Regulation of Bleomycin-Induced DNA Breakage and Chromatin Structure in Lung Endothelial Cells by Integrins and Poly(ADP-Ribose) Polymerase

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ABSTRACT

Activation of endothelial cell integrins inhibits DNA breakage by diverse agents, including the DNA-damaging agent bleomycin. DNA breaks activate nuclear poly(ADP-ribose) polymerase (PARP), which regulates chromatin structure and DNA repair. We determined the role of PARP in suppression of bleomycin genotoxicity by integrins using wild-type and PARP knockout mouse lung endothelial cells (MLEC), and the PARP inhibitor, 3-aminobenzamide (3AB). Activation of $\beta 1$ integrins by antibody clustering enhanced the sensitivity of wild-type nuclei to digestion with micrococcal nuclease and deoxyribonuclease I, indicating that chromatin structure was altered. 3AB blocked this effect. Knockout and 3AB-treated wild-type MLEC were hypersensitive to deoxyribonuclease I compared with wild-type cells, demonstrating that PARP regulates chromatin structure. Integrin clustering reduced the hypersensitivity of knockout cells, suggesting additional, PARP-independent mechanisms that inhibit nuclease interaction with chromatin. Bleomycin caused DNA breakage in wild-type and knockout MLEC. Breaks were eliminated after 60 min incubation of wild-type cells in drug-free medium, whereas 3AB or PARP knockout inhibited DNA repair. Integrin clustering protected wild-type cells from DNA breakage, and 3AB and PARP knockout inhibited this protection. Bleomycin caused large increases in PARP activity in wild-type but not knockout MLEC, and integrin clustering inhibited the activation of PARP. The results indicate that the antigenotoxic effects of integrin activation require PARP and that integrins alter chromatin structure by PARP-dependent and -independent mechanisms.

Anticancer drugs, radiation, and many environmental chemicals are recognized genotoxins, and any agent that activates oxygen has the potential to cause DNA breakage (Szabo and Dawson, 1998). Endothelial cells in the lung and other organs are common targets of DNA damage, which may lead to pulmonary and cardiovascular disease. The direct DNA cleaving antitumor antibiotic bleomycin (BLM), for example, is known to cause pulmonary fibrosis after a period of acute injury to lung endothelial and epithelial cells, limiting its use against cancer (Adamson, 1976). Inhibition of acute DNA damage in endothelium, or the response to it, could reduce pulmonary and cardiovascular diseases caused by BLM and other agents.

Previously, we found that activation of integrin cell adhesion receptors inhibited acute DNA breakage caused by BLM, bacterial endotoxin, and etoposide (Hoyt et al., 1996a,b; Hoyt et al., 1997). Evidence for the role of integrins in promoting endothelial cell survival in the face of different types of stress has accumulated in recent years (Meredith et al., 1993; Brooks et al., 1994; Ilic et al., 1998). Integrins are heterodimeric receptors that mediate adhesion of endothelial cells to extracellular matrix, largely by interaction with arginine-glycine-aspartate sequences in the matrix proteins. Signal transduction by integrins activated with peptide ligands or by clustering with antibodies may mediate their effects (Aplin et al., 1998; Ilic et al., 1998).

BLM, endotoxin, and etoposide cause DNA breakage by different mechanisms. The topoisomerase II inhibitor, etoposide, causes DNA breaks in response to stabilization of DNA-topoisomerase complexes in cell nuclei. Bacterial endotoxin also causes acute, reversible DNA breakage that greatly precedes apoptosis in endothelial cells. Endotoxin-induced DNA breakage may be caused by reactive oxygen species and peroxynitrite (Szabo and Dawson, 1998). The mechanism of BLM is very well characterized and depends on the ability of the antibiotic to bind iron and to activate oxygen in the vicinity of DNA (Wu et al., 1985). Integrin-mediated protection of endothelial cells from these diverse agents could re-

ABBREVIATIONS: BLM, bleomycin; PARP, poly(ADP-ribose) polymerase; ADPR, ADP-ribose; 3AB, 3-aminobenzamide; DNase, deoxyribonuclease; VCAM-1, vascular cell adhesion molecule-1; PECAM, platelet endothelial cell adhesion molecule; MLEC, mouse lung endothelial cells; ISNT, in situ nick translation; MNase, micrococcal nuclease; PARSIS, poly(ADPR) synthesis in situ.

sult from increased resistance of DNA to breakage, which may occur if nuclear structure is altered, or from activation of DNA repair.

