Adhesion of ST6Gal I-mediated human colon cancer cells to fibronectin contributes to cell survival by integrin ß1-mediated paxillin and AKT activation

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Received October 22, 2009; Accepted December 4, 2009

DOI: 10.3892/or_00000695

Abstract. We have recently demonstrated that ionizing radiation (IR) of cells increased the expression of ß-galactoside α-(2,6)-sialyltransferase (ST6Gal I) and the level of glycoprotein sialylation, especially for the key adhesion molecule integrin ß1. In addition, ST6Gal I-mediated sialylation of integrin ß1 contributed to cell adhesion-mediated radioresistance in colon cancer cells. In this study, we examined IR-induced cell adhesion to the extracellular matrix and evaluated the role of integrin ß1-associated downstream signaling molecules, such as paxillin and AKT. IR exposure and ST6Gal I overexpression increased adhesion of SW480 colon cancer cells to fibronectin and contributed to cell survival through the activation of paxillin and AKT. In contrast, knockdown of ST6Gal I or paxillin reduced the level of radiation-induced cell adhesion and increased the level of cell death. These results suggest that integrin ß1 sialylation may play a critical role in promoting adhesion of cancer cells by integrin ß1-mediated paxillin and AKT activation.

Introduction

Radiotherapy is an important modality for the treatment of cancer. However, acquired radioresistance during radiotherapy is the main obstacle for therapeutic efficiency due to the ability of most cancer cells, including colon cells, to metastasize to different sites (1,2). This response represents the major cause of relapse after radiotherapy. Previous studies have reported that ionizing radiation (IR) increases the invasiveness of malignant tumors, which is the limiting factor of the therapeutic value of the use of IR (3-5). Although several studies have contributed to a better understanding of the mechanisms for radioresistance and the occurrence of metastases, the signaling mechanisms that promote this occurrence remain elusive. Deciphering of the signaling pathways of the metastatic phenotype is the main target for the advancement to combat cancer progression.

Adhesion, migration and invasion of the surrounding extracellular matrix (ECM) by cancer cells are predominantly mediated by cell surface receptors, including integrins and other associated adhesion molecules (6). Integrins modulate the cellular response to IR and decrease radiation-induced cell death, which has been termed cell adhesion-mediated radioresistance (7-10).

ß-galactoside α-(2,6)-sialyltransferase (ST6Gal I), which adds α-2-6-linked sialic acids to glycoproteins, is up-regulated in colon adenocarcinoma and expression positively correlates with tumor cell invasiveness and metastasis (11-13). Previous studies have demonstrated that integrins are a substrate for ST6Gal I (11,14). In colon epithelial cells, oncogenic *ras* has been shown to induce up-regulation of ST6Gal I expression, leading to increased α-2,6 sialylation of ß1 integrin (15). Hypersialylation of integrin ß1 has been shown to augment colon tumor progression by altering cell preference for certain extracellular matrix milieu as well as by the stimulation of cell migration (11). Integrins also regulate cellular functions including survival, proliferation and cell spreading through the function of signaling molecules co-localized in the focal adhesion complex (16,17). One of the main focal adhesion proteins of integrin signaling is paxillin. Paxillin is a multi-domain adaptor protein that is localized in the focal adhesion complex. For adhesion, paxillin is phosphorylated and is subsequently activated by focal adhesion kinase (FAK) (18-21). Therefore, paxillin can serve as an adaptor between integrins and other signal transduction molecules for adhesion and survival of cancer cells. Another key player of anti-apoptotic molecules that induce cell survival is AKT. For radiation response, paxillin and AKT have been shown to induce radioresistance in various cancer cells (21-25).

We have previously reported that exposure to IR increased the expression of ST6Gal I and increased the level of glycoprotein sialylation (26,27). In this study, we have demonstrated that an increase of integrin ß1 sialylation by exposure of cells to IR increased the adhesion of SW480 colon cancer cells by integrin ß1-mediated paxillin and AKT activation. Therefore, sialylation of integrin ß1 and the subsequent activation of paxillin and AKT signaling may be one of the mechanisms involved in cell adhesion-mediated radioresistance and metastasis.

