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The CD44 standard isoform contributes to radioresistance of pancreatic cancer cells

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ABSTRACT

Resistance to chemoradiotherapy is one reason for the increased recurrence rate of pancreatic cancer after these therapies. These cells change the expression levels of several proteins, such as epithelial–mesenchymal transition (EMT), while acquiring the chemo- or radio-resistance. In this study, we focused on CD44, a pancreatic cancer stem cell marker. CD44 has isoforms with different functions: standard isoform (CD44s) and several variant isoforms (CD44v). However, little is known about the roles of these isoforms after ionizing irradiation. The purpose of this study was to investigate the role of CD44 isoforms in radioresistance of pancreatic cancer cells. AsPC-1 (a human pancreatic cancer cell line) was irradiated with 4 MV X-rays. The mRNA and protein levels of CD44s were strongly upregulated, dose dependently, compared with CD44v after irradiation. Thus, we further investigated CD44s at the point of cell proliferation. We evaluated cell proliferation and survival, using CD44s knockdown cells. CD44s knockdown did not change the proliferation rate for up to 72 h after the irradiation, but decreased cell viability in the colony formation assay. As one of the reasons for these effects, we found downregulation of phosphorylated extracellular signal–regulated kinase (Erk; which is involved with cell proliferation) by CD44s knockdown, time dependently. Moreover, radiation-induced EMT-like expression changes were detected and suppressed by CD44s knockdown. In conclusion, our work demonstrated that CD44 standard isoform was especially upregulated after high-dose X-ray irradiation in several isoforms of CD44 and contributed to longer-term cell survival after the irradiation through the maintenance of Erk phosphorylation and radiation-induced EMT.

KEYWORDS: CD44, X-ray, radioresistance, epithelial–mesenchymal transition, EMT, cancer stem cell

INTRODUCTION

Pancreatic cancer is a common malignant tumor and is often in an advanced state when detected, due to difficulty of diagnosis in early stages. The prognosis for this cancer is unfavorable, and the 5-year relative survival rate in Japan is <10% [1]. In advanced cases, chemoradiotherapy, using drugs such as Gemcitabine and 5-FU with X-ray radiation of ~50 Gy, is a treatment available in Japan [2]. However, the recurrence rate after chemoradiotherapy is still high. One of the reasons for tumor recurrence is the existence of chemo- and radio-resistant tumor cells. Therefore, new therapeutic strategies are necessary for killing these resistant cells, and it is essential to explore the character of these cells.

These resistant cells often change the expression of several proteins while acquiring resistance to the current therapies. One of the common changes is epithelial–mesenchymal transition (EMT). EMT is a biological process that allows epithelial cells to undergo multiple changes

that enable them to assume mesenchymal cell phenotypes. Through EMT, the expression of epithelial marker proteins, such as E-cadherin, are downregulated, while that of mesenchymal marker proteins, such as vimentin, and EMT markers, such as Slug and Zeb1, are upregulated [3, 4]. EMT is associated with the malignant nature of cancer cells, and their invasiveness, motility and resistance to apoptosis.

Recently, cancer stem cells (CSCs) have been proposed as the candidate minor cell population resistant to chemotherapy and radiation therapy. In addition to the resistance to the current therapy, CSCs have the ability for self-renewal and tumor initiation and contribute to tumor survival and recurrence [5–7]. Moreover, cancer cells are thought to achieve stemness through EMT [8, 9]. CSCs are identified by cell surface antigens (such as CD44, CD24 and CD133), and pancreatic CSCs are identified as CD44+/CD24+/ESA+ cells in general [5, 10]. Recently, it has been reported in a number of articles that these antigens play a role in determining the characteristics of CSCs. However, these roles are little understood. It has been reported that the expression of CD24 and ESA is limited to some cell lines of pancreatic cancer. Furthermore, CD24 has been reported to depress the invasive and migration capabilities of pancreatic cancer cells through maintaining the epithelial phenotype, which has characteristics opposite to those of CSCs [5, 11]. Therefore, in this study we focused on the role of CD44 in pancreatic cancer cells.

CD44 is a transmembrane glycoprotein and adhesive molecule to hyaluronic acid, a component of the extracellular matrix. It has been used as a CSC marker for several types of cancers, such as breast, prostate, colorectal, head and neck, ovarian, gastric and pancreatic cancer [10, 12–17]. CD44 has been reported to have the potential to be a marker of poor prognosis and to be involved in resistance to chemotherapy and radiation therapy in several cancers [14, 18–22].

CD44 is composed of 20 exons, and different isoforms of CD44 (the standard isoform and several variant isoforms) are produced through alternative splicing. CD44 with no variant exon (exons 6–15) is called the standard isoform (CD44s). The variant isoforms (CD44v) can have several combinations of variant exons (e.g. CD44v3–10 includes exons 8–15). The different functions of the various isoforms have been reported in several articles.

CD44s has been reported to be associated with EMT [23–26], and CD44s-positive pancreatic cancer cells are more malignant and more resistant to chemotherapy and radiation therapy than CD44s-negative cells [25, 27]. However, pancreatic cancer cells that are both CD44s-negative and CD44v (v6, v9)-positive have also been demonstrated to be more malignant [18]. Moreover, it is proposed that CD44v (v8–v10) is involved in metastasis and resistances to reactive oxygen species (ROS) and chemotherapy, which cause poor prognosis in some other cancer cells [28–31]. Thus, there are inconsistencies concerning the roles of the CD44 isoforms. In addition, the roles of these isoforms after X-ray irradiation have been hardly assessed.

