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CD44 regulates cell migration in human colon cancer cells via Lyn kinase and AKT phosphorylation

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Abstract

Colon cancer is among the leading causes of cancer death in North America. CD44, an adhesion and antiapoptotic molecule is overexpressed in colon cancer. Cofilin is involved in the directional motility of cells. In the present study, we looked at how CD44 might modulate cell migration in human colon cancer via cofilin. We used a human colon cancer cell line, HT29, which expresses CD44, HT29 where CD44 expression was knocked down by siRNA, SW620, a human colon cancer cell line which does not express CD44, stably transfected exons of CD44 in SW620 cells and the colon from CD44 knockout and wild-type mouse. Western blot analysis of siRNA CD44 lysates showed increased level of AKT phosphorylation and decreased level of cofilin expression. Similar results were also observed with SW620 cells and CD44 knockout mouse colon lysates. Experiments using the AKT phosphorylation inhibitor LY294002 indicate that AKT phosphorylation downregulates cofilin. Immunoprecipitation studies showed CD44 complex formation with Lyn, providing an essential link between CD44 and AKT phosphorylation. LY294002 also stabilized Lyn from phosphorylated AKT, suggesting an interaction between Lyn and AKT phosphorylation. Immunocytochemistry showed that cofilin and Lyn expression were downregulated in siRNA CD44 cells and CD44 knockout mouse colon. siRNA CD44 cells had significantly less migration compared to HT29 vector. Given the well-defined roles of CD44, phosphorylated AKT in apoptosis and cancer, these results indicate that CD44-induced cell migration is dependent on its complex formation with Lyn and its consequent regulation of AKT phosphorylation and cofilin expression.

Keywords: CD44; AKT phosphorylation; Lyn kinase; Cofilin; Cell migration; HT29; SW620 colon cancer cells

Introduction

Colon carcinogenesis involves the transformation of the normal colonic epithelium to an adenoma and then to a carcinoma. This sequence is characterized by a large number of molecular alterations, converging to alterations in epithelial cell differentiation, proliferation, migration and apoptosis. The maintenance of the colonic epithelium involves important cellular interactions with its environment and this is facilitated by adhesion molecules. CD44 is a unique adhesion molecule in that it facilitates both cell–cell and cell–matrix interactions (reviewed in Rudzki and Jothy, 1997; Ponta et al., 2003). For invasion to proceed, a subpopulation of cancer cells must interact, modify, and actively migrate through the barrier of extracellular matrix, proliferate and establish a colony in the new ectopic location (Chambers et al., 2002). The migrations of tumor cells within tissues were thought to employ a similar mechanism as in normal non-neoplastic cells which have a motile phenotype. However, recent studies have started to cast a doubt on this assumption (Chambers et al., 2002; Friedl and Wolf, 2003. A common feature is that cell migration is a process controlled by both internal and external signals (Dormann and Weijer, 2006). Dysregulation of these signals could underlie the aberrant cell migration observed with cancer cells.

Actin cytoskeleton directs a number of cellular events related to cell motility as well as maintenance of specialized structures in the cell under differentiated environment. A number of cell

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adhesion proteins bind directly or indirectly to actin, among them are E-cadherin and CD44 (Ponta et al., 2003). CD44 linkage to actin as well as to various types of structural and signaling proteins leads to dynamic interactions of migrating cells with ECM substrates. CD44 is thus not only a receptor for the ECM molecules but is also a platform for the generation of signal transmission by assembling bioactive molecules on the cell surface like growth factors (bFGF, HEGF, FGF8, cytokines (osteopontin), receptors (ERb4) and matrix metalloproteinases (MMPs) (Wolff et al., 1999; Mori et al., 2002; Yu et al., 2002; Ponta et al., 2003). Remarkably, numerous splice variants of CD44 are markedly overexpressed in a large number of malignancies, especially carcinomas. In a pancreatic cancer animal model, it was observed that overexpression of a specific splicing variant isoform of CD44 was involved in the production of metastasis (Gunthert et al., 1991).

Src family of non-receptor tyrosine kinases (Lyn, Fyn, c-Src) modulate several cellular processes, viz., cell adhesion, migration, proliferation, and differentiation (Thomas and Brugge, 1997; Bjorge et al., 2000; Summy and Gallick, 2003). Fyn has been shown to promote differentiation of oligodendrocytes (Osterhout et al., 1999; Wolf et al., 2001) and hemidesmosome disassembly in squamous carcinoma cells (Mariotti et al., 2001). c-Src controls the cytoskeletal reorganization of fibroblasts (Summy et al., 2003). Lyn promotes migration in human glioblastoma cells (Ding et al., 2003). Lyn has also been implicated in offering chemoresistance in colon carcinoma cells via AKT phosphorylation triggered by a CD44 survival pathway (Bates et al., 2001).