The nuclear enzyme poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) helps to orchestrate the acute reaction to DNA damage. PARP responds immediately to genotoxic stress by binding to DNA strand breaks via two C-terminal zinc fingers. This binding allosterically activates the enzyme, which uses NAD to synthesize polymers of ADP-ribose (ADPR) on glutamate residues in its own structure and in other nuclear proteins. Histones are major targets for modification (Boulikas, 1991; D'Amours et al., 1999). The dynamic balance of poly(ADPR) synthesis by PARP and its degradation by poly-(ADP-ribose) glycohydrolase may govern the final consequences of PARP activation (D'Amours et al., 1999). Topoisomerases and the tumor suppressor p53, which regulate nuclear function, are among the other targets of PARP that have been identified in genotoxin-treated cells (Scovassi et al., 1993; Simbulan-Rosenthal et al., 1999; Smith and Grosovsky, 1999). PARP has a role in DNA repair, in that such inhibitors as 3-aminobenzamide (3AB) slow the removal of DNA breaks (Althaus, 1992). Thus, PARP regulates chromatin structure and is necessary for DNA repair (D'Amours et al., 1999).

PARP is activated in a range of pathophysiologic situations, including oxidant stress (Szabo and Dawson, 1998). BLM-induced DNA breakage is a powerful PARP activator, and several studies implicate PARP in pulmonary fibrosis caused by this drug. The enzyme is activated in vivo by BLM, and niacin, an inhibitor of PARP and precursor of NAD, reduces fibrosis (Hussain et al., 1985; Wang et al., 1990). Activation of PARP and depletion of NAD correlate with the sensitivity of lung slices from different murine strains to the cytotoxicity of BLM. Furthermore, PARP activation, NAD depletion and cytotoxicity caused by BLM are inhibited by preincubation of lung slices with 3AB (Hoyt and Lazo, 1992, 1993).

Because PARP regulates chromatin structure and DNA repair, we hypothesized that integrin-induced protection of endothelial cells from BLM requires PARP. Here we investigated the ability of integrins to suppress BLM-induced DNA breakage and to alter chromatin in lung endothelial cells from wild-type and PARP knockout mice.

Experimental Procedures

Materials. Cell culture media and trypsin were purchased from Life Technologies (Gaithersburg, MD). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). BLM, 3AB, goat anti-rat IgG, goat and rat serum, NAD, and PBS were obtained from Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* DNA polymerase I, Micrococcal Nuclease (S1), and bovine serum albumin were from Roche Diagnostics (Nutley, NJ). Deoxyribonuclease I (DNase I) was from Cooper Biomedical (Freehold, NJ). Fluorescein-12-dUTP was purchased from Stratagene (La Jolla, CA). Rat anti-mouse $\beta 1$ integrin, vascular cell adhesion molecule-1 (VCAM-1), and platelet endothelial cell adhesion molecule (PECAM) were purchased from PharMingen (San Diego, CA), and rabbit anti-poly(ADPR) was obtained from Biomol Research Laboratories, Inc., (Plymouth Meeting, PA). Cy3-conjugated goat anti-rabbit IgG was from Jackson Immunoresearch Laboratories (West Grove, PA).

Cells. Murine lung microvascular endothelial cells (MLEC) were isolated from wild-type $(+/+)$ and PARP knockout $(-/-)$ mice (Wang et al., 1995) as described previously (Gerritsen et al., 1995).

