dependent pathway in MDA-MB-231 breast cancer cells.

Introduction

Extracellular matrix (ECM), including basement membrane (BM), is a network of interacting molecules such as collagens, fibronectin and laminin, which mediates cellular processes like migration, growth and differentiation (McClay and Ettensohn, 1987; McDonald, 1988). BM is the first barrier that cells must degrade to produce metastasis, which is composed of entactin, proteoglycans and other glycoproteins. It is also rich in laminin and type IV collagen and regulates epithelial cell morphology, growth, differentiation and apoptosis in mammary epithelial cells, whereas it promotes adhesion and motility of various normal and transformed cells (Aumailley and Gayraud, 1998; Boudreau et al., 1995; Muschler et al., 1999; Weaver et al., 2002). Type IV collagen is a trimer composed of three monomeric chains with the usual composition of two α1(IV) and one α2(IV) (Gunwar et al., 1998; Timpl et al., 1985), provides the structural framework of all BMs and interact with cell surface biomolecules including integrins, proteoglycans and discoidin domain receptors (Miles et al., 1994; Vogel et al., 2006; Wisdom et al., 1992; Yoshinaga et al., 1993).

Discoidin domain receptors (DDRs) are a family of receptor tyrosine kinases (RTKs) that are characterized by an extracellular domain of strong homology to the *Dictyostelium discoideum* protein discoidin (Vogel et al., 2006; Alves et al., 1995). There are two known members of the DDR family, namely DDR1 and DDR2. DDR1 is activated by collagen types I–V and is overexpressed in ovary, esophagus and brain cancers, suggesting a role in tumor progression (Vogel et al., 2006; Johnson et al., 1993; Laval et al., 1994; Ram et al., 2006; Alves et al., 1995). DDR2 is activated by fibrillar collagens (types I and III), and its transcripts are present in heart, lung, brain, kidney, skeletal muscle and in the surrounding stromal cells of tumors. DDRs activation is a slow process, sustained for several days and requires the native triple-helical conformation of collagens, whereas they are not activated for other members of the ECM, including fibronectin and laminin (Vogel, 1999; Vogel et al., 2006).

Tetraspanins are a large family of ubiquitously expressed integral membrane proteins characterized by the presence of four transmembrane domains delimiting two extracellular regions of unequal size and three short intracellular regions. Tetraspanins are

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0171-9335/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.ejcb.2010.07.004

implicated in a large variety of physiological and pathological processes, and there are at least 32 distinct members in mammals, CD9, CD37, CD53, CD81, CD151, TM4SF2 and ROM being the best characterized (Maecker et al., 1997; Hemler, 2005; Hemler et al., 1996; Boucheix et al., 2001; Levy and Shoham, 2005). CD9 is a 24- to 27-kDa protein that is widely expressed in the plasma membrane of several cell types and in most of the tumoral cell lines. CD9 is able to associate with other transmembrane proteins and forms functional complexes that facilitate diverse functions, including cell signaling, adhesion, motility, proliferation, differentiation, sperm–egg fusion and metastasis (Berditchevski and Odintsova, 1999; Hemler, 2003; Boucheix et al., 2001; Miyake et al., 1991, 2000; Miyado et al., 2000).

In the present study, we report that native type IV collagen induces a transient increase of CD9-cell surface levels and cell migration through a DDR1 and CD9-dependent pathway in MDA-MB-231 breast cancer cells.

Materials and methods

Materials

Triton X-114 and X-100, recombinant epidermal growth factor (EGF), bovine serum albumin (BSA), chlorpromazine, ammonium chloride and chloroquine diphosphate salt were obtained from Sigma (St. Louis, MO). ECL reagent, CD9 monoclonal antibody (Ab) C-4, DDR1 polyclonal Ab H-126, major histocompatibility complex class I (MHC-I) monoclonal Ab BRA23/9, DDR1 siRNA, CD9 siRNA and scramble siRNA kits were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). CD9 monoclonal Ab MM2/57 was obtained from Biosource (Camarillo, CA). β-Subunit of Na^+/K^+ ATPase monoclonal Ab was kindly provided by Dr. Gerardo Contreras (Cinvestav-IPN, Mexico). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or rabbit was from Zymed (San Francisco, CA). Complete protease inhibitors cocktail was obtained from Roche (Mannheim, Germany). Micro Bradford protein assay reagent was from Bio-Rad (Hercules, CA). All other reagents used were of the highest grade available.

