Base Excision Repair Proteins Are Required for Integrin-Mediated Suppression of Bleomycin-Induced DNA Breakage in Murine Lung Endothelial Cells

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ABSTRACT

Engagement of integrin cell adhesion receptors suppresses bleomycin (BLM)-induced DNA strand breakage in endothelial cells. Previous investigation of cells from poly(ADP-ribose) polymerase (PARP)-1 knockout mice and with an inhibitor of the enzyme indicated that this facilitator of base excision repair (BER) is required for integrin-mediated suppression of DNA strand breakage. Here, small inhibitory RNA (siRNA) was used to assess the requirement for the BER proteins, DNA ligase III (Lig3) α, PARP-1, and X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1), and for the long-patch BER ligase, DNA ligase I (Lig1), in integrin-mediated protection from BLM-induced DNA breakage. Murine lung endothelial cells (MLECs) were transfected with siRNA, treated with anti-β1 integrin antibody, and then BLM. 3’-OH in DNA and accumulation of phosphorylated histone H2AX (γH2AX), which reflects double-strand breakage, were measured. Integrin antibody inhibited the increases in 3’-OH caused by BLM in MLECs transfected with either control or Lig1 siRNA. However, after knockdown of Lig3α, PARP-1, or XRCC1, suppression of DNA breakage by integrin antibody was limited. BLM increased γH2AX levels, and integrin treatment inhibited this by 57 to 73% in MLECs transfected with control siRNA. Integrin engagement also inhibited increases in γH2AX in BLM-treated cells transfected with Lig1 siRNA. In contrast, Lig3α, PARP-1, and XRCC1 siRNAs prevented integrin-mediated inhibition of BLM-induced γH2AX levels. The results suggest that the BER proteins, Lig3α, PARP-1, and XRCC1, are required for integrin-mediated suppression of BLM-induced DNA breakage.

Pharmacological manipulation of DNA damage may be important in the understanding, medical application, and suppression of genotoxicity. Previously, we found that engagement of β1 integrin cell adhesion receptors with antibodies inhibited acute, reversible DNA strand breakage induced by bleomycin (BLM), bacterial endotoxin, and etoposide in endothelial cells (Hoyt et al., 1996, 1997; Jones et al., 2001; Huang et al., 2003). Integrins are a family of heterodimeric cell surface proteins, composed of α and β subunits that mediate adhesion of cells to each other, the extracellular matrix, and additional ligands. Integrin engagement initiates intracellular signals that can promote endothelial cell survival, whereas a lack of ligation or inappropriate engagement can induce apoptosis (Meredith et al., 1993; Stupack et al., 2001; Juliano, 2002).

The ability of integrin engagement to inhibit DNA breakage caused by several unrelated agents suggested that integrins act through mechanisms, such as altered nuclear architecture or enhanced DNA repair, that could affect DNA breaks arising from diverse causes (Jones et al., 2001; Huang et al., 2003; Rose et al., 2005). In fact, integrin-dependent protection of mouse lung endothelial cell (MLEC) DNA was abolished in cells lacking the DNA repair facilitator, poly(ADP-ribose) polymerase (PARP)-1, and in cells treated with a PARP-1 inhibitor (Jones et al., 2001; Huang et al., 2003).

ABBREVIATIONS: BLM, bleomycin; MLEC, mouse lung endothelial cell; PARP, poly(ADP-ribose) polymerase; BER, base excision repair; XRCC1, X-ray repair complementing defective repair in Chinese hamster cells 1; Lig3, ligase III; Lig1, ligase I; NHEJ, nonhomologous end joining; DNA-PKcs, DNA-dependent protein kinase catalytic subunit DNA; HRP, horseradish peroxidase; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; ET2, ethidium homodimer; siRNA, small inhibitory RNA; PCR, polymerase chain reaction; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ISNT, in situ nick translation; H33342, 2’-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5’-bis-1H-benzimidazole.
BLM is an anticancer antibiotic that cleaves DNA by iron-mediated activation of oxygen, resulting in single- and double-strand DNA breaks (Chen and Stubbe, 2005). These breaks require distinct pathways of repair. In base excision repair (BER) of single-strand breaks, PARP-1 is activated by 3'-OH of broken DNA in a complex with X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) and DNA ligase III (Lig3) α (Masson et al., 1998; Caldecott, 2003; Fan and Wilson, 2005). In short-patch BER, DNA polymerase β, interacting with XRCC1, replaces a single nucleotide, and Lig3α seals the nicked DNA (Caldecott, 2003; Dianoova et al., 2004). Strand displacement replacing 2 to 12 bases can also occur in long-patch BER, with ligation mediated by Lig3α or DNA ligase I (Lig1) (Caldecott, 2003; Fan and Wilson, 2005). Lig3α is recruited to sites of damage in association with XRCC1, whereas a slower recruitment of Lig1 depends on its association with proliferating cell nuclear antigen (Mortusewicz et al., 2006). XRCC1 level and Lig3α activity regulate the degree of short versus long-patch repair (Petermann et al., 2006).