Integrin Clustering. $\beta 1$ integrins were activated with anti-integrin antibodies as described previously (Hoyt et al., 1996a). MLEC were transferred to microscope slides at 80% confluence and cultured for 24 h. The cells were rinsed and treated 0, 0.1, or 1 μg anti-$\beta 1$ integrin antibody/ml for 1 h at 4°. Some MLEC were treated with 1 μg of rat anti-mouse PECAM or VCAM per milliliter, or preimmune rat serum (1/5000 dilution, which approximates a 1 μg/ml concentration), rather than anti-integrin antibody. Goat anti-rat IgG was added to all cells at a concentration of 2 μg/ml and the temperature was raised to 37° for 2 or 4 h. After clustering, cells were treated with 0 or 0.2 mg BLM/ml for 45 min. Some MLEC were rinsed with fresh medium and allowed an additional drug-free period of 60 min (washout). Where indicated, the PARP-inhibitor, 3AB, was included at a concentration of 2.5 mM 18 h before clustering (Hoyt and Lazo, 1992).

In Situ Nick Translation (ISNT) to Quantify DNA Strand Breaks. Cells were washed three times with PBS at 4° after treatment with BLM. They were fixed with 1% formaldehyde in PBS and made permeable with 70% ethanol at −20°. DNA breaks were labeled by ISNT with the substitution of fluorescein-12-dUTP (Gorczyca et al., 1993; Hoyt et al., 1997). Cells were then incubated at 37° for 90 min with ISNT buffer (2.5 mM MgCl$_2$, 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, 16 μM fluorescein-12-dUTP, and 2 U/ml *E. coli* DNA polymerase. The labeling was stopped by rinsing with PBS.

Nuclease Digestion. Relative sensitivity of nuclear DNA to digestion with exogenous nucleases was used to determine whether integrins or PARP could alter chromatin structure (von Hippel and Felsenfeld, 1964; Hewish and Burgoyne, 1973). MLEC were fixed with formaldehyde, permeabilized with ethanol, then rinsed with PBS. For DNase digestion, cells were rinsed in ISNT buffer and then digested 25 min at room temperature with 0 to 48 U/ml DNase I in ISNT buffer. The cells were rinsed three times with PBS and subjected to ISNT as described above. For digestion with micrococcal nuclease (MNase), MLEC were rinsed in MNase buffer (10 mM Tris, pH 7.4, 1 mM CaCl$_2$, 2.5 mM MgCl$_2$) and then digested 25 min at room temperature with 0 to 55 U/ml MNase in MNase buffer. The cells were then rinsed three times with PBS before ISNT.

Poly(ADPR) Synthesis In Situ (PARSIS). MLEC were treated, rinsed three times with PBS and fixed in acetone at 4°C. Cells were then rinsed in PARSIS buffer (100 mM Tris, pH 8, 10 mM MgCl$_2$, 1 mM dithiothreitol), and then incubated 0 to 20 min in PARSIS buffer containing 0 to 200 μM NAD (Iseki, 1987). The reaction was stopped by rinsing with PBS. Poly(ADPR) was detected by fluorescence immunostaining (Kupper et al., 1996). Cells were blocked with 10% goat serum in PBS and then incubated with rabbit-anti poly(ADPR) at 4° for 18 h. After rinsing, Cy3-conjugated goat-anti-rabbit IgG (Fab2′) in 10% goat serum in PBS was added for 1 h at 37°. Cells were then rinsed with PBS.

Data Analysis. An Olympus BX60 fluorescence microscope and 20× objective were used to capture digital images of fluorescein- or Cy3-labeled MLEC. Fluorescence intensity of nuclei from 100 to 500 cells was determined by image analysis (Scanpro, SPSS Science, Chicago, IL). Data were analyzed by Student's *t* test or by ANOVA with Bonferroni correction for multiple comparisons (Snedecor and Cochran, 1980).