The purpose of this study was to investigate the role of CD44 isoforms in irradiated pancreatic cancer cells, especially with respect to radioresistance.

MATERIALS AND METHODS

Cell culture

The human pancreatic cancer cell line AsPC-1 was obtained from ECACC (Salisbury, UK) and maintained in RPMI-1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS, Biowest, Nuaillé, France), 300 mg/l L-glutamine and 1% penicillin streptomycin (Nacalai tesque, Kyoto, Japan) at 37°C and 5% CO_2 in a humidified chamber. This cell line has been reported to express CD44 isoforms at intermediate level in several pancreatic cancer cell lines [25].

Irradiation

Cells were irradiated with 4 MV X-rays in a linear accelerator (EXL-6SP; Varian Medical Systems, Palo Alto, CA) at Osaka University Graduate School of Medicine. The dose rate was 100 cGy/min. Serum-free medium containing 1% penicillin streptomycin was added to a cell culture dish for build-up and was changed to the culture medium immediately after irradiation.

Flow cytometry

Cells were washed with phosphate buffer solution (PBS) and harvested with ethylenediaminetetraacetic acid (EDTA). Cells were washed with Eagle's minimal essential medium (EMEM) containing 0.1% NaN_3 and 1% FBS (wash buffer), and suspended in the wash buffer at a density of 1×10^6 cells/100 µl. Cells were labeled with monoclonal mouse anti-human CD44-fluorescein isothiocyanate (FITC, Miltenyi Biotec, Auburn, CA, USA). Isotype-matched immunoglobulin (FITC, DAKO, Denmark) served as a control. Cells were incubated at 4°C for 1.5 h for labeling. The labeled cells were washed twice in the wash buffer, then they were resuspended in the wash buffer (100 µl) and PBS (900 µl). The samples were analyzed using a FACSCalibur™ (Beckton Dickson, Heidelberg, Germany) with CellQuest™ software (Beckton Dickson).

Extraction of total RNA

Cells were lysed in Sepasol®—RNA I Super G (Nacalai tesque, Kyoto, Japan) and chloroform was added. After centrifuging at 12 000 rpm for 15 min at 4°C, the aqueous layer containing the total RNA was transferred to a fresh tube. Then isopropanol was added and it was incubated for 10 min at room temperature. After centrifugeing at 12 000 rpm for 10 min at 4°C, the supernatant was removed. The total RNA pellet was washed twice with 75% ethanol. The RNA pellet was air-dried and dissolved in RNase-free water. The RNA was quantitated using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, USA).

Quantitative RT-PCR

Reverse transcription of total RNA was performed. RNA liquid (15 µl) containing the same amount of total RNA was denatured at 95°C for 5 min. The reaction regent was composed of 5 × AMV buffer (5 µl) (Promega, Madison, USA), dNTP (2 µl, 10 mM) (Promega), Oligo dT (1 µl, 0.5 µg/µl) (FASMAC Co., Atsugi, Japan), RNasin® (1 µl, 20 u/µl) (Promega) and AMV reverse transcriptase (1 µl) (Promega). Reverse transcription was performed for 1 h at 42°C and 10 min at 65°C. The PCR reaction regent consisted of THUNDERBIRD® SYBR qPCR mix (10 µl) (TOYOBO Co., Osaka, Japan), forward primer (0.6 µl, 10 µM), reverse primer (0.6 µl, 10 µM), cDNA mixture (0.2 µl) and sterile water (8.6 µl). Quantitative PCR was performed using a LightCycler 480 System

(Roche Diagnostics, Basel, Switzerland). Values were calculated using the ΔΔCt method and normalized to those of GAPDH. The sequences of the forward and reverse primers are described in Table 1 and Supplementary Figure 1.

Western blotting

The cells were lysed in sample buffer [not containing 2-mercaptoethanol or bromophenol blue, but containing a protease inhibitor cocktail (Nacalai tesque), for the detection of CD44 protein] or RIPA buffer [(Wako) containing a protease inhibitor cocktail, NaF (100 μM) and Na_3VO_4 (1 mM), for the detection of other proteins]. The cell lysates for CD44 were sonicated, and the concentrations of all proteins were measured using a Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific); equivalent amounts of the proteins were prepared in sample buffer, containing 2-mercaptoethanol and bromophenol blue, and heated to 95°C for 5 min. Samples were run on 7–10% SDS polyacrylamide gels and separated. The proteins were transferred to PVDF membranes (Millipore, Bedford, USA), and the membranes were blocked in PBS-T (phosphate buffer solution with 0.2% Triton X) containing 5% skim milk or Blocking One (Nacalai tesque). The membranes were incubated overnight with primary antibodies at 4°C. The list of primary antibodies is described in Table 2. Subsequently, the membranes were washed in PBS-T three times and incubated with isotype-matched secondary antibody [anti–mouse IgG (HRP): ab97023 or anti–rabbit IgG (HRP): ab97051; Abcam, Cambridge, UK] for 45 min at room temperature. Membranes were washed in PBS-T three times, and luminescence from Horse Radish Peroxidase was detected with X-ray film (Fuji Photo Film Ltd, Tokyo, Japan) or CCD camera (ImageQuant LAS 4000mini, GEhealth care UK Ltd, Little Chalfont, UK), using Pierce™ ECL Western Blotting