The majority of double-strand DNA breaks are repaired by nonhomologous end joining (NHEJ) of DNA termini with final ligation by DNA ligase IV (Weterings and van Gent, 2004). Upon double-strand breakage the three phosphatidylinositol 3-kinase-like kinases, ataxia-telangiectasia mutated, ataxia-telangiectasia mutated Rad3-related, and DNA-dependent protein kinase catalytic subunit (DNA-PKCS) are activated and can phosphorylate the histone variant, H2AX, at a conserved C-terminal SQ motif that is not present in H2A (Valerie and Povirk, 2003). Immunocytochemical analysis demonstrated that phosphorylated H2AX, called γH2AX, accumulates adjacent to double-strand breaks and facilitates recruitment of repair proteins (Fernandez-Capetillo et al., 2004; Drouet et al., 2005). Interestingly, recent studies indicate that BER proteins also play a role in repairing double-strand breaks (Audebert et al., 2004, 2006; Wang et al., 2005; Levy et al., 2006).

Although integrin engagement suppresses 3'-OH induced by several DNA-damaging agents, it is not known whether double-strand breakage is reduced. Furthermore, the sensitivity of integrin-mediated suppression of DNA breakage to PARP-1 inhibition or deletion suggested that PARP-1, or associated repair factors, may be required for integrin action. Given the role of BER proteins in single- and double-strand repair, we investigated their role in integrin-mediated inhibition of DNA damage.

Materials and Methods

Endothelial cell growth supplement, heparin, phenylmethylsulfonyl fluoride, goat anti-rat IgG, phenol-chloroform-isoo amyl alcohol, BLM, and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Rat anti-mouse β1 integrin antibody was from PharMingen (San Diego, CA). DNA polymerase I was from New England Biolabs (Beverly, MA). Mouse monoclonal DNA ligase I antibody was from Novus Biologicals (Littleton, CO). Rabbit polyclonal XRCC1 antibody was purchased from Abcam (Cambridge, MA). Monoclonal PARP-1 antibody (clone A6.4.12) was from Servette (Raleigh, NC). Anti-phosphohistone H2AX (Ser139), clone JBW301, was purchased from Upstate Biotechnologies Inc. (Charlotteville, VA). Cy3 and horseradish peroxidase (HRP)-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Renaissance Enhanced Chemiluminescence Reagent was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Bovine serum albumin, fluorescein-12-2'-deoxy-uridine-5'-triphosphate, and anti-Lig3α antibody were obtained from Roche Applied Science (Indianapolis, IN). Silica chromatography filters were purchased from Qiagen (Valencia, CA). Dulbecco’s modified Eagle’s medium (DMEM), trypsin, TRIZol, Superscript Reverse Transcriptase, Platinum TaqDNA polymerase, Platinum PFX, pcDNA3.1 CT-green fluorescent protein (GFP) plasmid, RNase-free DNase, dCTP, dGTP, dATP nucleotides, ethidium homodimer (ET2), and H33342 were purchased from Invitrogen (Carlsbad, CA). Micron YM100 molecular cutoff filters were from Millipore (Billerica, MA). Microspin G-25 columns were purchased from Amersham (Uppsala, Sweden). T7 RNA polymerase and ribonucleotides (RNA Maxx kit) were from Stratagene (La Jolla, CA). Recombinant Dicer was purchased from Ambion Corp. (Austin, TX). Tris-base, EDTA, NaCl, Na3VO4, NaF, Tween 20, formaldehyde, SDS, and formaldehyde were from Fisher Scientific (Fair Lawn, NJ). Triton X-100 was purchased from Pierce (Rockford, IL). Female 129/Sv × C57BL/6 mice were kindly donated by Dr. Csaba Szabo (Inotek Corporation, Beverly, MA; current affiliation: CellScreen Applied Research Center, Semmelweis University Medical School, Budapest, Hungary).