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Key words: sialyltransferase, ß-galactoside α-(2,6)-sialyltransferase, integrin ß1, paxillin, radioresistance, cell adhesion

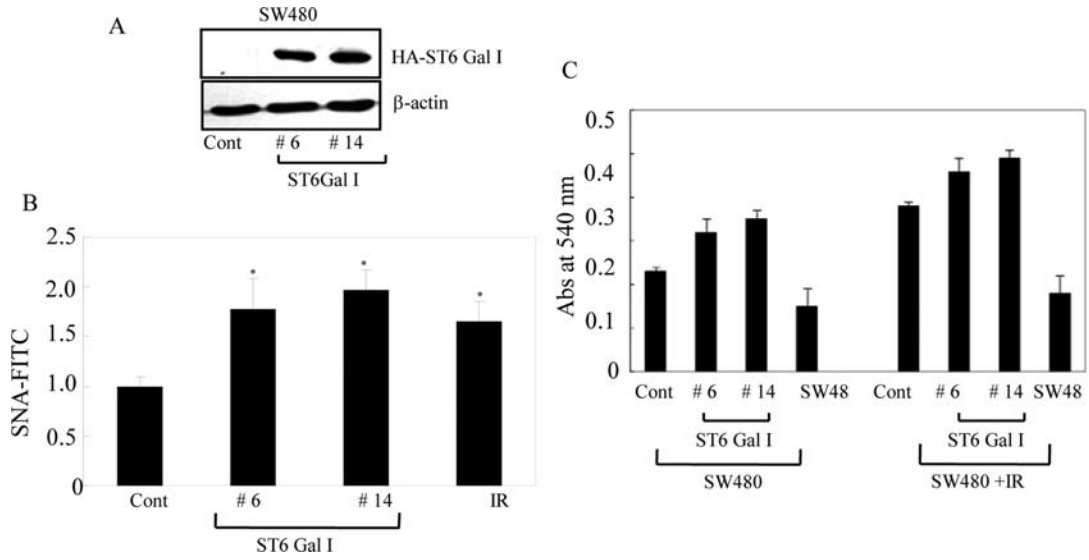

Figure 1. Stable expression of ß-galactoside α-2,6 sialyltransferase (ST6Gal I) increased the adhesion of SW480 human colon cancer cells to fibronectin. (A) SW480 human colon cancer cells that were stably expressed control and HA-ST6Gal 1 vectors (clones #6 and #14) were performed Western blotting using HA antibody. (B) Ten Gy radiation irradiated SW480 cells or ST6Gal I stably expressed clones of SW480 were probed with sialic acid-specific FITC-SNA. Sialylation levels were detected by FACS analysis. (C) Cells containing a control (vector) and the two stable clones that expressed ST6Gal I were irradiated and adhesion to fibronectin was quantified using an *in vitro* adhesion assay. The degree of adhesiveness of the ST6Gal I knockout cell lines and SW48 human colon cancer cells were also analyzed after irradiation. *P<0.05 vs. control (mean ± SD).

Materials and methods

Cell culture, small interfering RNA treatment and irradiation. SW480 and SW48 (ST6Gal I -/-) human colorectal carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum and antibiotics (Gibco BRL, Eggenstein, Germany). Cells were treated with ST6Gal I siRNA, integrin ß 1 siRNA (Dharmacon, Lafayette, CO, USA) and paxillin siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were exposed to γ-rays with a ^{137}Cs γ ray source (Atomic Energy of Canada, Mississauga, ON, Canada) at a dose rate of 3.81 Gy/min.