Table 1. Sequence of CD44total, v6, v9, v10, s and GAPDH PCR primers

Target gene	Primer sequence
CD44total	F: TGCCGCTTTGCAGGTGTATT
	R: GGCAAGGTGCTATTGAAAGCCT
CD44v6	F: CAGAAGGAACAGTGGTTTGGCA
	R: GTCTTCTTTGGGTGTTTGGCGA
CD44v9	F: GAGCTTCTCTACATCACATGAAGGC
	R: GTCAGAGTAGAAGTTGTTGGATGGTC
CD44v10	F: ACCTCTCATTACCCACACACGA
	R: TAGCTGAGGTCACTGGGATGAA
CD44s	F: AAAGGAGCAGCACTTCAGGA
	R: TGTGTCTTGGTCTCTGGTAGC
GAPDH	F: CAATGACCCCTTCATTGACC
	R: TTGATTTTGGAGGGATCTCG

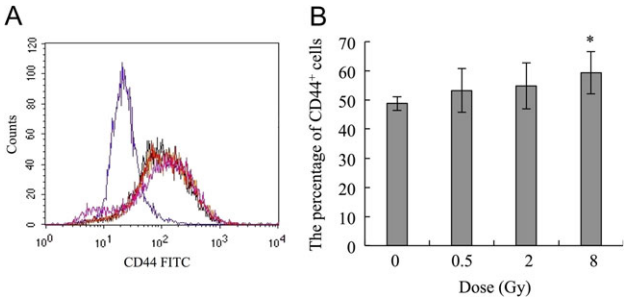

Fig. 1. The result of flow cytometric analysis of CD44 24 h after X-ray irradiation. (A) Each line represents the fluorescence of isotype control (purple) and CD44 after 0 Gy (black), 0.5 Gy (orange), 2 Gy (red) and 8 Gy (pink) irradiation. The vertical axis shows counts of cells and the horizontal axis shows the fluorescence of CD44-FITC at logarithmic scale. The horizontal bar with M3 means the threshold of CD44-positive cells. (B) The graph shows the percentage of CD44-positive ($CD44^+$) cells. Each bar represents the average ± standard deviation. (*$P < 0.05$ vs 0 Gy).

Table 2. List of primary antibodies for western blotting

Target	Class	Host	Source	Cat#
CD44	monoclonal	mouse	R&D systems	BBA10
Erk1/2	polyclonal	rabbit	Cell Signaling Technology	9102
p-Erk1/2 (Thr202/Thr204)	monoclonal	rabbit	Cell Signaling Technology	4370
E-cadherin	monoclonal	mouse	Thermo fisher scientific	33-4000
Vimentin	monoclonal	mouse	Abcam	ab8978
Slug	monoclonal	rabbit	Cell Signaling Technology	9585
Zeb1	monoclonal	rabbit	Cell Signaling Technology	3396
beta-actin	monoclonal	mouse	Abcam	ab6276

Substrate (Thermo Fisher Scientific). For reblotting, WB Stripping Solution (Nacalai tesque) was used to detach the antibody from the PVDF.

siRNA transfection

siRNA against CD44 standard isoform (siCD44s, sense: ccugcuaccagagaccaagTT, anti-sense: cuuggucucugguagcaggTT) and Silencer® select negative control No. 2 siRNA (siNC, Thermo Fisher Scientific) were transfected into cells using Lipofectamine™ RNAiMAX and Opti-MEM® (Thermo Fisher Scientific). siCD44s was obtained from the NIPPON GENE Co. (Tokyo, Japan).

WST-1 assay

Immediately after irradiation, the cells were trypsinized, and 5×10^3 cells/100 μl were seeded into 96-well cell culture plates. At 3 h after seeding and 24, 48 and 72 h after irradiation, WST-1 solution (10 μl) was added to each well and incubated for 2 h. After the incubation, the absorbance of 450 nm were measured with

