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Title page

Integrin $\beta 1$ -dependent invasive migration of irradiation-tolerant human lung adenocarcinoma cells in 3D collagen matrix

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Abstract

Radiotherapy is one of the effective therapies used for treating various malignant tumors. However, the emergence of tolerant cells after irradiation remains problematic due to their high metastatic ability, sometimes indicative of poor prognosis. In this study, we showed that subcloned human lung adenocarcinoma cells (A549P-3) that are irradiation-tolerant indicate high invasive activity *in vitro*, and exhibit an integrin $\beta 1$ activity-dependent migratory pattern. In collagen gel overlay assay, majority of the A549P-3 cells displayed round morphology and low migration activity, whereas a considerable number of A549P-3IR cells surviving irradiation displayed a spindle morphology and high migration rate. Blocking integrin $\beta 1$ activity reduced the migration rate of A549P-3IR cells and altered the cell morphology allowing them to assume a round shape. These results suggest that the A549P-3 cells surviving irradiation acquire a highly invasive integrin $\beta 1$ -dependent phenotype, and integrin $\beta 1$ might be a potentially effective therapeutic target in combination with radiotherapy.

Keywords

lung adenocarcinoma, irradiation, 3D culture, collagen gel, invasion, integrin $\beta 1$

1. Introduction

Radiotherapy is commonly used for treating cancerous tumors. However, it has been reported that local tumor irradiation can lead to enhancement of metastases [1]. Recent studies indicate that tumors surviving irradiation acquire high metastatic ability *in vivo* [2], and the irradiation-tolerant cancer cells enhance cellular physiological activities, such as invasion, migration, and adhesion *in vitro* [3]. These results suggest that irradiation of tumors could induce malignant transformation and result in poor prognosis, although the detailed mechanism is not well understood.

Integrins are a family of heterodimeric transmembrane proteins, involved in the adhesion of cells to the extracellular matrices (ECMs), such as fibronectin, vitronectin, laminin, and collagen [4]. They also regulate cell proliferation, differentiation, and apoptosis [5], and mediate intracellular signals that control cytoskeletal organization [6]. Especially, integrin $\beta 1$ is one of the probable key players in regulating cell invasion. Human fibrosarcoma HT1080 cells overexpressing membrane type 1-matrix metalloproteinase (MT1-MMP) elongate a leading edge and invade into 3D collagen matrix by coclustering MT1-MMP and integrin $\beta 1$ in the direction of cell migration [7].

Several studies revealed that irradiation of cancer cells affects integrin expression. For instance, x-ray irradiation increases expression of functional integrins $\beta 1$ and $\beta 3$ in glioma cell lines in a dose-dependent manner [8]. In lung adenocarcinoma A549 and SKMES1 cells, x-ray irradiation also induces expression of functional integrin $\beta 1$ [9]. Furthermore, a signal through

the cytoplasmic domains of integrin $\beta 1$ contributes to cell survival after irradiation [10].

Previous studies on irradiation effects in cancer cells mainly focused on phenomena just after irradiation, or within a few days after irradiation. In this study, we established new cancer cell lines that survive after irradiation and investigated the properties of these irradiation-tolerant cells. We also showed that the irradiation-tolerant cells exhibit a highly invasive integrin $\beta 1$ -dependent phenotype in 3D collagen matrix.

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). A549 cells were cloned by limiting dilution, and a subclonal cell line (A549P-3) was established. 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 (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in a humidified incubator with 5% CO₂.

2.2. Irradiation of A549P-3 cells

Semi-confluent A549P-3 cells were irradiated at room temperature at a dose of 10 Gy. The cells were immediately dispersed with trypsin-EDTA and 5×10^6 cells were seeded into a culture flask (75 cm²-culture area). Cells were cultured for 30 days, and the surviving cells were harvested and designated as A549P-3IR.