Western blotting. For polyacrylamide gel electrophoresis (PAGE), immunoblotting and lectin affinity assays, the cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.25% sodium deoxycholate, 0.5% sodium lauryl sulfate, 1 mM EDTA). The total and phosphorylated proteins were detected using the following commercial antibodies: anti-HA, anti-phospho-paxillin, AKT, phospho-AKT, poly (ADP-ribose) polymerase (PARP) and caspase 3 (Cell Signaling Technology, Danvers, MA, USA), anti-integrin ß1 (BD Biosciences, Franklin Lakes, NJ, USA) and anti-FAK and anti-paxillin (Santa Cruz Biotechnology). ß-galactoside α-2,6 sialyltransferase (ST6Gal I) was detected using a polyclonal rabbit antiserum, as described previously (26).

Flow cytometry. Cells were detached with trypsin/EDTA, washed with phosphate-buffered saline and were stained with propidium iodide (1 μg/ml) according to the manufacturer's protocol for cell death analysis or with fluorescein isothiocyanate (FITC)-conjugated lectins (FITC-SNA and FITC-MAL, Vector Laboratories, Burlingame, CA, USA) for sialylation detection, as previously described (28,29). The stained cells were then analyzed using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Clonogenic survival assay. Clonogenicity was examined with the use of a colony-forming assay, as previously described (30).

In vitro adhesion assay. The adhesion of cells was assayed as previously described (31). Briefly, cultured cells were washed twice with serum-free media and were added to microwell plates, which were precoated with collagen, fibronectin or laminin at a concentration of 2 μg/ml. In addition, bovine serum albumin (2%) was also coated onto the plates as controls. After a 60-min incubation, non-adherent cells were removed and adherent cells were stained with crystal violet. Absorbance was measured at 540 nm.

Statistical analysis. Statistical significance was determined using the Student's t-test to compare mean values. Values were expressed as the mean ± standard deviation (SD). A null hypothesis was rejected whenever a P-value was <0.05.

Results

We have previously reported that exposure of cells to IR increased ST6Gal I expression and sialylation of integrin ß1 (26). As SW480 colon cancer cells show low levels of α-2,6 sialylation (32), an adhesion assay using ST6Gal I-over-expressing SW480 cells (clones #6 and #14) (Fig. 1A) was performed. Total sialylation levels were measured using FITC-conjugated *Sambucus negra* agglutinin (SNA), which is specific for the detection of α-2,6 sialylation. Stable transfectants of SW480 cancer cells expressing ST6Gal I and irradiated SW480 cancer cells showed a specific increase of

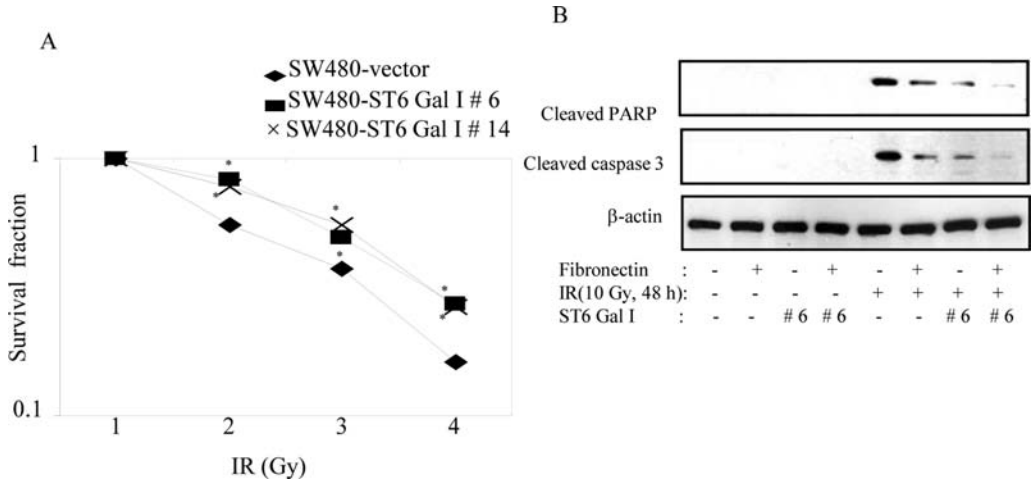

Figure 2. Adhesion to fibronectin increased cell survival after irradiation. (A) Survival curves of the SW480 cells transfected with a control vector and a stable clone that expressed ST6Gal I (clone #6) were grown on fibronectin-coated dishes and were assessed by the use of a clonogenic survival assay. (B) The SW480 cells transfected with a control vector and the two stable clones that expressed ST6Gal I, with or without culture on fibronectin-coated dishes, were exposed to 10 Gy IR. After 48 h, cell lysates were immunoblotted to detect PARP and caspase 3 cleavage. *P<0.05 vs. control.