AKT, a serine/threonine kinase is a well-known mediator for cell survival in response to growth factor signaling and cellular adhesion (Dudek et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kelley et al., 1999). Akt has also been reported to suppress apoptosis through several mechanism, through BAD (Datta et al., 1997), caspase 9 (Cardone et al., 1998) and GSK-3 (Pap and Cooper, 1998). More recently, it has been implicated as a negative regulator of cell migration and invasion in breast cancer cells (Yoeli-Lerner et al., 2005).

Cofilin is the key regulator required for actin polymerization/depolymerization. Cofilin contributes to directional motility of cells (Ghosh et al., 2004), thus, could have important implications to the motility and invasion of cancer cells. There is no direct evidence to suggest that the CD44 induces selection of metastatic prone cells. Our previous work has shown that the variant CD44 3–10v isoform modulates cofilin expression and phosphorylation (Subramaniam et al., 2005).

In this study, we used human colon cancer cells that highly express CD44, stable cell lines generated where CD44 was inhibited by siRNA, and murine colon from CD44 knockout and wild-type mice, to examine the role of CD44 in modulating cell migration.

Materials and methods

siRNA

siRNAs were synthesized by Dharmacon Inc. (Lafayette, CO). siRNA sequences targeting human CD44 was used in this study.

Antibodies

Mouse anti-human CD44 (MEM 263, Monosan, Sanbio, Uden, The Netherlands), mouse anti-human β-actin (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-Lyn and CD44 (HCAM DF1485) (Santa Cruz biotechnology, Santa Cruz, CA, USA), rabbit anti-phosphorylated AKT and total AKT (Cell Signaling technology, Beverly, MA, USA), rabbit anti-cofilin (Cytoskeleton, Denver, CO, USA) were used in the Western blot and immunohistochemistry studies. Secondary antibodies were goat anti-mouse and anti-rabbit FITC (Jackson Immunological Research Inc., PA, USA), goat anti-rabbit HRP and goat anti-mouse HRP (Promega, Madison, WI, USA).

Cell culture

HT29 and SW620 human colon cancer cells (ATCC, Manassas, VA) were grown using DMEM medium (Invitrogen, Burlington, ON, Canada) containing 10% FBS with penicillin/streptomycin antibiotics (100 U of penicillin, and 100 μg of streptomycin per milliliter). SW620 transfected with CD44 isoforms (standard isoform), 3–10v and 8–10v isoform and empty vector construct (V) were maintained as described (Wong et al., 2003). Optimem1, oligofectamine, glutamine, penicillin and streptomycin were obtained from Invitrogen.

Mice

CD44 −/− mice (C57BL/6J) were obtained from Dr. Tak Mak (Ontario Cancer Institute, Toronto, Canada). Age- and sex-matched wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA).

In vitro transfection of HT29 cells with siRNA

For stable transfection, HT29 cells were trypsinized and resuspended in fresh DMEM medium without antibiotics. $1–3\times10^4$ Cells (500 μl/well) were plated in 24-well plates a day prior to transfection, corresponding to a density of 30–50% at the time of transfection and transfected with lipofectamine and plasmid DNA vector that has the CD44 siRNA insert as per the manufacturer's protocol (Ambion, Austin, TX) with 1000 μg/ml of hygromycin as the antibiotic of choice for the selection of stable clones. After the selection process, the clones were maintained in 10% serum medium containing 500 μg/ml of hygromycin.

Detection of siRNA-mediated gene silencing by Western blot analysis

Cell lysates were prepared and Western blotting performed as described (Subramaniam et al., 2003). Briefly, the cells were lysed in lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% NP-40) containing the cocktail of protease inhibitors (PMSF, leupeptin, pepstatin and aprotinin). 2× Concentrated Laemmli sodium dodecyl sulfate (SDS) sample buffer was added to the cell lysates and incubated for 5 min in a boiling water bath, vortexed and appropriate amount loaded onto a 12% SDS-PAGE for Western blot analysis with appropriate primary antibody for CD44. Western blot analysis as outlined above was similarly done for AKT phosphorylation (AKT-P), total AKT, cofilin, and Lyn kinase. Representative Western blots from one of at least three similar experiments are shown.

PI3K inhibitor studies

HT29 vector and siRNA CD44 cells were cultured as described above. 2×10^6 Cells were plated in DMEM containing 10% FBS and 500 μg/ml of hygromycin. Twenty-four hours after the cells were seeded in a 10-cm culture dish, 20 μm of LY294002 (Calbiochem, San Diego, CA, USA) was added to the serum-free medium for a further 24 h or the carrier DMSO as control. Cells were then washed in PBS and lysed and used in Western blot analysis as described above.

Isolation of mouse colonic crypts

Two to three centimeters of mouse colon segments above the rectum was excised and protein isolation was performed for immunoblotting as previously described (Lakshman et al., 2004a). Colonic crypts were isolated from both