2.3. Reagents

Binding of integrin β1 to ECM was inhibited by the anti-human integrin β1 monoclonal antibody—AIIB2 [11] purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. For immunofluorescence staining of integrin β1, AIIB2 was used as a primary antibody at a concentration of 350 ng/ml (1:80 dilution), and AlexaFluor-594 goat

anti-rat IgG (H+L) (Invitrogen) was used as a secondary antibody at a concentration of 10 µg/ml. AlexaFluor-488 phalloidin (Invitrogen) was used for F-actin staining. Cellmatrix Type I-P (Nitta Gelatin Inc., Osaka, Japan) was used at a concentration of 1.6 mg/ml to make the collagen gel, and Cellmatrix Type I-C (Nitta Gelatin) was used for collagen-coating on a glass substrate.

2.4. siRNA transfection

The 21-nucleotide siRNA duplex was synthesized using an *in vitro* Transcription T7 kit (TAKARA, Otsu, Japan). The target sequence to silencing integrin β1 was 5'-AACAACTGTGATAGATCCAAT-3' (sense sequence). Cells were transfected with the siRNA or a Random RNA duplex by Lipofectamine RNAiMAX Reagent (Invitrogen). Knockdown of integrin β1 mRNA was confirmed by RT-PCR. Cells, transfected with siRNA or random RNA, were fixed after 2 days and roundness index analysis was performed as described later.

2.5. Collagen gel overlay assay and time-lapse observation

A glass dish of 8.0 mm radius was filled with 400 µl of collagen gel, and 4×10^3 cells were seeded onto the collagen gel. After 24 h, 200 µl of collagen sol solution were poured onto the cells, and incubated for 60 min at 37°C to complete gelation. This method allows the cells to be cultured in a 3D environment. Then, the dish was filled with culture medium, and sealed with silicone grease to avoid changes in pH of the medium. A phase-contrast microscope, (TE300,

Nikon Instech., Tokyo, Japan) equipped with a 10 \times objective and kept at 37°C in an acrylic resin box, was used for time-lapse observations. Image-Pro software (Media Cybernetics Inc., Silver Spring, MD) was used for time-lapse observation by capturing images every 5 min. After 24 h, AIIB2 treatment (300 ng/mL) was performed, and the observation was continued for 24 h. Movies were edited from a series of capture images. Cell migration rate was calculated by measuring the displacement of the cell center every 30 min.

2.6. Fluorescence microscopy

A glass dish of 8.0 mm radius was filled with 100 μ l of collagen gel, and 4×10^3 cells were seeded onto the collagen gel. After 24 hours, 50 μ l of collagen sol were poured onto the cells and gelled at 37°C. After 24 h, cells were fixed with 4% paraformaldehyde in PBS and blocked with 0.5% bovine serum albumin in PBS (Sigma). For staining of integrin β 1 and F-actin, reactions of primary and secondary antibodies were performed at 37°C for 3 h, where dilution of AlexaFluor-488 phalloidin was 1:500. Fluorescence images were obtained by using confocal laser scanning microscopy (C1 confocal imaging system; Nikon Instech.).

2.7. Roundness index analysis

A glass dish of 17.5 mm radius was filled with 250 μ l of collagen gel, and 10^4 cells were seeded onto the collagen gel. After 24 h, 125 μ l of collagen sol were poured onto the cells and gelled at 37°C. Then, cells were cultured for 24 h in the 3D environment. For inhibition of integrin β 1 activity, 30 ng/ml AIIB2 monoclonal antibody was used. After culturing with AIIB2

for 24 h, cells were fixed with 4 % paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS and blocked with 0.5% bovine serum albumin in PBS. F-actin staining was performed for 1 h at room temperature, where dilution of AlexaFluor-488 phalloidin was 1:200. Fluorescence images were obtained by using confocal laser scanning microscopy, and roundness index was calculated by the Image-Pro software (Media Cybernetics Inc.). Briefly, the roundness index is calculated with the equation $4\pi S/L^2$, where S = surface area and L = perimeter length of the cell. If the roundness index is larger than 0.5, we categorized the cell as “round”, whereas if the roundness index is less than 0.5, the cell was considered “spindle”.