binding to SNA (Fig. 1B). Next, we performed an adhesion assay using the two stable clones that expressed ST6Gal I and SW48 colon cancer cells that were knockout cell lines for ST6Gal I. Cell adhesion to fibronectin was increased in the stable clones that expressed ST6Gal I when compared to cells transfected with a control vector. As SW48 colon cancer cells are deficient for ST6Gal I, an increase of cell adhesion to fibronectin after exposure to IR was not seen (Fig. 1C). These results suggest that radiation-induced cell adhesion to fibronectin is dependent on sialylation by up-regulation of ST6Gal I activity.

To examine the role of ST6Gal I on cell adhesion-mediated radioresistance, we performed a clonogenic survival assay. The two stable clones that expressed ST6Gal I showed increased cell survival following radiation exposure (Fig. 2A). Caspase 3 activation and PARP cleavage following exposure to 10 Gy were also inhibited by ST6Gal I overexpression (Fig. 2B).

Integrins are cell surface receptors that connect cells to extracellular matrices and exert effects that promote cell proliferation, adhesion and metastasis (6). Moreover, both integrins and receptor tyrosine kinases share cellular signaling pathways that involve a variety of integrin-associated signaling proteins including paxillin and AKT (25,33,34). Exposure of SW480 cells to IR increased the level of the phosphorylated forms of paxillin (paxillin Y118) and AKT (AKT S473), which are the downstream molecules of integrin ß1 (Fig. 3A). To examine further whether radiation-induced activation of these proteins was mainly regulated by ST6Gal I, the two stable clones that expressed ST6Gal I were subjected to immunoblotting. The phosphorylated forms of paxillin and AKT were detected in extracts of the two stable clones that expressed ST6Gal I (Fig. 3B and C). Moreover, the activation of these signaling molecules by radiation exposure of cells was inhibited by treatment with Si-ST6Gal I (Fig. 3D). These results suggest that paxillin and AKT are activated by radiation exposure with ST6Gal I activation.

Since we observed that paxillin was activated during radiation-induced adhesion with ST6Gal I activation, we tested whether ST6Gal I functions as an inducer of cell adhesion-mediated radioresistance. IR-induced cell adhesion was prominently inhibited by treatment of cells with Si-paxillin or Si-ST6Gal I (Fig. 4A and B). A cell death assay and Western blotting analysis performed at 48 h after exposure of cells to 10 Gy IR demonstrated the knockdown of paxillin and treatment of SW480 cells with ST6Gal I siRNA significantly increased the level of cell death (Fig. 4C). Caspase 3 activation and cleavage of PARP were also shown with knockdown of paxillin and ST6Gal I (Fig. 4D). Based on these results, we conclude that the activation of paxillin by ST6Gal I is possibly a mediator of IR-induced cell adhesion and radioresistance.

Discussion

We have identified that sialylation of integrin ß1 by ST6Gal I, which is up-regulated by radiation exposure of cells, increased cellular adhesion to fibronectin and cell survival. Increased adhesion and cellular survival due to the action of ST6Gal I is mediated by integrin ß1-induced signaling activation by the proteins paxillin and AKT. We have previously reported that ST6Gal I expression and integrin ß1 sialylation are up-regulated in cells after radiation exposure (26). One important goal of this study was to determine the function of ST6Gal I for radiation-induced adhesion, with a specific focus on integrin ß1-mediated signaling pathways.

ST6Gal I overexpression was found to induce increased cellular adhesion and survival after radiation exposure, a finding that is consistent with results of our previous study (26). Since we determined that ST6Gal I induced sialylation of integrin ß1, we further examined the downstream proteins in a pathway involving integrin ß1 affected by ST6Gal I.