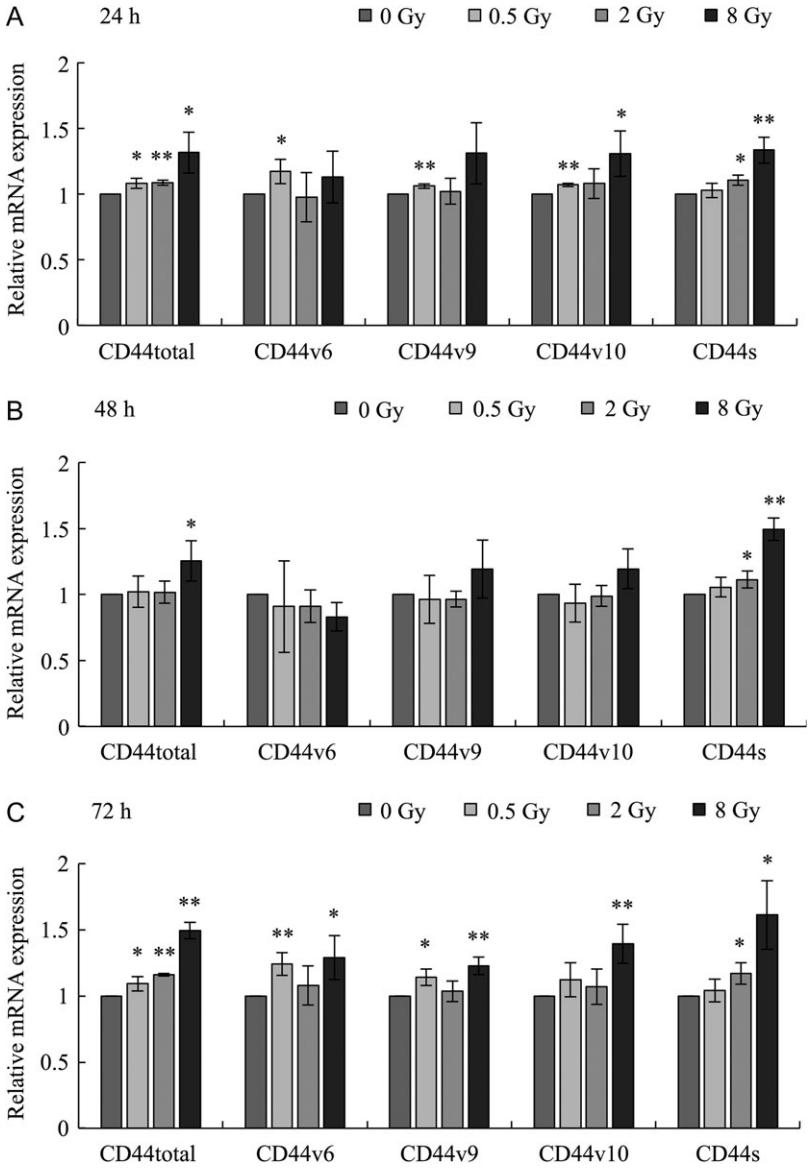

Fig. 2. The result of quantitative RT-PCR. Each graph shows the expression (A) 24 h, (B) 48 h and (C) 72 h after X-ray irradiation. The mRNA expressions of all isoforms of CD44 (CD44total), exon 11 (CD44v6), exon 14 (CD44v9), exon 15 (CD44v10) of CD44 and CD44 standard isoform are normalized by the expression of non-irradiated cells (0 Gy). The values were calculated by the ΔΔCt method based on the expression of GAPDH. Each bar represents the average ± standard deviation. (*$P < 0.05$, **$P < 0.01$ vs 0 Gy).

Multiscan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific).

Colony formation assay

Immediately after irradiation, the cells were trypsinized and seeded into culture dishes. After incubation for 12–17 days, the cells were fixed with 10% formalin and stained with crystal violet solution. The surviving fraction was calculated as: (colony number of irradiated cells)/(colony number of non-irradiated cells) for each set of conditions.

Statistics

Each result was expressed as an average with a standard deviation. The statistical significance was tested by means of Student's *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

The expression of CD44, especially CD44 standard isoform, increased after X-ray irradiation

First, we confirmed that the percentage of cells that had CD44 protein on the cell surface increased after X-ray irradiation, as several articles had reported [5, 27, 32, 33]. The expression of CD44 protein on the cell surface had already tended to increase 24 h after X-ray irradiation, as the dose increased, and had significantly increased after 8 Gy irradiation (Fig. 1).

Second, we tried to investigate which isoform of CD44 increased after X-ray irradiation by quantitative RT-PCR. As for the cell surface CD44 protein, the expression of total CD44 mRNA (CD44total) was upregulated dose dependently 24, 48 and 72 h after X-ray irradiation. Also, CD44s mRNA expression increased dose dependently after X-ray irradiation, and was significantly upregulated 24, 48 and 72 h after 2 Gy and 8 Gy irradiation. As for the CD44 variant isoforms, the expression of CD44v9 and CD44v10 was not changed by 2 Gy irradiation, although it tended to increase dose dependently. While the expression of CD44v6 increased dose independently 72 h after X-ray irradiation, it was not upregulated by 2 Gy irradiation, only by 0.5 Gy and 8 Gy irradiation (Fig. 2).

Next we conducted western blotting to find which isoform of CD44 protein was upregulated by X-ray irradiation. The CD44 antibody reacting with all isoforms was used, and the identification of isoforms was based on molecular weight: CD44s is 85–90 kDa, and CD44v is heavier [34]. The expression of CD44s tended to increase 24 h after X-ray irradiation, and this change was maintained 48 h after the irradiation. Moreover, CD44s was significantly upregulated by 8 Gy irradiation, while CD44v didn't increase significantly (Fig. 3).

Summarizing these results, CD44 was upregulated by X-ray irradiation from 24 h after the irradiation; in particular, both mRNA and protein expression of CD44s significantly increased dose dependently in several isoforms of CD44.

CD44s contributed to cell survival for a longer time after X-ray irradiation

Considering the above results, we thought CD44s might affect cell survival of irradiated cells in several isoforms of CD44. Therefore,

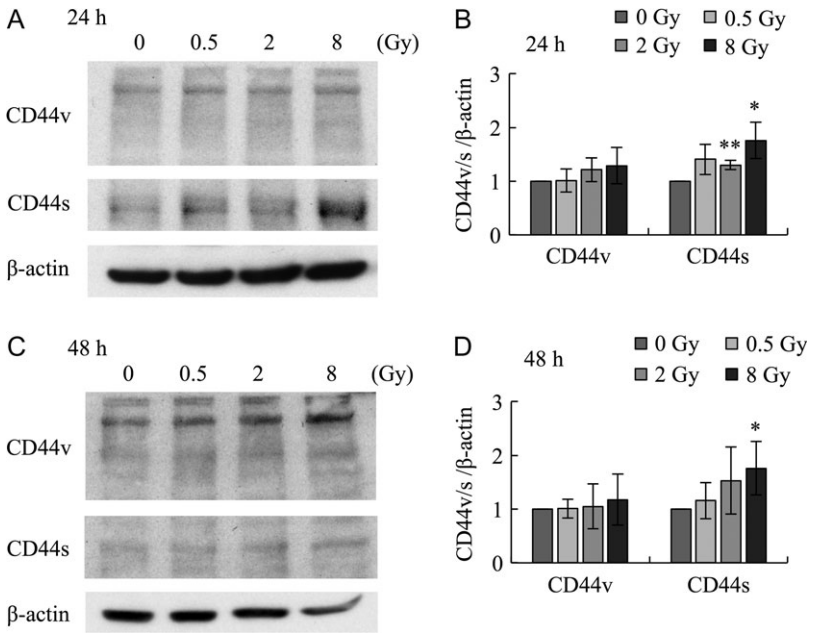

Fig. 3. Result of Western blotting of CD44. The expression of CD44 protein (A) 24 h and (C) 48 h after X-ray irradiation is shown. β-actin was used for the control. Each graph shows the density of each band normalized by that of beta-actin (B) 24 h and (D) 48 h after X-ray irradiation. The values were normalized by the density of non-irradiated cells (0 Gy). Each bar represents the average ± standard deviation. (*$P < 0.05$, **$P < 0.01$ vs 0 Gy).