Results

Nuclease Sensitivity. PARP is known to alter chromatin structure and to facilitate DNA repair. To determine whether integrin activation or PARP altered chromatin structure, exogenous nucleases were used to probe the accessibility of DNA (von Hippel and Felsenfeld, 1964; Hewish and Burgoyne, 1973). Breaks in DNA were produced with MNase or DNase I in formaldehyde-fixed +/+ MLEC. MNase is a cal-

cium-dependent endonuclease that prefers to cut between nucleosomes in chromatin (Hayes and Lee, 1997), whereas DNase I cuts more randomly throughout (Suck, 1997). Enzyme-generated DNA breaks were detected by ISNT and fluorescence microscopy.

Figure 1, A and B, show that digestion with increasing concentrations of either enzyme caused an increase in DNA breaks. In Fig. 1A, a drop in signal was seen with the highest concentration of MNase used. This could have been caused by excessive release of DNA from the cells. Integrin activation enhanced the level of DNA breakage produced by both nucleases. The effect of the two highest concentrations of MNase (18 and 55 U/ml) was enhanced, and the DNase concentration-response relationship was shifted to the left. DNase (0.75 U/ml) was used to examine the dependence of nuclease sensitivity on anti-$\beta1$ integrin antibody concentration. Activation of $\beta1$ integrin for 4 h caused DNase hypersensitivity that increased as anti-$\beta1$ antibody was increased from 0.01 to 1.0 μg/ml (Fig. 2). These results suggest that integrin activation increased the access of exogenous nucleases to DNA.

The effect of integrin activation on nuclease sensitivity in +/+ and −/− MLEC was compared. Figure 3 depicts the effect of integrin activation on DNase sensitivity in +/+ and −/− MLEC in representative images. The results of image analysis are presented in Fig. 4. As in the previous experiments, integrin activation increased the digestion of DNA by DNase in +/+ cells. In contrast, −/− MLEC were DNase-hypersensitive compared with +/+ cells, and integrin activation reduced the digestion of −/− cell DNA. Furthermore, an 18-h preincubation of +/+ cells with 3AB alone moderately increased the sensitivity to DNase. The larger increase caused by integrin clustering alone was not seen in +/+ cells when they were treated with both integrin antibody and 3AB.

BLM-Induced DNA Strand Breakage. We previously showed that BLM caused acute, reversible DNA strand breaks that were inhibited by integrin activation (Hoyt et al., 1997). DNA strand breaks were measured here to determine the effect of BLM, 3AB, PARP knockout, and integrin clustering on breakage and repair (Fig. 5). As expected, BLM caused DNA strand breakage within 45 min. Prior activation of $\beta1$ integrin for 4 h inhibited BLM-induced DNA breakage. The inhibition was specific for the $\beta1$ integrin antibody, because neither rat anti-mouse PECAM, VCAM, nor preimmune rat serum inhibited BLM-induced DNA damage (not shown). DNA breaks were repaired, as they disappeared after an additional 60-min incubation in drug-free medium. Preincubation with 2.5 mM 3AB for 18 h did not affect BLM-induced DNA breakage but, as expected, DNA repair was completely inhibited. The combination of 3AB and integrin antibody resulted in significant DNA breakage that was less than 3AB alone and more than $\beta1$ clustering alone (not indicated by a symbol).