Paxillin, which is a downstream adaptor protein for integrin ß1, is a multi-domain protein that is localized in cultured cells and localization occurs primarily at sites of cell adhesion to the ECM, termed focal adhesions (21,35). Focal adhesions form structural links between the ECM and the actin cytoskeleton and are important sites for signal transduction (16,19,20). In effect, focal adhesion components propagate

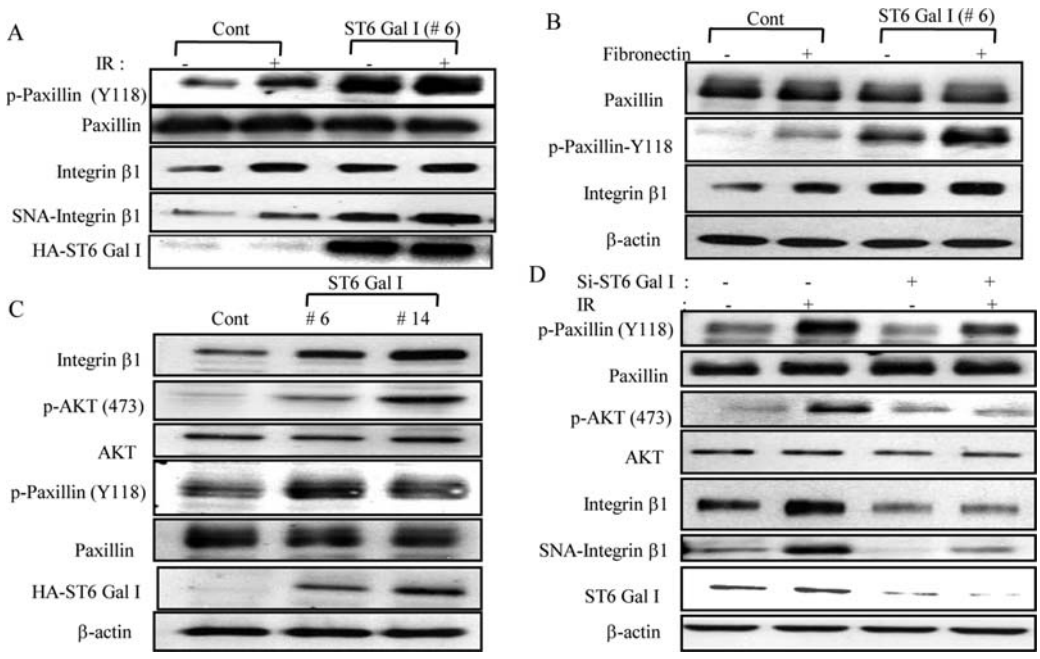

Figure 3. Ionizing radiation (IR) activates paxillin and AKT by ST6Gal I. SW480 cells transfected with a control vector and a stable clone that expressed ST6Gal I (clone #6) were exposed to 10 Gy IR (A) or were adhered to fibronectin. (B) Cell lysates were analyzed by the use of Western blotting with a specific antibody for phosphor-paxillin (Y118). (C) SW480 cells transfected with a control vector and the two stable clones that expressed ST6Gal I (clones #6 and #14) were harvested and were subjected to immunoblotting to detect the phospho-forms of paxillin, phosphor-AKT (S473) and integrin ß1. (D) After pretreatment with an siRNA for ST6Gal I, cells were exposed to IR. Cell lysates were probed with specific antibodies and were subjected to Western blotting.