the knockdown of CD44s by siRNA targeting only CD44s (siCD44s) was performed to investigate the role of CD44s in cell survival and proliferation of pancreatic cancer cells after X-ray irradiation. The knockdown and the confirmation of that was performed according to the schedule described in Fig. 4A. It was confirmed that siCD44s knocked down only CD44s, not CD44v, at both mRNA and protein level [by quantitative RT-PCR and western blotting, respectively (Fig. 4)]. Though CD44v10 was slightly upregulated after CD44s knockdown compared with in control cells, there was no significant difference between siNC transfected cells and CD44s knockdown cells (Fig. 4).

First, we performed the WST-1 assay to evaluate the effect of CD44s knockdown on cell proliferation and cell survival in the short term, according to the schedule described in Fig. 5A. The relative growth was measured up to 72 h after irradiation. As for the control cells, we detected a significant difference between that of non-irradiated cells and irradiated cells only 72 h after 0.5, 2 and 8 Gy irradiation and 48 h after 8 Gy irradiation (Fig. 5B). The CD44s knockdown cells did not show a decrease in the relative growth of either non-irradiated cells or irradiated cells for up to 72 h, compared with siNC cells. In addition, low-dose irradiation (0.5 and 2 Gy) didn't suppress the relative growth of CD44s knockdown cells compared with non-irradiated cells. Therefore, CD44s knockdown could not suppress cell proliferation or survival in the short term after X-ray irradiation (Fig. 5C and D).

Second, we conducted a colony-formation assay to evaluate the effect of CD44s knockdown on cell survival in the longer term according to the schedule described in Fig. 5A. CD44s knockdown did not change the survival rate of cells after 2 Gy irradiation; however, the survival rate after 8 Gy irradiation was significantly suppressed by CD44s knockdown compared with control and siNC transfected cells (Fig. 5E).

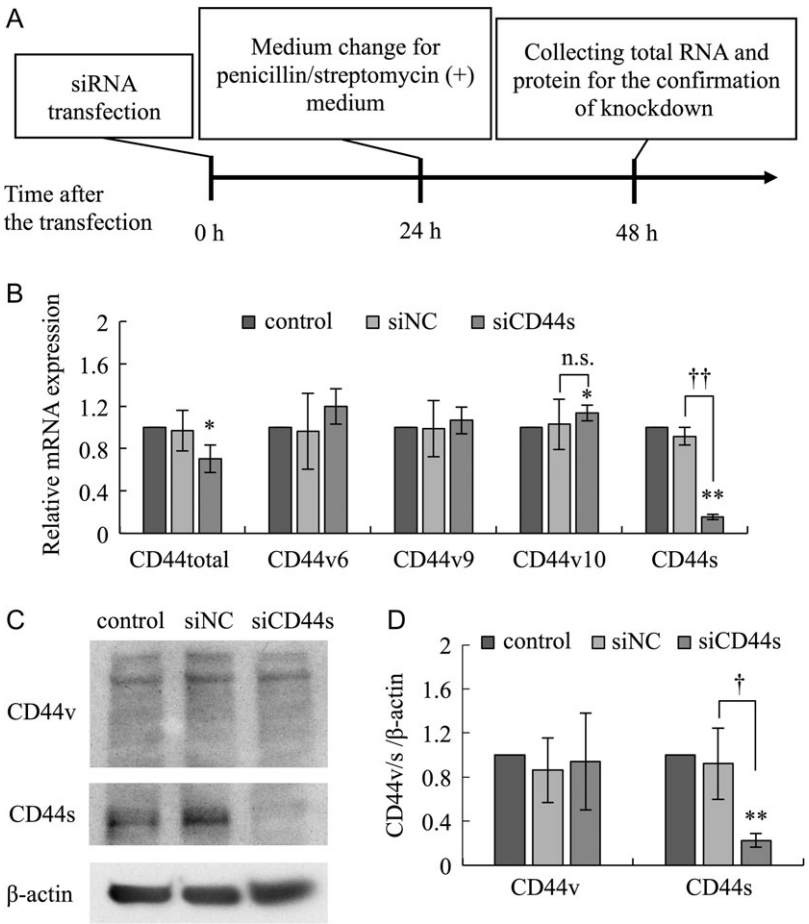

Fig. 4. The confirmation of CD44s knockdown. (A) The schedule of the confirmation. (B) The result of quantitative RT-PCR after siRNA knock down. The mRNA expressions are normalized by the expression of control cells. The values was calculated by ΔΔCt method based on the expression of GAPDH. Each bar means the average ± standard deviation. (C, D) The result of Western blotting after siRNA knockdown. Beta-actin (β-actin) was used for control. The graph shows the density of each band normalized by the density of beta-actin. The values were normalized by that of control cells. Each bar means the average ± standard deviation. (*$P < 0.05$, **$P < 0.01$ vs control †$P < 0.05$, ††$P < 0.01$ vs siNC, n.s. = not significant).