BLM also caused DNA breakage in −/− MLEC, although it was less than in +/+ cells. As with +/+ cells treated with 3AB, −/− MLEC did not effectively repair DNA breaks during washout. In contrast to the protection of +/+ MLEC,
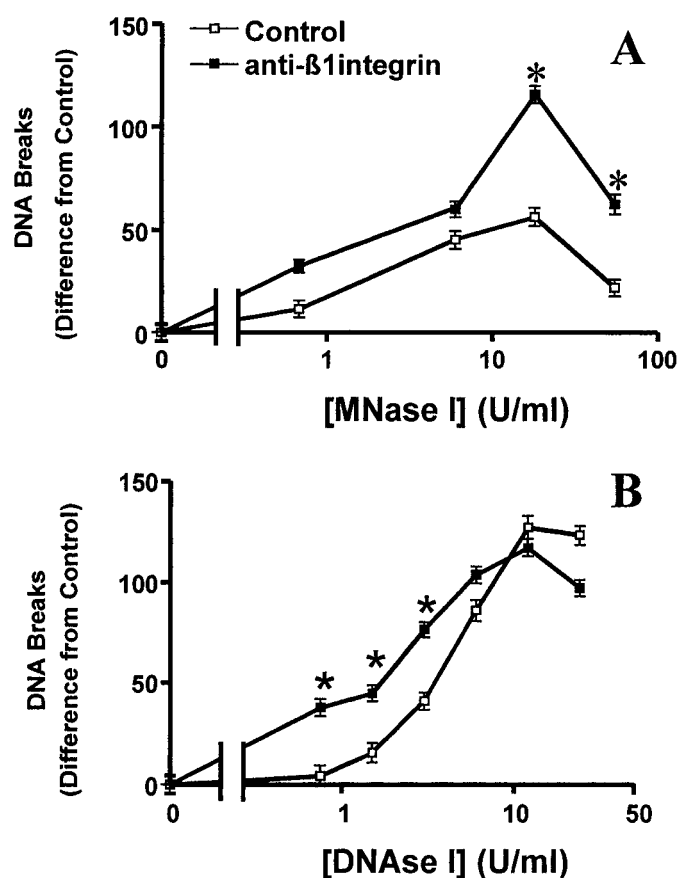

Fig. 1. Effect of integrin activation on the sensitivity of nuclei to nucleases. +/+ MLEC $\beta1$ integrins were activated by 1 h incubation with 0 or 1 μg anti-$\beta1$ integrin antibody/ml at 4°C followed by addition of goat-anti rat IgG (2 μg/ml final concentration) and incubation at 37°C for 2 h. Cells were fixed, permeabilized with 70% ethanol and digested with the indicated concentrations of MNase (A) or DNase (B) for 25 min. Breaks in DNA were fluorescently labeled by ISNT. Bars represent the mean difference in nuclear fluorescence intensity between cells incubated with or without nuclease ± S.E. of the difference for 100 to 500 cells in each experimental group. *$p < 0.05$ for comparison with 0 μg/ml anti-$\beta1$ integrin antibody.
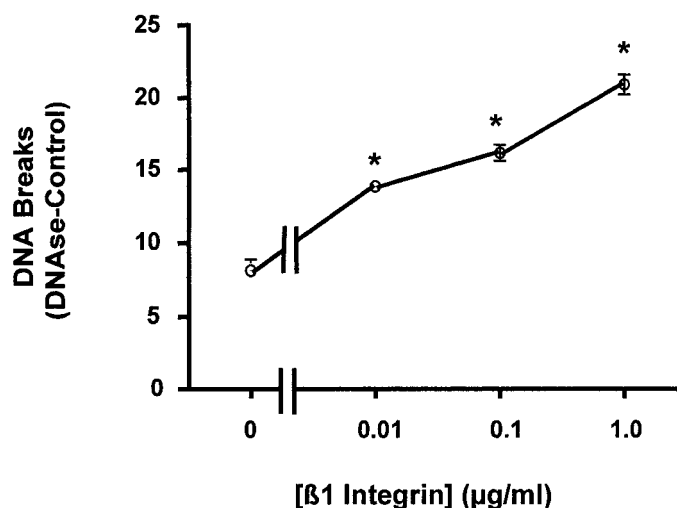

Fig. 2. Effect of increasing concentration of integrin antibody on the sensitivity of wild-type nuclei to DNase digestion. +/+ MLEC $\beta1$ integrins activated by 1 h incubation with 0, 0.1, or 1 μg anti-$\beta1$ integrin antibody/ml at 4°C followed by addition of goat-anti rat IgG (2 μg/ml final concentration) and incubation at 37°C for 4 h. Cells were processed and digested with 0.75 U/ml DNase I for 25 min and breaks in DNA were labeled by ISNT. Bars represent the mean difference in nuclear fluorescence intensity between cells incubated with or without nuclease ± S.E. of the difference for 100 to 500 cells in each experimental group. *$p < 0.05$ for comparison with 0 μg/ml anti-$\beta1$ integrin antibody.