siRNA. MLEC RNA was isolated by TRIZol extraction. cDNA was synthesized using Superscript Reverse Transcriptase. Amplification of the cdna by polymerase chain reaction (PCR) was then performed with pairs of 46-base primers containing a T7 RNA polymerase promoter sequence (5'-CGC TAA TAC GAC TCA CTA GGA GA-3') at the 5' end, followed by 20 bases of cdna-specific sequence. The cDNA-specific sequences in Lig1, Lig3α, PARP-1, and XRCC1 were designed from accession numbers NM010715, NM010716, BC012041, and U02887, respectively. The following primer sets for PCR were therefore generated: Lig1, sense, 5'-CGC TAA TAC GAC TCA CTA GGA GAA AGC GGT CAC GTA GCG GAT G-3', antisense, 5'-CGC TAA TAC GAC TCA CTA GGA GAG CTC GTG CTA AGT CAG CTC-3'; PARP-1, sense, 5'-CGC TAA TAC GAC TCA CTA GGA GAT ATC GTC TCT GAC AGC TCA C-3', antisense, 5'-CGC TAA TAC GAC TCA CTA GGA GAG GAT ATC GTC TCT GAC AGC TCA C-3'; XRCC1, sense, 5'-CGC TAA TAC GAC TCA CTA GGA GAG CTC GTA AGT CAG CTC-3', antisense, 5'-CGC TAA TAC GAC TCA CTA GGA GAG CTC GTA AGT CAG CTC-3'. All PCRs were carried out with high-fidelity Platinum PFX polymerase. PCR products were verified for size by agarose electrophoresis and purified from PCR products by silica chromatography. The concentration of these transcription templates was determined from absorbance at 260 nm. The PCR products (0.4 μg), having a T7 promoter at each 5' end, were transcribed in vitro with T7 RNA polymerase (8 U/μl) and 4 μM each ribonucleotide. The long double-stranded RNA was treated with RNase-free DNase, precipitated with ethanol, redissolved in water, and measured by absorbance at
260 nm. One microgram of long RNA was digested to approximately 21 bases by incubation for 18 h with 1 U of recombinant Dicer in a 10-μl reaction containing 20 mM Tris, pH 8, 150 mM NaCl, and 2.5 mM MgCl₂. The small RNA was desalted with a Microspin G-25 column and purified by spin filtering with a Micron YM 100 molecular cutoff filter. siRNA and its large precursor RNA were run in agarose gels and stained with ethidium bromide. Fluorescent digital images were recorded and quantified with image analysis software. The concentration of siRNA was calculated by comparison with the known amount of large RNA, assuming an average double-stranded RNA molecular weight of 13,629 g/mol.

Treatment of Cells. Endothelial cells were obtained from mouse lungs as described previously (Gerritsen et al., 1995; Jones et al., 2001; Huang et al., 2003; Rose et al., 2005). MLEC were cultured at 50% confluence in DMEM containing 20% FBS for 24 h. Cells were removed from flasks with trypsin, centrifuged (250g), washed once by resuspension and centrifugation in DMEM/20% FBS, and resuspended in 400 μl of DMEM without serum. The cell suspension was incubated with 200 nM siRNA for 5 min and placed in an aluminum-lined 1-ml cuvette with a 0.4-cm gap, and electroporated with a Linarator (Bio-Rad, Hercules, CA). MLECs were then cultured in DMEM/20% FBS for 24 h before protein extraction or further treatment. For integrin engagement, cells were treated with 0 or 1 μg of anti-β1 integrin antibody/ml for 1 h and then 2 μg of goat anti-rat IgG for 4 h as described previously (Hoyt et al., 1996, 1997; Jones et al., 2001; Huang et al., 2003; Rose et al., 2005). Medium or BLM was then added for 15 or 45 min.

Western Blotting. MLEC were washed three times with ice-cold PBS and lysed with lysis buffer containing 1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM Na₃VO₄, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Extracts were sonicated, and protein concentration was determined by the Bradford assay as described previously (Rose et al., 2005). Ten micrograms of protein was incubated with protein loading buffer and incubated for 10 min at 95°C. Proteins were separated on 4 to 20% Tris-glycine polyacrylamide gels and transferred to nitrocellulose.

After transfer, the blots were blocked with 3% nonfat dry milk in Tris-buffered saline buffer and Tween 20 (0.1% Tween 20, 10 mM Tris, pH 7.5, and 150 mM NaCl) and probed with primary antibody for 1 h at room temperature. After blots were washed, appropriate HRP-conjugated secondary antibody was added and incubated for 1 h. Proteins were visualized using enhanced chemiluminescent reagents and captured on X-ray film. Films were scanned to produce digital images for analysis.

