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Irradiation-tolerant lung cancer cells acquire invasive ability dependent on dephosphorylation of the myosin regulatory light chain

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ABSTRACT

Radiotherapy is one of the major treatment modalities for malignancies. However, cells surviving irradiation often display high levels of invasiveness. This study shows that irradiation-tolerant lung adenocarcinoma demonstrates high invasive capability depending on dephosphorylation of the myosin regulatory light chain (MRLC). In a collagen gel overlay condition, low-invasive subclones of lung adenocarcinoma (A549P-3) showed a round morphology and diphosphorylation of MRLC. In contrast, irradiation-tolerant A549P-3 cells (A549P-3IR) displayed high invasiveness and a lower level of MRLC diphosphorylation. In addition, inhibition of MRLC phosphatase activity decreased the invasive activity. These findings suggest that A549P-3IR cells acquire high invasiveness through MRLC dephosphorylation.

1. Introduction

Radiotherapy is often the primary treatment for many types of malignancies. However, it has been reported that growth and metastasis of solid tumors are induced after local tumor irradiation [1]. Recent studies have revealed that some cancer cells show higher motility and invasiveness after irradiation than prior to irradiation [2,3]. It has also been reported that irradiation of cancer cells leads to increased expression of adhesion molecules, integrins [4,5], or matrix metalloproteinases [6]. These findings indicate that irradiation of tumors can evoke malignant properties, although the details of the underlying mechanism is not well understood.

Contractile force is important for many physiological functions such as cell migration, cytokinesis, and morphological change. Contractile force is generated by the contractility mediated by actomyosin, composed of actin filaments and myosin II. In this process, the myosin regulatory light chain (MRLC) is essential for myosin motor activity, and phosphorylation of Ser19 and/or Thr18 of MRLC is crucial for its activation [7]. Contractile forces also play an important role in cell migration on a 2D substrate. MRLC diphosphorylation induced by RhoA-dependent ROCK activity triggers cellular contractile forces [8,9]. On the other hand, in a 3D environment, the relationship between cell migration and cellular contractile forces is not well studied.

In this study, we investigated the contribution of contractile forces generated by diphosphorylation of MRLC to the invasive behavior of irradiation-tolerant cancer cells in a 3D environment. Our previous study revealed that irradiation-tolerant lung adenocarcinoma cells show integrin β1-dependent invasive activity in a 3D collagen matrix [10]. We show here that high-invasive irradiation-tolerant lung cancer cells demonstrate a lower level of MRLC diphosphorylation than that of low-invasive lung cancer cells in a 3D collagen matrix, and that the invasiveness is dependent on integrin β1 activity. We also show that constitutive phosphorylation of MRLC, by use of a phosphatase inhibitor, decreases the invasive activity of irradiation-tolerant cells.

2. Materials and methods

2.1. Cell culture

The lung adenocarcinoma A549 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). A

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subclonal A549 cell line (P-3) and irradiation-tolerant P-3 cell line (IR) were established, as previously reported [10]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences Inc., Lenexa, KS) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA). Cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

2.2. Reagents

We used Y27632 (Sigma) to inhibit Rho kinase (ROCK) activity and to reduce MRLC phosphorylation. We used calyculin A (CA; Sigma) to inhibit phosphatase activity of myosin light chain phosphatase (MLCP) [11]. To inhibit integrin β1 activity, we used monoclonal antibody AIIB2 [12] (purchased from the Developmental Studies Hybridoma Bank at the University of Iowa). We used rat serum IgG (I8015, Sigma) as a control antibody. We made a 1.6 mg/ml collagen type I gel with Cellmatrix Type I-P (Nitta Gelatin Inc., Osaka, Japan). For immunofluorescence staining of F-actin, AlexaFluor-488 phalloidin (Invitrogen) was used at a 1:1000 dilution. Phospho-myosin light chain 2 (Ser19/Thr18) antibody (#3674; Cell Signaling Technology, Beverly, MA) was used as a primary antibody for staining of diphosphorylated MRLC (PP-MRLC) at a 1:150 dilution. AlexaFluor-594 goat anti-rat IgG (H + L; Invitrogen) was used as a secondary antibody for PP-MRLC staining at a 1:500 dilution.