β1-integrin clustering did not inhibit BLM-induced DNA breakage in $-/-$ cells. This suggested that PARP was required for the anti-genotoxic action of integrin activation.

Poly(ADP-Ribose) Polymerase (PARP). PARSIS was developed as a convenient microscopic method to investigate genotoxic stress and to characterize knockout cells. Based on a microautoradiographic method (Iseki, 1987), acetone-fixed cells were supplemented with various concentrations of NAD and ADPR polymers were detected by fluorescence immunostaining (Kupper et al., 1996). Figure 6 shows the effect of BLM and PARP knockout on ADPR immunofluorescence. BLM caused a large increase in poly(ADPR) that was enhanced by NAD added to the in situ reaction with $+/+$ cells (Fig. 6, A and D versus C and F) but not $-/-$ cells (Fig. 6, B and E). Inclusion of 3AB in the in situ reaction abolished labeling in $+/+$ cells treated with BLM and incubated with 20 μM NAD (Fig. 6G). Image analysis of BLM-treated $+/+$ MLEC showed that poly(ADPR) levels were dependent on increasing concentrations of NAD and maximal incorporation was seen at 10 min in $+/+$ cells (Fig. 7). Based on these results, a 10-min reaction with 0 to 200 μM NAD was used to assess the effects of BLM, β1-integrin clustering, and 3AB on PARP activity. BLM significantly increased the level of poly(ADPR) detected after in vitro incubation of fixed cells with 0, 2, 20, and 200 μM NAD compared with cells that were not treated with BLM (Fig. 8). $-/-$ MLEC synthesized far less poly(ADPR) than $+/+$ MLEC in all conditions. A small BLM-dependent increase in poly(ADPR) was seen in $-/-$ cells incubated with 200 μM NAD, however.

These results indicate that BLM increased basal poly(ADPR) content (signal with 0 μM NAD), and that PARP was greatly activated (reaction in the presence of NAD).

β1-integrin clustering for 4 h in $+/+$ MLEC blocked both the BLM-stimulated basal polymer level and the BLM-stimulated in vitro synthesis in the presence of 20 μM NAD (Fig. 9). Preincubation of $+/+$ MLEC with 2.5 mM 3AB for 18 h alone, or followed by integrin clustering, also prevented increases in poly(ADPR) caused by BLM.

Discussion

Integrin cell-adhesion receptor activation inhibits acute DNA breakage caused by a variety of unrelated agents (Hoyt et al., 1996a,b; Hoyt et al., 1997). Integrins may protect cells by multiple mechanisms that are specific to each agent. Alternatively, one or a few general mechanisms could account for broad protection by integrin activation.

Integrin activation could affect cell nuclei, enhancing repair or making DNA resistant to damage. Here we demonstrated for the first time that integrin activation altered the structure of MLEC chromatin, as indicated by changes in sensitivity to nucleases (von Hippel and Felsenfeld, 1964; Hewish and Burgoyne, 1973). The sensitization may have resulted from dissociation or movement of proteins that restricted the access of nucleases to DNA. Although the exact mechanism for altered nuclease sensitivity is not fully understood, we can conclude that integrin activation affected the MLEC nucleus. A fundamental change in the nucleus could affect DNA breakage or repair in response to many agents.

It is possible that integrins reduce the cellular accumulation of chemicals. However, altered transport did not account for integrin-induced resistance of myeloma cells to doxorubicin and melphalan (Damiano et al., 1999). The effect of integrins on transport in MLEC has not been investigated.

The nuclear enzyme, PARP, is activated by DNA strand breaks and it alters chromatin structure by modification of nuclear proteins (Das and Kanungo, 1986a,b; Boulikas, 1991; D'Amours et al., 1999). Thus, inhibition or genetic deletion of PARP should affect MLEC nuclei. Indeed, 3AB increased DNase digestion of $+/+$ cells, and knockout of PARP rendered $-/-$ cells more sensitive to digestion with DNase than wild-type MLEC (Fig. 4).