2.8. RT-PCR

Cells were lysed with ISOGEN (Wako, Osaka, Japan) for RNA extraction, and reverse transcription reaction was performed by ReverTra Ace qPCR RT kit (TAKARA). PCR reaction was performed with Taq Polymerase in ThermoPol Buffer (NEB, Ipswich, MA). Primers were as follows; GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (upper) and 5'-TCCACCACCCTGTTGCTGTA-3' (lower); and integrin β 1, 5'-AATGAAGGGCGTGTGGTAG-3' (upper) and 5'-CCTCGTTGTTCCCATTCACT-3' (lower).

3. Results

3.1. IR cells showed spindle morphology and high invasive activity in collagen gels

Subcloned adenocarcinoma cells (A549P-3; abbreviated as P-3) and irradiation-tolerant cells (A549P-3IR; abbreviated as IR) were cultured on a collagen-coated glass. There was no notable phenotypic difference between the two cell lines (Figure 1A). We then used collagen gel overlay method, which better mimics the *in vivo* environment than collagen-coated glass. This method allowed cells to be surrounded by the collagen matrix. Under the collagen gel overlay condition, we performed time-lapse observation of cells. P-3 cells displayed a spheroid morphology and low migration activity in the collagen gel (Figure 1B). On the other hand, IR cells showed spindle morphology and rapid changes in cell surface, alternating phases of elongation and retraction of protrusions, and significant increase in migration rate.

3.2. Invasiveness of IR cells was dependent on integrin $\beta 1$ activity

The localization of integrin $\beta 1$ was different in P-3 and IR cells in collagen gels. Immunofluorescence indicated that integrin $\beta 1$ in P-3 cells was diffused on the cell surface, whereas accumulation of integrin $\beta 1$ at the head of elongated cell surface was observed in IR cells (Figure 1C). Therefore, we predicted that integrin $\beta 1$ activity induced morphological changes and high migration activity in IR cells. Thus, in order to inhibit integrin $\beta 1$ activity, we treated cells with the monoclonal antibody AIIB2. Under the collagen gel overlay condition, IR cells treated with AIIB2 showed morphological changes allowing them to assume a round shape,

and cell migration was suppressed (Figure 2A), whereas P-3 cells treated with AIIB2 did not show any changes in cell shape and movement. Results from the motility assay suggested that treatment with the AIIB2 antibody reduces the migration rate of IR cells significantly (Figure 2B).

3.3 Spindle morphology was induced by integrin $\beta 1$ activity

We calculated the cell roundness index from F-actin stained images to quantify the proportion of cells exhibiting a particular morphology (Figure 3A). The results indicated that more than 70% of P-3 cells showed round morphology, whereas almost 70% of IR cells showed spindle morphology (Figure 3B). Furthermore, in IR cells, AIIB2 treatment induced changes in shape from spindle to round, and washout treatment subsequently recovered the spindle morphology.

siRNA transfection also induced round morphology in IR cells (Figure 4A). The results of RT-PCR indicated that collagen-coated glass and collagen gel induced the same levels of integrin $\beta 1$ - mRNA expression (Figure 4B). The siRNA transfection reduced mRNA expression of integrin $\beta 1$, and random RNA did not (Figure 4C). The proportion of IR cells with a round morphology was significantly reduced by siRNA transfection (Figure 4D). These results indicate that spindle morphology of IR cells was strongly dependent on integrin $\beta 1$ expression.

4. Discussion

Both P-3 and IR cells did not show marked difference in phenotype on a collagen-coated glass. However, when these cells were buried in a collagen gel, only IR cells indicated spindle morphology and migrated through the collagen matrix, depending on the activity of integrin $\beta 1$ that clustered in the direction of migration. These results demonstrate that irradiation-tolerant cells can acquire integrin $\beta 1$ activity and exhibit integrin $\beta 1$ -dependent increased invasiveness in a 3D environment.

As reported previously, co-expression of integrin $\beta 1$ and MT1-MMP in the direction of the migrating site were observed in highly invasive HT1080/MT1 cells [7]. Thus, it is possible that not only integrin $\beta 1$, but also MT1-MMP is activated by irradiation, resulting in increased migration of IR cells. Furthermore, the formation of membrane protrusions at the leading edge of migrating cells, as a result of actin reorganization, is regulated by one of the small GTPases, Rac [12], and activated Rac1 induces integrin-dependent migration in mammary epithelial cells [13]. Therefore, Rac activity might be necessary to induce invasiveness in IR cells.