Extraction and purification of type IV collagen

Type IV collagen was extracted and purified from human placenta as described previously (Munoz et al., 1982; Santoro and Cunningham, 1982). Purity of type IV collagen was verified by SDS-PAGE and Coomassie staining. Our preparation showed a typical pattern of type IV collagen without any contaminant bands (Timpl et al., 1985).

Cell culture

MDA-MB-231 and MCF-7 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/l sodium bicarbonate, 5% fetal bovine serum (FBS), in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

The non-tumorigenic epithelial cell lines MCF10A and MCF12A were cultured in DMEM/F12 (3:1 and 1:1, respectively). Medium was supplemented with 5% FBS, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant EGF and antibiotics, in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

For experimental purposes, MDA-MB-231, MCF-7, MCF10A and MCF12A cells were starved in DMEM without FBS, insulin, hydrocortisone and EGF for 12 h before treatment with inhibitors, Abs and/or type IV collagen.

Cell stimulation

Cell stimulation was performed as described previously with some modifications (Shrivastava et al., 1997; Vogel et al., 1997). Briefly, confluent cultures were washed twice with DMEM without serum, equilibrated in the same medium at 37 °C for at least 30 min and then treated with inhibitors, Abs and/or native type IV collagen in solution for the times or concentrations indicated. Stimulation was terminated by aspirating the medium, and then integral membrane proteins were obtained or flow cytometry analysis was performed.

Separation of integral membrane proteins

Separation of integral membrane proteins was performed as described previously with some modifications (Friedrichson and Kurzchalia, 1998; Bordier, 1981). Briefly, 2×10^6 cells were washed twice with ice-cold PBS and lysed for 20 min at 4 °C and 10 min at 37 °C in 1 ml of Triton X-114 lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-114 and protease inhibitors). Lysates were collected, chilled on ice and cleared by 15 min centrifugation at 15,000 × *g*. Cleared lysates were subjected to temperature-induced phase separation for 5 min at 37 °C. Aqueous and detergent phases were separated by centrifugation for 3 min at 13,000 rpm at room temperature. To the detergent phases, 0.9 ml Triton X-114 wash buffer (150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.06% Triton X-114 and protease inhibitors) was added and vortexed before centrifugation for 15 min at 15,000 g (at 4 °C) and an other two rounds of phase separation were performed. Proteins were precipitated from detergent phases with cold acetone for 3 h at −20 °C. Pellets of integral membrane proteins were reconstituted in radioimmune precipitation assay (RIPA) buffer containing 50 mM HEPES (pH 7.4), 150 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1.5 mM $MgCl_2$, 0.1% SDS and protease inhibitors.

Western blotting

Equal amounts of protein were separated by SDS-PAGE using 12% separating gels or by 6–12% gradient gels, followed by transfer to nitrocellulose membranes. After transfer, membranes were blocked using 5% non-fat dried milk in phosphate buffered saline (PBS) (pH 7.2)/0.1% Tween 20 and incubated overnight at 4 °C with the primary Ab as indicated. The membranes were washed three times with PBS/0.1% Tween 20 and then incubated with secondary Abs (HRP-conjugated goat anti-mouse or rabbit, 1:5000) for 2 h at 22 °C. After washing three times with PBS/0.1% Tween 20, the immunoreactive bands were visualized using ECL detection reagents. Autoradiograms were scanned and the labeled bands were quantified using the Kodak image analysis software (Eastman Kodak Company).

Immunoprecipitation

MDA-MB-231 cells were washed twice with DMEM without serum, equilibrated in the same medium at 37 °C for at least 30 min and then treated with type IV collagen in solution for the times indicated. The stimulation was terminated by aspirating the medium and solubilizing the cells in 1 ml of Triton X-114 lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-114 and protease inhibitors). Integral membrane proteins were obtained from cell lysates and then reconstituted in ice-cold RIPA buffer. The protein level of each sample was determined by the micro Bradford protein assay. Equal amounts of protein (500 μg) were immunoprecipitated at 4 °C overnight with protein A/G-agarose linked to 1 μg polyclonal anti-DDR1 Ab. Immunoprecipitates were