In Situ Nick Translation. Relative levels of 3’-OH in DNA were labeled by in situ nick translation (ISNT) with a fluorescein nucleotide as described previously (Gorczyca et al., 1993; Hoyt et al., 1997; Jones et al., 2001; Huang et al., 2003). After treatments, cells were fixed in 95% ethanol at −20°C. Cells were washed three times with PBS at 4°C and then incubated at 37°C for 90 min with ISNT buffer (2.5 mM MgCl₂, 50 mM Tris, pH 7.8, 10 mM β-mercaptoethanol, and 10 μg/ml bovine serum albumin) containing 16 μM each dGTP, dATP, and dCTP, fluorescein-12’-deoxygenated-5’-triphosphate, and 2 U/ml E. coli DNA polymerase. Labeling was stopped by rinsing with PBS. An Olympus BX60 fluorescence microscope and 20× objective were used to image fluorescein (blue excitation/green emission) and to record digital microscopic images.

Immunofluorescence for γH2AX. After treatments, MLEC were fixed in 95% ethanol/5% acetic acid for 5 min at 4°C and rinsed three times with Tris-buffered saline (10 mM Tris, pH 7.5, and 150 mM NaCl). MLEC were blocked with 10% goat serum in Tris-buffered saline for 1 h and then incubated with 1:500 dilution of anti-γH2AX antibody/ml for 1 h and with 1:100 dilution of goat anti-mouse conjugated Cy3 secondary antibody in 10% goat serum/Tris-buffered saline for 1 h. Medium or BLM was then added for 15 or 45 min.

Western Blotting. MLEC were washed three times with ice-cold PBS and lysed with lysis buffer containing 1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM Na₃VO₄, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Extracts were sonicated, and protein concentration was determined by the Bradford assay as described previously (Rose et al., 2005). Ten micrograms of protein was incubated with protein loading buffer and incubated for 10 min at 95°C. Proteins were separated on 4 to 20% Tris-glycine polyacrylamide gels and transferred to nitrocellulose.

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In Situ Nick Translation. Relative levels of 3’-OH in DNA were labeled by in situ nick translation (ISNT) with a fluorescein nucleotide as described previously (Gorczyca et al., 1993; Hoyt et al., 1997; Jones et al., 2001; Huang et al., 2003). After treatments, cells were fixed in 95% ethanol at −20°C. Cells were washed three times with PBS at 4°C and then incubated at 37°C for 90 min with ISNT buffer (2.5 mM MgCl₂, 50 mM Tris, pH 7.8, 10 mM β-mercaptoethanol, and 10 μg/ml bovine serum albumin) containing 16 μM each dGTP, dATP, and dCTP, fluorescein-12’-deoxygenated-5’-triphosphate, and 2 U/ml E. coli DNA polymerase. Labeling was stopped by rinsing with PBS. An Olympus BX60 fluorescence microscope and 20× objective were used to image fluorescein (blue excitation/green emission) and to record digital microscopic images.

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Cytotoxicity. Cytotoxicity of BLM was assessed, as described previously (Huang et al., 2003), by cell permeability to ET2, which fluoresces when bound to DNA after entering cells. MLEC were electroporated with GFP or XRCC1 siRNA, cultured for 24 h, incubated with antibodies, and treated with 0 to 500 μg of BLM/ml for 4 h. Cells were washed and incubated 20 min in medium containing 4 μM ET2 and 310 μM H33342, a cell-permeable bisbenzamide DNA stain. UV excitation and blue emission for H33342 were used to

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Fig. 1. Effect of siRNA on Lig1, PARP-1, Lig3α, and XRCC1 protein levels. MLEC were transfected with 200 nM GFP (open bars), XRCC1 (solid bars), Lig3α (checked bars), PARP-1 (vertical striped bar), or Lig1 (diagonal striped bars) siRNA, and protein was extracted after 24 h. Images are representative western blots for Lig1 (A), Lig3α (B), PARP-1 (C), or XRCC1 (D) proteins. Bars, mean ± S.E. of densitometric analysis of Western blots of three independent transfections of each siRNA. *p < 0.05 for comparison with GFP siRNA.
objectively identify nuclei in microscopic fields, and digital images of ET2 (green excitation/red emission) were recorded.

**Data Analysis.** Protein levels in Western blots and average intensity of nuclei in microscopic images were determined with image analysis software (Scanpro; SPSS Science, Chicago, IL) as described previously (Hoyt et al., 1997; Jones et al., 2001; Huang et al., 2003; Rose et al., 2005). Data were analyzed by Student’s *t* test or by analysis of variance with Bonferroni correction for multiple comparisons (Snedecor and Cochran, 1980).