Several studies show that irradiation augments the invasive character of some kinds of cancer cells. Mouse fibrosarcoma QRsP cells exhibit an enhanced invasive activity after survival through 10 Gy irradiation [14]. Moreover, in human non-small lung cancer H1299 cells, invasiveness is enhanced after 10 Gy irradiation [3]. Increase in invasive activity after irradiation might be a common phenomenon in cancer cells making it difficult to use radiotherapy as a single modality treatment for the complete cure of many malignant cancers.

It is also reported that a combined therapy of CNTO 95, the anti-human integrin αv monoclonal antibody and fractionated radiation is more effective than radiation therapy only [15]. Moreover, integrin $\beta 1$ plays an essential role in mediating post-radiation cancer cell survival [16]. Taken together, our results suggest that integrin $\beta 1$ can serve as a potentially effective therapeutic target in combination with radiotherapy.

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Figure Legends

Figure 1.

(A) Phase contrast images of A549P-3 (P-3) and A549P-3IR (IR) cells cultured on a collagen-coated glass. Bar = 250 μm . (B) Temporal sequence of phase contrast micrographs of P-3 and IR cells cultured in a collagen gel. Numbers in the images denote relative time from the start of observation. The white arrow indicates the direction of cell movement. Bar = 50 μm . (C) Fluorescent images of F-actin and integrin $\beta 1$ in P-3 and IR cells cultured in a collagen gel. Cross-sectional views of X-Z and Y-Z directions (axes) are shown together. The white arrow shows the accumulation site of integrin $\beta 1$. Bar = 20 μm .

Figure 2.

(A) Time-lapse phase contrast images of P-3 and IR cells cultured in a collagen gel treated with AIIB2. Numbers in the micrographs represent the observation time. Bar = 50 μm . (B) Statistical analysis of the migrating speed with which cells invade the collagen gel. The mean values of more than 12 cells are shown with S.D. (shown as error bars) from 3 independent experiments.

* $P < 0.01$.

Figure 3.

(A) Fluorescent images of F-actin in P-3 and IR cells cultured in a collagen gel. Numbers in the images show the roundness index (R.I.) of the cell morphology. Bar = 20 μm . (B) The

proportion of cells categorized as round and spindle. P-3 and IR cells were treated with AIIB2 or washed out after AIIB2 treatment. The mean values of more than 150 cells are shown with S.D. calculated from at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$.

Figure 4.

(A) Fluorescent images of F-actin in P-3 and IR cells cultured in a collagen gel transfected with random RNA or integrin $\beta 1$ siRNA. Bar = 20 μm . (B) mRNA expression of integrin $\beta 1$ detected by RT-PCR. P-3 and IR cells were cultured under 2 different conditions (glass; on a collagen-coated glass, gel; in a collagen gel) (C) mRNA expression in IR cells cultured in a collagen gel transfected with random RNA or integrin $\beta 1$ siRNA. (D) The proportion of cell morphology of P-3 and IR cells. The mean values of more than 150 cells are shown with S.D. calculated from at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$.

Supplementary Material

IR cells invading into a collagen gel. This movie was edited from the time-lapse micrographs captured every 5 min, where 1 s in the movie corresponds to 150 min in real time. IR cells were treated with AIIB2, inhibitor of integrin $\beta 1$ activity, during the observation, and then washed out twice. Screen width = 650 μm .