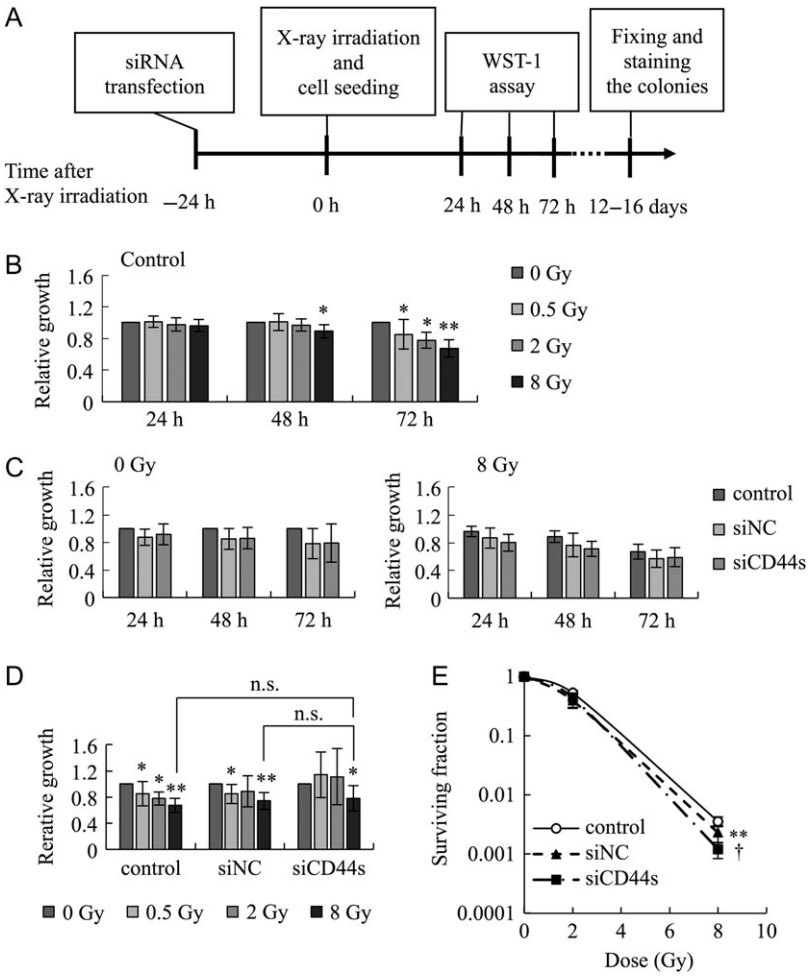

Fig. 5. The assessment of the effect of CD44s knockdown on cell proliferation and survival after X-ray irradiation. (A) The schedule of WST-1 assay and colony formation assay. The results of WST-1 assay. Each bar represents the average ± standard deviation. (B) The relative growth of control cells up to 72 h. The values were normalized by non-irradiated cells. (0 Gy) (*$P < 0.05$, **$P < 0.01$ vs 0 Gy). (C) The relative growth of non-irradiated cells and 8 Gy–irradiated cells. The values were normalized by non-irradiated cells of control cells (0 Gy, control). (D) The relative growth up to 72 h. The values were normalized by non-irradiated cells of each condition. (0 Gy) (*$P < 0.05$, **$P < 0.01$ vs 0 Gy of each condition, n.s. = no significant difference). (E) The survival curves of each condition based on colony-formation assay. The surviving fraction (vertical axis) was calculated by (colony number of irradiated cells)/(non-irradiated cells) for each set of conditions and described at logarithm scale. Each plot represents the average ± standard deviation. ($P < 0.01$ siCD44s vs control, †$P < 0.05$ siCD44s vs siNC)**

CD44s knockdown changed the expressions of several proteins involved with cell proliferation and epithelial–mesenchymal transition

Western blotting of several proteins involved with cell proliferation, survival and EMT, the latter of which is associated with resistance to apoptosis, was conducted in order to survey the effect of CD44s knockdown on their expression, according to the schedule described in Fig. 6A.

First, we evaluated the phosphorylation of extracellular signal–regulated kinase (Erk), the protein involved with cell proliferation. Phosphorylated Erk was upregulated in all conditions 24 h after 8 Gy irradiation (Fig. 6B). However, phosphorylated Erk was decreased by CD44s knockdown and was not upregulated by 8 Gy irradiation 72 h after X-ray irradiation (Fig. 6C).

Second, we evaluated the expression of epithelial marker, E-cadherin, mesenchymal marker, vimentin, and EMT markers Slug and Zeb1, 72 h after X-ray irradiation. The expression of E-cadherin in control and siNC transfected cells was slightly downregulated after 8 Gy irradiation, while the expression of vimentin in these cells was upregulated after 8 Gy irradiation. However, expression of these proteins in CD44s knockdown cells were hardly changed. In accord with these changes, the expressions of EMT markers Slug and Zeb1 in control and siNC transfected cells were upregulated after 8 Gy