**Results**

Figure 1 demonstrates the depletion of Lig1, PARP-1, Lig3α, and XRCC1 protein 24 h after transfection of MLECs with siRNAs. Lig1 protein was reduced to 7% GFP control siRNA levels in Lig1 siRNA-transfected MLECs (Fig. 1A). Lig3α siRNA depleted Lig3α protein to 34% of control levels (Fig. 1B). PARP-1 siRNA preferentially depleted PARP-1 protein to 20% of control levels (Fig. 1C), whereas XRCC1 siRNA reduced XRCC1 protein to 34% control siRNA (Fig. 1D). XRCC1 siRNA also reduced the levels of Lig3α (Fig. 1B).

This result is consistent with the ability of XRCC1 protein to stabilize Lig3α protein through their physical interaction (Caldcott et al., 1994) and was observed with a different XRCC1 siRNA in another cell type (Petermann et al., 2006). The effect of knockdown of Lig1 and Lig3α on BLM-in-
duced DNA strand breakage was assessed. In MLECs transfected with the control siRNA, 0.2, 0.5, and 1.0 mg BLM/ml significantly increased DNA strand breakage measured by ISNT of 3′-OH, and reductions in 3′-OH after a 1 h drug-free recovery period were observed (Fig. 2A). In cells transfected with either Lig1 or Lig3α siRNAs, the removal of 3′-OH was antagonized (Fig. 2, B and C), confirming the activity of these siRNAs. Anti-β1 integrin antibody suppressed 3′-OH induced by 0.2, 0.5, and 1.0 BLM by 71, 48, and 66%, respectively, in cells transfected with control siRNA (Fig. 3A). Integrin-mediated protection was also observed with Lig1 siRNA, where 3′-OH labeling in cells treated with 0.2, 0.5, and 1.0 BLM was reduced, respectively, by 61, 66, and 58% (Fig. 3B). However, no inhibition of BLM-induced DNA breakage by the integrin antibody was seen in cells transfected with Lig3α siRNA (Fig. 3C). In MLECs treated with anti-β1 integrin antibody and 0.5 mg BLM/ml and allowed a washout period (Fig. 3, A–C, right), 3′-OH labeling was not different from the level in antibody-treated cells before wash-out. In particular, 3′-OH remained elevated following wash-out in cells treated with Lig3α siRNA and anti-β1 integrin antibody (Fig. 3C).

Knockdown of XRCC1 increased the 3′-OH labeling caused by 0.2, 0.5, and 1.0 mg BLM/ml in comparison with control siRNA-treated cells (Fig. 4, open bars). With control siRNA, elevated 3′-OH labeling was reduced to insignificant levels after 1-h incubation in BLM-free medium (Fig. 4A, washout, single hatched bars). Each group of XRCC1 siRNA-treated cells (Fig. 4B) exhibited increased 3′-OH labeling in comparison with the same group treated with control siRNA (p < 0.05 for each comparison of groups in Fig. 4, B with A). Thus, XRCC1 siRNA increased the level of breakage during incubation with BLM and after a 1-h washout with or without integrin engagement. β1 Integrin antibody suppressed DNA breaks caused by 0.5 to 1.0 mg BLM/ml in control siRNA-transfected cells (Fig. 4A). With 1.0 mg BLM/ml, the reduction was by 94%, for example. However, in cells transfected with XRCC1 siRNA, 3′-OH labeling induced by 0.2 to 1.0 mg BLM/ml was partially reduced by β1 integrin antibody but was still significant (Fig. 4B, solid bars). Here, the reduction by integrin engagement was 51% in XRCC1 siRNA-transfected cells treated with 1.0 mg BLM/ml. Recovery after washout was inhibited by XRCC1 siRNA; although 3′-OH labeling decreased after washout of 0.5 and 1.0 mg BLM/ml by 66 and 75% of the unwashed groups, respectively, in control siRNA-treated cells, the decreases were only by 9 and 34% with XRCC1 siRNA. Furthermore, although recovery was complete after 0.2 mg of BLM/ml in XRCC1 siRNA plus β1 integrin antibody-treated cells, it was completely blocked in those MLECs treated with 0.5 or 1.0 mg BLM/ml. Integrin-mediated inhibition of DNA strand breaks was also prevented by PARP-1 siRNA in comparison with control siRNA in another independent experiment (Fig. 5).