Fig. 1 S. Ishihara *et al.*

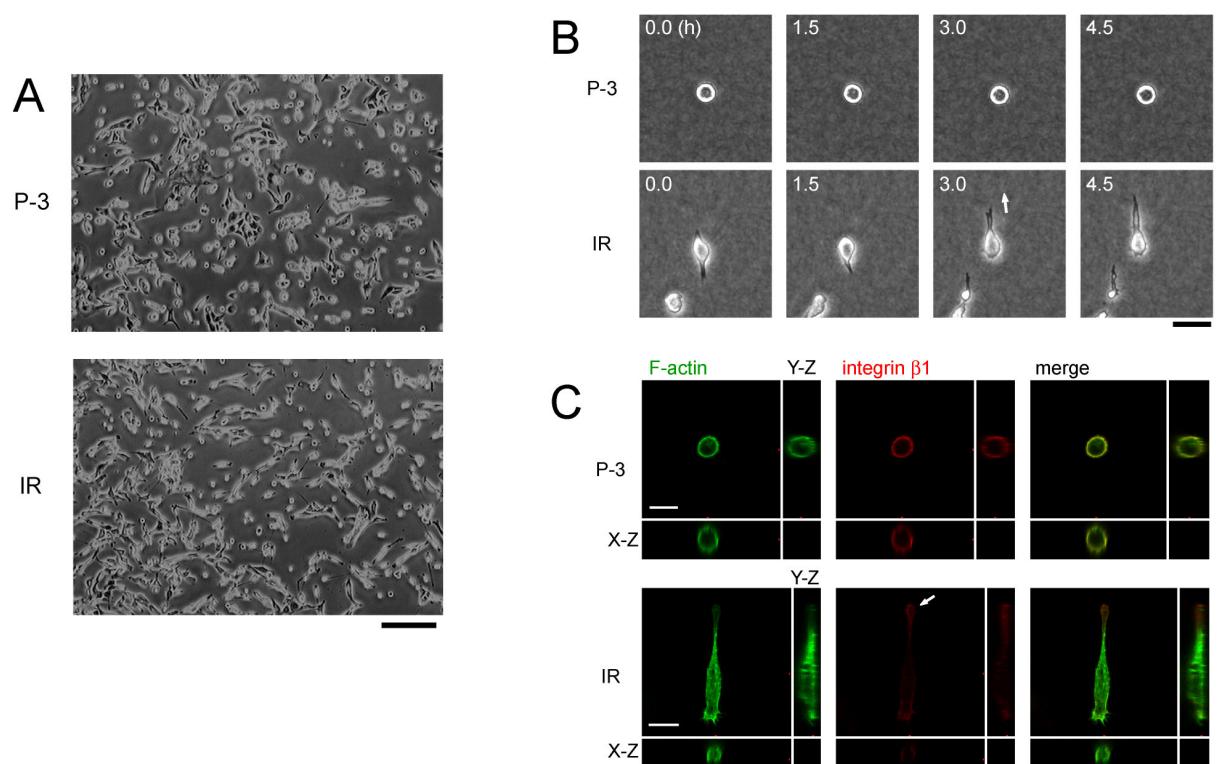


Fig. 2 S. Ishihara *et al.*

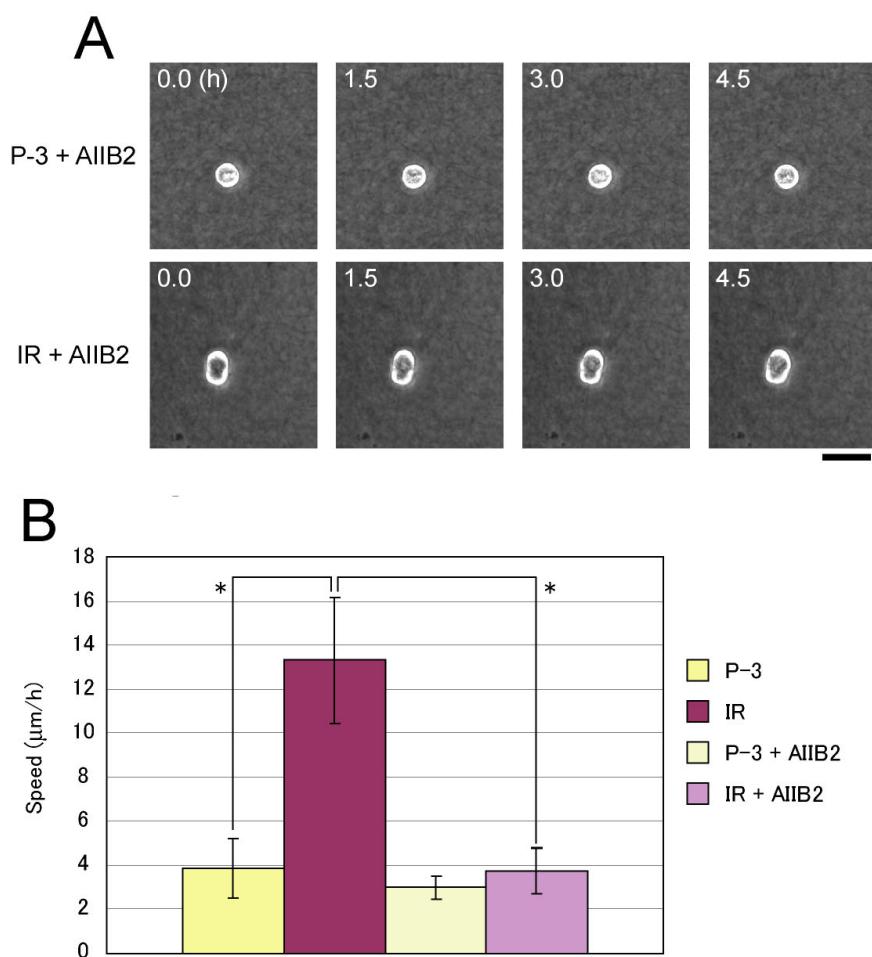