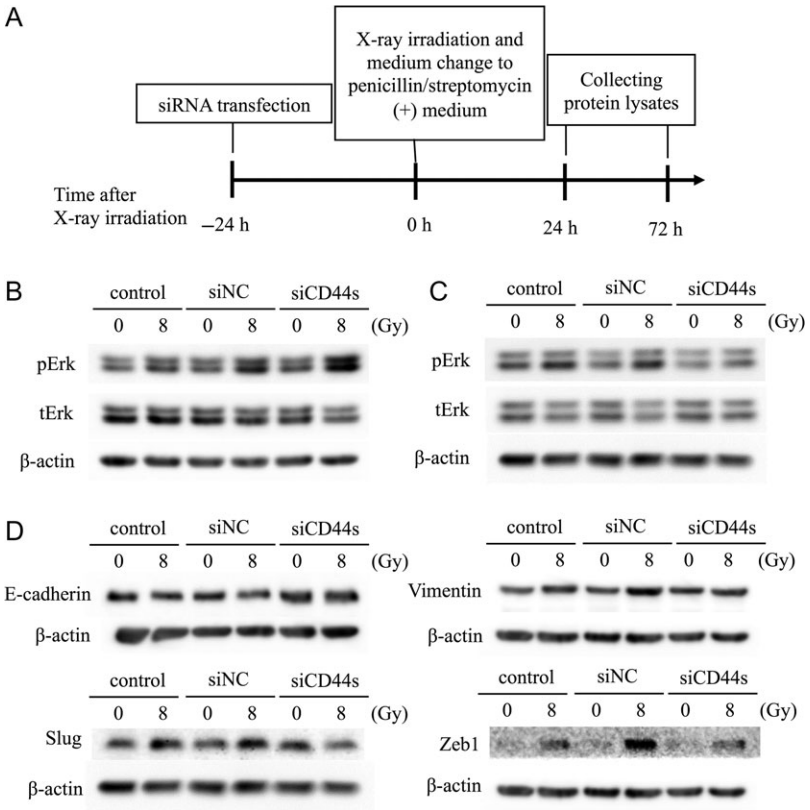

Fig. 6. The assessment of the effect of CD44s knockdown on several proteins related to cell proliferation and survival. (A) The schedule of collecting protein lysates. The results of western blotting of Erk (B) 24 h and (C) 72 h after X-ray irradiation. β-actin was used for a control. (D) The results of Western blotting of epithelial–mesenchymal transition (EMT)-associated proteins 72 h after X-ray irradiation. β-actin was used for a control. E-cadherin was used as an epithelial marker, vimentin as a mesenchymal marker and Slug and Zeb1 as EMT markers.

irradiation, while Slug in CD44s knockdown cells was downregulated, and the upregulation of Zeb1 in CD44s knockdown cells was suppressed compared with in siNC transfected cells (Fig. 6D). Summarizing these results, CD44s knockdown suppressed the EMT-like expression changes induced by the irradiation.

DISCUSSION

CD44 standard isoform is strongly upregulated after high-dose X-ray irradiation in several isoforms of CD44

CD44 was upregulated by X-ray radiation (Figs 1–3). These results were consistent with several articles reporting CD44+/CD24+ CSCs were resistant to X-ray irradiation, and the percentage of these marker-positive cells increased after irradiation because of the cell death of non-CSCs [5, 34]. However, CD44 had already been upregulated 24 h after X-ray irradiation, at which time cell death had not yet occurred according to the results of the WST-1 assay (Fig. 5B). Our results suggested that CD44 might be upregulated in response to DNA damage caused by X-rays, and play a role in the acquisition of radioresistance.

As for isoforms of CD44, CD44s was significantly upregulated dose dependently at both mRNA level and protein level (Figs 2 and 3). It has been reported in several articles that the ratio CD44s to CD44v6 (CD44s/v6) is one of the indicators for assessing whether CD44s or CD44v was upregulated more strongly [23, 24]. This ratio was increased dose dependently by high-dose X-ray irradiation (8 Gy) in our study (Fig. 7). These results suggested CD44 upregulation after high-dose X-ray irradiation was mainly due to CD44s upregulation. It has been reported that several mRNA are involved with the alternative splicing of CD44, which determines whether CD44s or CD44v are produced [23–25, 29]. These mRNAs might be one of the reasons why CD44s was especially upregulated after X-ray irradiation. Further study is needed about the effects of X-ray irradiation on these mRNAs.

CD44 standard isoform contributes to cell survival for the long term through the maintenance of Erk signaling activation

The knockdown of CD44s was performed to examine whether CD44s contributed to the radioresistance of pancreatic cancer cells.

First, the radiosensitising effect of CD44s knockdown was not seen in the WST-1 assay after high-dose irradiation, and surprisingly, low-dose irradiation could not suppress the cell proliferation of CD44s knockdown cells (Fig. 5C and D). One of the reasons for

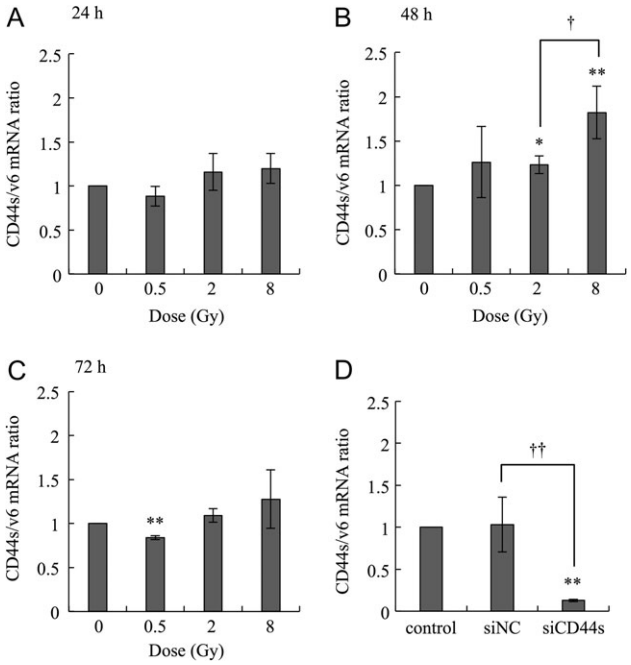

Fig. 7. The ratio of CD44s mRNA to CD44v6 mRNA (A) 24 h, (B) 48 h and (C) 72 h after X-ray irradiation or (D) 24 h after siCD44s transfection. The values were calculated from the results of quantitative RT-PCR (Figs 2 and 4). Each bar represents the average ± standard deviation. [(A–C) *$P < 0.05$, **$P < 0.01$ vs 0 Gy, †$P < 0.05$ 2 Gy vs 8 Gy, (D) **$P < 0.01$ vs control, ††$P < 0.01$ vs siNC.]