γH2AX, or H2AX phosphorylated at Ser139 in its C-terminal domain, is an indirect indicator of double-strand DNA breaks in cells exposed to genotoxic agents (Valerie and Povirk, 2003). A specific antibody revealed the concentration-dependent effect of BLM on H2AX phosphorylation (Fig. 6). Image analysis indicated that exposure to BLM for 15 min significantly increased the level of γH2AX in MLECs (Fig. 6B).

Based on these results, MLECs were transfected with siRNA, treated with integrin antibody, and then challenged with 200 nM GFP or PARP-1 siRNA. Twenty four hours later, β1 integrins were engaged as in Fig. 3. Cells were then treated with 0 or 1 mg BLM/ml for 45 min and processed for ISNT. Bars, mean difference in nuclear fluorescence intensity between BLM- and vehicle-treated cells + S.E. for 400 to 2000 cells in each experimental group. *, p < 0.05 for comparison with 0 mg BLM/ml (or a mean difference of 0 on the vertical axis). +, p < 0.05 for comparison with no anti-β1 integrin antibody. #, p < 0.05 for comparison with GFP siRNA-transfected cells.

**Fig. 5.** Effects of knockdown of PARP-1 on integrin-mediated suppression of BLM-induced DNA strand breakage measured by ISNT. MLECs were transfected with 200 nM GFP or PARP-1 siRNA. Twenty four hours later, β1 integrins were engaged as in Fig. 3. Cells were then treated with 0 or 1 mg BLM/ml for 45 min and processed for ISNT. Bars, mean difference in nuclear fluorescence intensity between BLM- and vehicle-treated cells + S.E. for 400 to 2000 cells in each experimental group. *, p < 0.05 for comparison with 0 mg BLM/ml (or a mean difference of 0 on the vertical axis). +, p < 0.05 for comparison with no anti-β1 integrin antibody. #, p < 0.05 for comparison with GFP siRNA-transfected cells.

**Fig. 4.** Effects of knockdown of XRCC1 and drug washout on integrin-mediated suppression of BLM-induced DNA strand breakage measured by ISNT. MLECs were transfected with 200 nM GFP (A) or XRCC1 siRNA (B). Twenty four hours later, β1 integrins were engaged as in Fig. 3. Cells were then treated with 0 to 1 mg BLM/ml for 45 min and incubated for 0 (“Treatment”) or 1 (Washout) h in BLM-free medium and processed for measurement of 3′-OH DNA strand breaks by ISNT. Bars, mean difference in nuclear fluorescence intensity between BLM- and vehicle-treated cells + S.E. for 400 to 2000 cells in each experimental group. *, p < 0.05 for comparison with 0 mg BLM/ml (or a mean difference of 0 on the vertical axis). +, p < 0.05 for comparison with no anti-β1 integrin antibody. #, p < 0.05 for comparison with no washout (Treatment) group treated in the same manner. All groups in B were significantly elevated in comparison with the same group in A (p < 0.05).
with BLM for 15 min. As expected, in the absence of integrin engagement, BLM increased γH2AX levels in cells transfected with control siRNA (Fig. 7A). Pretreatment with anti-β1 integrin antibody reduced γH2AX fluorescence intensity by 73% in MLECs treated with 25 μg of BLM/ml and 57% in cells exposed to 100 μg of BLM/ml (Fig. 7A). In Lig1 siRNA-transfected MLECs, integrin antibody also reduced γH2AX immunofluorescence by 58 and 44% after treatment with 25 and 100 μg of BLM/ml, respectively (Fig. 7B). However, depletion of Lig3α, PARP-1, or XRCC1 severely compromised this integrin-mediated inhibition (Fig. 7, C to E). In the case of XRCC1 siRNA, the induction of γH2AX by BLM was actually enhanced by treatment with anti-β1 integrin antibody.

Given the differing effect of XRCC1 siRNA on integrin modulation of 3′-OH and γH2AX, the effect of this siRNA on acute cytotoxicity of 100 and 500 μg of BLM/ml BLM was measured. BLM caused a concentration-dependent increase in permeability to ET2 in MLECs electroporated with control siRNA, and engagement of β1 integrins prevented it (Fig. 8E). XRCC1 siRNA alone reduced the permeability caused by 100 μg of BLM/ml, and integrin engagement did not further reduce it (Fig. 8F). Although the increased permeability caused by 500 μg of BLM/ml was not affected by XRCC1 siRNA, integrin-mediated protection from this concentration was partially inhibited (Fig. 8F).