Fig. 3 S. Ishihara et al.

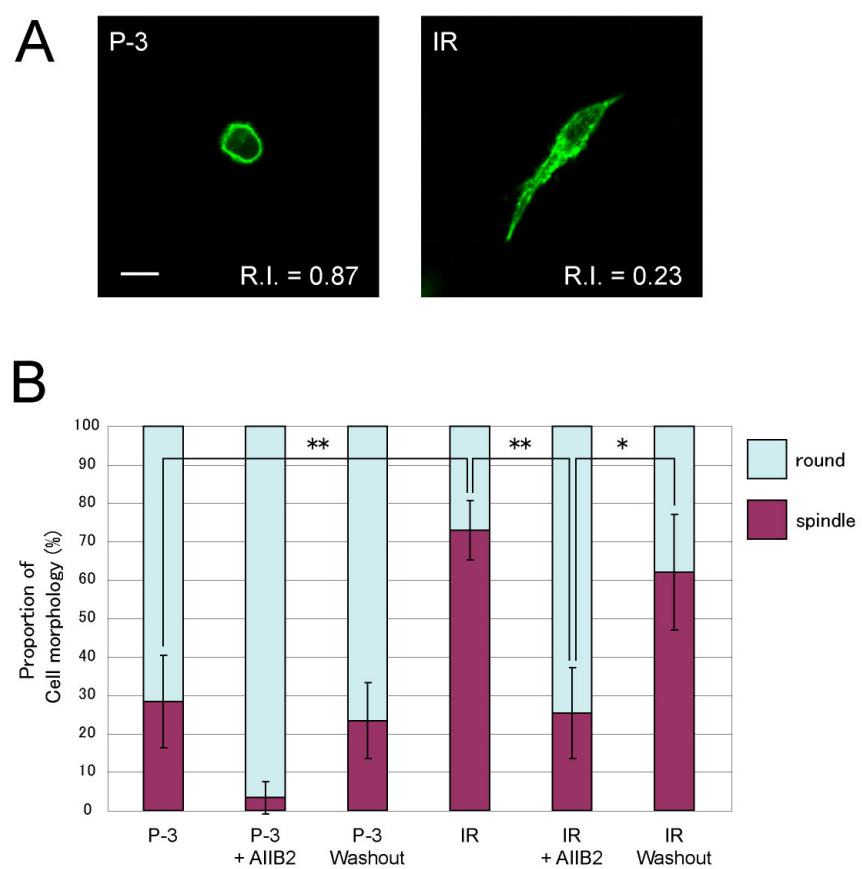


Fig. 4 S. Ishihara *et al.*