these results after low-dose irradiation could be the CD44s/v6 ratio. After low-dose irradiation, the CD44s/v6 ratio did not increase, and it significantly decreased 72 h after 0.5 Gy irradiation (Fig. 7A–C). These results suggested that CD44s might not be the main isoform of CD44 after low-dose X-ray irradiation. Moreover, CD44v6, CD44v9 and CD44v10 were upregulated after 0.5 Gy irradiation, and CD44v10 was slightly upregulated after CD44s knockdown compared with in control cells (Figs 2 and 4). It has been reported in several articles that CD44v (v8–v10) is involved with resistance to ROS, which are produced by X-ray irradiation [27, 28]. These variant isoforms might enhance the radioresistance and blur the radiosensitizing effect of CD44s knockdown. However, these articles did not assess directly the resistance to X-ray irradiation of these variant isoforms. Moreover, they assessed other types of cancer cells [28, 29]. Further study is needed about these CD44 variant isoforms of pancreatic cancer cells after different doses of X-ray irradiation.

Second, CD44s knockdown decreased the surviving fraction after high-dose irradiation in the colony-formation assay, but not in the WST-1 assay (Fig. 5D and E). These results mean that CD44s knockdown affected cell survival after high-dose X-ray irradiation, time dependently. We focused on Erk signaling to approach this time-dependent change. Erk signaling is activated by phosphorylation of Erk 1/2 and is associated with cell proliferation. The activation of Erk signaling after X-ray irradiation has been reported in several articles [35, 36]. Phosphorylated Erk 1/2 was also upregulated 24 h after X-ray irradiation in our study. Moreover, this upregulation was independent of CD44s knockdown. This might be one of the reasons why the relative growth up to 48 h after X-ray irradiation was almost the same as that of non-irradiated cells, and why there were no significant differences between CD44s knockdown cells and siNC transfected cells (Fig. 5B–D). However, phosphorylated Erk 1/2 of CD44s knockdown cells was downregulated 72 h after X-ray irradiation, while the upregulation had been maintained in control and siNC transfected cells. These results suggested that CD44s was not associated with radiation-induced phosphorylation of Erk 1/2, but with the maintenance of the phosphorylation. CD44 has been reported to be involved with activation of the Erk signaling pathway [20, 37]. Our study suggested CD44s contributed to cell survival for a longer term through the maintenance of Erk 1/2 phosphorylation.

Third, we would like to discuss the radiosensitizing effects from another point of view. CD44 is used as a pancreatic cancer stem cell marker that is resistant to X-ray irradiation. It has been reported that CD44 high population of AsPC-1 represents a high expression of CD44s and a low expression of CD44v [25]. Therefore it was suggested that CD44 was not merely a marker, and that CD44s rather than CD44v was involved in the intrinsic radioresistance of cancer stem cells. This might be another reason why CD44s was especially upregulated after X-ray irradiation.

The CD44 standard isoform is involved in radiation-induced EMT

We have demonstrated that the CD44s/v6 ratio was upregulated after high-dose X-ray irradiation (Fig. 7A–C). This upregulation has been thought to bring about several changes similar to EMT [23–26]. Recently, it has been proposed that high-dose X-ray irradiation induces changes in the genes involved in EMT in several types of cancer cells, including pancreatic cancer cell line Panc1 [38–40]. We also detected the expression changes suggesting this radiation-induced EMT resulting from 8 Gy irradiation: the downregulation of E-cadherin and the upregulation of vimentin, Slug and Zeb1 in AsPC-1 cells. Moreover, CD44s knockdown seemed to suppress these changes (Fig. 6D). CD44s knockdown significantly decreased the CD44s/v6 ratio (Fig. 7D). However, morphological change like EMT was hardly observed after 8 Gy irradiation due to the morphological heterogeneity of AsPC-1 (data not shown). According to the previous reports, a higher dose and a longer time after the irradiation might be needed to detect morphological changes in this cell line [38–40]. In the clinical setting, the prescribed dose for pancreatic cancer is much higher (~50 Gy) [2]. Our results suggested that the CD44s/v6 ratio might be associated with radiation-induced EMT-like changes in protein expressions, through which CD44s might contribute to the acquiring of resistance to apoptosis after X-ray irradiation.

In summary, our study is, to the best of our knowledge, the first to demonstrate that CD44s is especially upregulated after high-dose X-ray irradiation in several isoforms of CD44. Furthermore, our study revealed that CD44s contributed to long-term cell survival after the irradiation through the maintenance of Erk phosphorylation and radiation-induced EMT-like changes in protein

expressions. Our study suggested that CD44s played important roles in the development of radioresistance of pancreatic cancer cells, though the CD44 variant isoforms might affect the radiosensitivity to some extent.

SUPPLEMENTARY DATA

Supplementary data are available at the *Journal of Radiation Research* online.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest.

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