**Discussion**

BLM is a useful anticancer agent that has a relatively low potential for myelosuppressive side effects. However, it can cause lung disease, in part as a consequence of damage to pulmonary endothelial cells (Hoyt et al., 1997). Suppression of acute DNA damage in normal tissue might be a beneficial clinical adjunct. More generally, stimulation or inhibition of DNA damage via cell surface receptors may be particularly useful.

Engagement of β1 integrin receptors was found to inhibit endotoxin-, etoposide-, and BLM-induced DNA strand breakage as measured by 3′-OH labeling (Hoyt et al., 1996; Jones et al., 2001; Huang et al., 2003). Investigation with a PARP-1 inhibitor and in PARP-1 knockout MLECs indicated that integrin-dependent reduction in 3′-OH breaks requires the BER facilitator, PARP-1 (Jones et al., 2001; Huang et al., 2003). Roles for other single-strand repair proteins in integrin action had not been examined. siRNA techniques allow such investigations where useful antagonists or knockouts are unavailable.

Knockdown of Lig1 and Lig3α both antagonized the removal of 3′-OH during a 1-h drug-free recovery phase (Fig. 2). This inhibition of recovery is consistent with roles for both ligases in DNA repair (Leppard et al., 2003; Mortusewicz et al., 2006). Lig3α, but not Lig1, siRNA antagonized the ability of integrin engagement to suppress BLM-induced DNA breakage (Fig. 3). In addition, with integrin engagement, 3′-OH remained low after a 1-h washout in Lig1 siRNA-treated cells (Fig. 3B) but remained elevated with Lig3α siRNA (Fig. 3C). These results suggest that integrin action depends selectively on the short-patch BER ligase component and not all DNA ligases.

Knockdown of XRCC1 increased the level of 3′-OH DNA breaks caused by BLM (Fig. 4A). This result is similar to that of another XRCC1 siRNA on alkylating agent-induced DNA breakage in HeLa cells (Petermann et al., 2006) and presumably reflects a critical role of this protein in DNA repair. Integrin-mediated suppression of 3′-OH DNA breaks was partially antagonized by XRCC1 siRNA, and 3′-OH was still elevated 1 h after BLM-free washout with or without integrin engagement (Fig. 4). Because XRCC1 siRNA also reduced Lig3α levels (Fig. 1B) (Petermann et al., 2006), it was expected that integrin action would be extensively blocked as seen with Lig3α siRNA (Fig. 3C). Other evidence suggests that long-patch repair with ligation of DNA 3′-OH by Lig1 is facilitated when XRCC1 and Lig3α are deficient (Caldecott, 2003; Fan and Wilson, 2005; Petermann et al., 2006). Thus, we speculate that antagonism of integrin-mediated suppression of 3′-OH DNA breaks by XRCC1 siRNA might be limited to some extent by alternative repair mechanisms that become operational. In any case, BLM-induced DNA breakage and its inhibition by integrin engagement were sensitive to XRCC1 knockdown.

As expected, siRNA knockdown of PARP-1 inhibited integrin action, confirming the prior indications that PARP-1 is required for integrin-mediated protection (Fig. 5) (Jones et al., 2001; Huang et al., 2003). Although PARP-1 does not modify damaged DNA during repair, it binds to 3′-OH and is
allosterically activated to modify several repair proteins and histones with polymers of ADP-ribose. The potential for these polymers to stimulate ligation and the association of PARP-1 with XRCC1 and Lig3α are consistent with the requirement for PARP-1 in integrin action (Masson et al., 1998; Oei and Ziegler, 2000; Caldecott, 2003; Leppard et al., 2003; Fan and Wilson, 2005). Overall, the results indicate that integrin-mediated suppression of BLM-induced 3'-OH DNA breaks depends on PARP-1, XRCC1, and Lig3α, which are physically interacting BER components with a recognized role in short-patch repair of single-strand breaks (Masson et al., 1998; Caldecott, 2003; Fan and Wilson, 2005).

BLM can also generate double-strand breaks and activate ataxia-telangiectasia mutated (Chen and Stubbe, 2005; Fernandes et al., 2005). Radiation-induced double-strand breaks were originally correlated with phosphorylation of histone variant H2AX (Rogakou et al., 1998). Subsequent studies with γH2AX-specific antibodies correlated staining with double-strand DNA breaks assessed by other methods (Paull et al., 2000; Nazarov et al., 2003). As in other cell types (Banath and Olive, 2003), BLM caused a concentration-dependent increase in γH2AX in MLECs (Fig. 6).