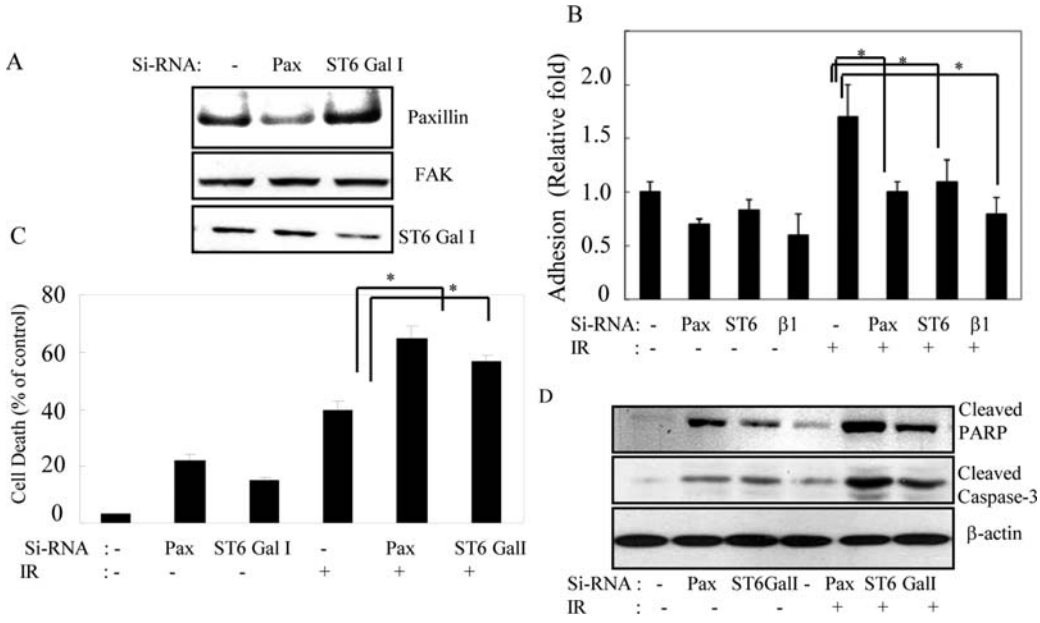

Figure 4. Knockdown of paxillin or ST6Gal I by RNA interference decreased adhesion to fibronectin and promoted radiation-induced cell death. (A) Cells were treated with Si-Paxillin or Si-ST6Gal I to assess the inhibition of protein levels by the use of Western blotting. SW480 cells were pretreated with siRNAs for paxillin or ST6Gal I. Cell adhesion (B) and cell death (C) were measured following exposure to 10 Gy IR. (D) At 48 h after IR exposure, cell lysates were subjected to Western blotting to detect PARP and caspase 3 cleavage. *P<0.05.

signals arising from the activation of integrins following engagement with ECM proteins, such as fibronectin, collagen and laminin.

In the stable clones that expressed ST6Gal I and irradiated SW480 cells, activation of paxillin and AKT occurred concurrently with integrin ß1 stabilization (Fig. 3), suggesting that ST6Gal I-mediated integrin ß1 sialylation induced the activation of the downstream pathways of integrin ß1, such as paxillin and AKT. Paxillin activation increased cellular adhesion and survival in cells that overexpressed ST6Gal I. Both cellular adhesion and survival, were inhibited by knockdown of paxillin (Fig. 4). Phosphorylation of AKT was also increased by ST6Gal I overexpression (Fig. 3). These findings suggest that ST6Gal I-induced cellular survival and

adhesion are partially mediated by integrin ß1 downstream pathways including proteins such as paxillin and AKT.

Modulation of adhesion and migration may have a role in tumor response by interfering with radioresistance, metastasis, angiogenesis, carcinogenesis and immunity through different signaling pathways (8,9,24). An increase of cellular adhesion and migration induced by radiation exposure of cells provides a potential method for the targeting of drug delivery (36,37). Based on our findings, ST6Gal I induced by IR exposure of cells resulted in sialylation of integrin ß1 and increased protein stability. The stabilization of integrin ß1 exhibited increased levels of cellular adhesion and survival after IR exposure of cells with activation of paxillin and AKT, which is suggested as a new possible model for radiation-induced adhesion and cellular survival.

Acknowledgements

This study was supported by the Nuclear Research and Development Program through a National Research Foundation of Korea (NRF) grant funded by the Korean government (Ministry of Education, Science and Technology, grant code: M2ANA001 and M2AMA006).

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