2.3. Immunofluorescence staining

A glass dish of 8.0 mm radius was filled with 100 μl of collagen gel and 4×10^3 cells were seeded onto the collagen gel. After 24 h, 50 μl of collagen sol were poured on the dish and incubated at 37 °C for 30 min for gelation. This method allowed the cells to be cultured in a 3D substrate. After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 5% skim milk in PBS for 1 h. For PP-MRLC staining, cells were incubated overnight with the primary antibody in 0.5% skim milk in PBS at room temperature. Then, the cells were rinsed 3 times with 0.5% skim milk in PBS and incubated with secondary antibody in 0.5% skim milk in PBS for 1 h at room temperature. After reaction with secondary antibody, cells were rinsed 3 times with 0.5% skim milk in PBS. Fluorescence images were obtained using confocal laser scanning microscopy (C1 confocal imaging system; Nikon Instech., Tokyo, Japan). To analyze the relative PP-MRLC fluorescence intensity, we calculated fluorescence intensity and analyzed the intensity ratio of PP-MRLC per F-actin by Image-Pro software (Media Cybernetics Inc., Silver Spring, MD).

2.4. Roundness index analysis

A glass dish of 12.5 mm radius was filled with 250 μl of collagen gel, and 1×10^4 cells were seeded onto the collagen gel. After 24 h, 125 μl of collagen sol was poured on the dish and incubated at 37 °C for 30 min for gelation. Then, the cells were incubated with or without Y27632 (20 μM), CA (1 nM), or AIIB2 (30 ng/ml) at 37 °C. After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. For F-actin staining, the cells were incubated with AlexaFluor-488 phalloidin in PBS for 20 min at 37 °C. Then, the cells were rinsed 3 times. Fluorescence images were obtained by confocal laser scanning microscopy and the roundness index was calculated using Image-Pro software, as previously reported [10].

2.5. Time-lapse observation

A glass dish of 12.5 mm radius was filled with 1000 μl of collagen gel, and 1×10^4 cells were seeded onto the collagen gel. After 24 h, 500 μl of collagen sol were poured onto the dish and incubated at 37 °C for 30 min for gelation. Then, the dish was filled with culture medium and sealed with silicone grease to avoid exposure to air and a change in the pH of the media. A phase-contrast microscope (TE300, Nikon Instech.), equipped with a 10 × objective and a 37 °C acrylic resin incubation box, was used for time-lapse observations of cells. Image-Pro software was used for time-lapse observation, which involved capturing images every 5 min. After 12 h, the cells were treated with CA at a concentration of 1.5 nM and the observation was continued. After 12 h, we removed the media and washed the cells 3 times with fresh DMEM to remove CA from the dish. The observation was continued for 27 h after removing CA. The movie was edited from a series of the captured images.

2.6. Statistical analysis

Mean and standard deviation (S.D.) of fluorescence intensities were calculated, and statistically significant differences were identified using Student's *t*-tests.

3. Results

3.1. Diphosphorylation level of MRLC in P-3 and IR cells

To investigate differences in the contractile force between low-invasive subclonal A549 lung adenocarcinoma cells (P-3) and high-invasive irradiation-tolerant P-3 cells (IR) cultured in a 3D collagen gel matrix, we performed immunofluorescent staining of diphosphorylated MRLC (PP-MRLC). PP-MRLC intensity in IR cells was higher than that of P-3 cells (Fig. 1A). Analysis of the PP-MRLC fluorescence intensities indicated that the PP-MRLC intensity in P-3 cells was significantly higher than that in IR cells (Fig. 1B). The S.D. of intensities (shown as error bars in Fig. 1B) in P-3 cells was larger than that in IR cells. This is because some of P-3 cells had low PP-MRLC fluorescence intensity, whereas that in IR cells consistently demonstrated low PP-MRLC fluorescence intensity. These results suggested that high-invasive IR cells consistently generate lower contractile force compared to low-invasive P-3 cells.

3.2. Spindle morphology induced by dephosphorylation of MRLC in P-3 cells

We treated P-3 cells with Y27632, an inhibitor of MRLC phosphorylation, and observed the morphological changes in a 3D matrix. Treatment with Y27632 significantly reduced PP-MRLC fluorescence intensity in P-3 cells (Fig. S1). Analysis of roundness index revealed that most of the untreated P-3 cells showed a round morphology in a collagen gel. In contrast, a significant number of P-3 cells treated with Y27632 displayed spindle morphology (Fig. 2A and B). These results suggested that dephosphorylation of MRLC induces invasive morphology in P-3 cells cultured in a 3D matrix.

3.3. Invasive behavior induced by diphosphorylation of MRLC in IR cells

Next, we promoted diphosphorylation of MRLC in IR cells with CA, known to be an inhibitor of protein phosphatase [11], and observed the morphological changes. IR cells treated with CA showed a higher intensity of PP-MRLC immunofluorescence than did untreated IR cells in a collagen gel (Fig. S2). Roundness index indicated that the proportion of IR cells with a round morphology after treatment with CA was significantly greater than that in untreated IR cells in a 3D matrix (Fig. 2C and D).

We also observed the invasive behavior of IR cells in a 3D matrix. Under untreated condition, IR cells displayed spindle