Although suppression of drug-induced DNA 3'-OH strand breaks by integrin engagement has been repeatedly seen (Figs. 3–5) (Hoyt et al., 1996, 1997; Jones et al., 2001; Huang et al., 2003), we found here for the first time that integrin engagement suppressed the induction of γH2AX by BLM (Fig. 7). Lig3α, PARP-1, or XRCC1, but not Lig1, siRNA antagonized integrin-mediated reductions. Integrin-mediated suppression of 3'-OH and acute cytotoxicity were each partially inhibited by XRCC1 siRNA (Figs. 4 and 8), suggesting a correlation between these two endpoints. Interestingly, the γH2AX response to 100 μg of BLM/ml was actually increased by integrin engagement after treatment with XRCC1 siRNA (Fig. 7D), whereas cytotoxicity was not (Fig. 8F). As discussed below, XRCC1 seems to function in both BER and...
NHEJ pathways. It is possible that BER and NHEJ may require different levels of XRCC1 or Lig3α for normal function and for the response to integrin engagement. Complete depletion or knockout of XRCC1 or Lig3α might also have different effects than the partial knockdowns attained with siRNA. Given that XRCC1 siRNA reduced both XRCC1 and Lig3α, whereas Lig3α siRNA selectively depleted Lig3α, we hypothesized that depletion of XRCC1 permits integrin engagement to facilitate increases in γH2AX in response to BLM. Because the level of γH2AX is actually a balance between kinase and phosphatase activities acting on this histone (Nazarov et al., 2003), H2AX kinases or phosphatases could be modulated by integrin engagement in a manner that is dependent on XRCC1. Nevertheless, the results suggest that PARP-1, XRCC1, and Lig3α, but not Lig1, are required for integrin-mediated suppression of BLM-induced double-strand breakage.

The involvement of PARP-1, XRCC1, and Lig3α in the suppression of double-strand breakage by integrin engagement might be due to possible effects of these BER components on conversion of isolated single-strand breaks, or clusters of them, to double-strand breaks. Recent investigations also indicate that PARP-1, XRCC1, and Lig3α contribute to repair of double-strand breaks in vitro and to cellular resistance to double-strand breaking agents (Audebert et al., 2004, 2006). In addition, ionizing radiation induced DNA-PKcs to phosphorylate XRCC1, and γH2AX accumulation was increased by expression of a nonphosphorylatable mutant of XRCC1 in comparison with wild type (Levy et al., 2006). Lig3 also associated with the NHEJ proteins Ku70 and Ku80, as indicated by their retention on a Lig3 affinity column (Leppard et al., 2003), and DNA ligase IV-deficient cells retained a substantial level of DNA end-joining activity that was reduced 80% by a Lig3α siRNA (Wang et al., 2005). Finally, EM9 and EM-C11 cell lines lacking XRCC1 displayed slower repair of double-strand breaks compared with cells containing XRCC1 (Schwartz et al., 1987; Nocentini, 1999). Thus, multiple functions of BER proteins in single- and double-strand DNA repair may account for their requirement in integrin-mediated suppression of DNA 3'-OH and γH2AX levels in BLM-treated MLEC.

Integrin engagement may increase PARP-1/XRCC1/Lig3α-dependent repair of single- and double-strand breaks. Mechanisms that could be responsible for this potential effect are not known, but integrin engagement could modify the repair proteins or the DNA substrate. For example, chromatin structural proteins may be altered so that repair is more efficient. Growing evidence implicates chromatin modifications in the repair of strand breaks (Bird et al., 2002; Peterson and Cote, 2004; Verger and Crossley, 2004). Efficient repair of double-strand breaks through NHEJ requires stabilization of the ends of DNA by Ku70/80 proteins and DNA-PKcs (Weterings and van Gent, 2004; Wang et al., 2005), and this step may be facilitated by chromatin structural proteins (Iliakis et al., 2004). Previously, we found that integrin engagement increased the sensitivity of MLEC DNA to nuclease digestion, increased the acetylation of histone 3, and generally reduced the association of linker histone 1 with DNA (Jones et al., 2001; Rose et al., 2005). Thus, integrin-mediated effects on chromatin structure may make DNA breaks more manageable by PARP-1/XRCC1/Lig3α in the repair process. Alternatively, integrin engagement may in-
crease BER protein activities that enhance repair of single- and/or double-strand breaks.

In summary, integrin engagement reduced both 3'-OH and yH2AX caused by BLM in MLECs. Protection was antagonized by suppression of the BER proteins, PARP-1, XRCC1, and Lig3α, but not Lig1. These results suggest potential targets for pharmacological intervention in genotoxicity through BER proteins and indicate a novel mechanism by which integrin engagement protects endothelial cells from DNA damage.

References