The critical role of PARP in DNA repair was confirmed by the observation that BLM-induced DNA breaks were not repaired in $-/-$ cells or in $+/+$ cells treated with 3AB (Fig. 5). This result was expected from previous studies by many
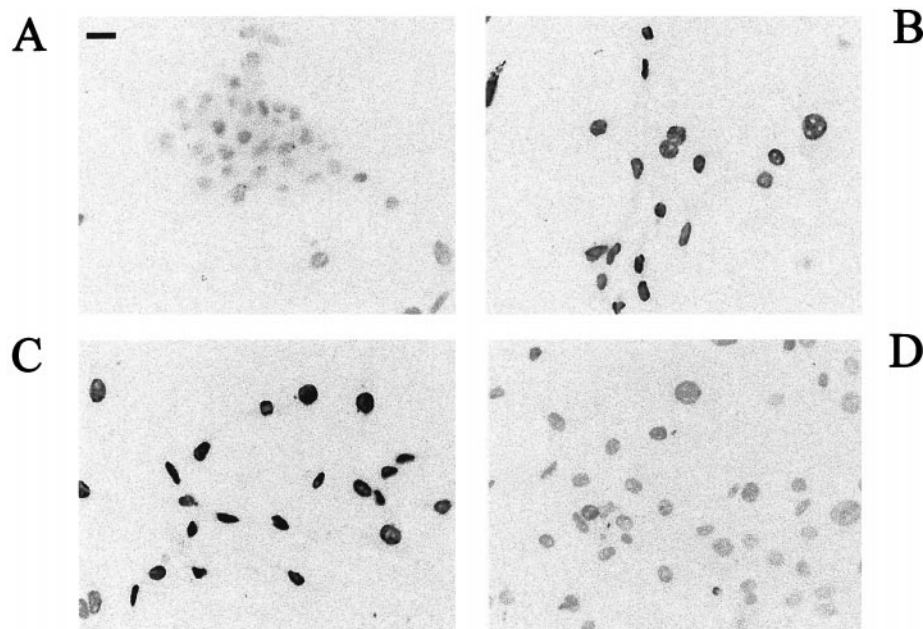
Fig. 3. DNase I Digestion of Wild-Type and PARP Knockout MLEC. Representative fluorescence images of $+/+$ (A and B) and $-/-$ MLEC (C and D) that were treated with 0 (A and C) or 1.0 μg anti-β1 integrin antibody/ml (B and D) at 4°C for 1 h and then with goat-anti rat IgG (2 μg/ml final concentration) at 37°C for 4 h, are shown. Cells were processed and digested with 0.75 U/ml DNase I for 25 min and breaks in DNA were labeled by ISNT. The bar in A represents 40 μm.

others (D'Amours et al., 1999). Furthermore, integrin clustering did not inhibit BLM-induced breakage in −/− or 3AB-treated cells as greatly as in +/+ cells. Thus, integrin-mediated protection from BLM requires PARP.

We measured basal and in vitro synthesized poly(ADPR) to determine the effect of BLM and integrin activation on PARP. Polymerization of ADPR is normally rapid, and begins within minutes of DNA damage. The majority of cellular NAD may even be consumed by the reaction. Degradation of poly(ADPR) by glycohydrolase is also immediate, so that a transient modification of nuclear proteins is seen (D'Amours et al., 1999). Consistent with these facts was our finding that poly(ADPR) was low in control MLEC and that BLM significantly increased the basal polymer level in +/+ cells (Figs. 8 and 9). Furthermore, in vitro reaction with 20 to 200 μM NAD in fixed +/+ cells greatly increased poly(ADPR) content. Thus, basal poly(ADPR) presumably reflects the steady

state condition in cells, whereas reaction with NAD in vitro, where initial velocity conditions outweigh degradation of poly(ADPR), reflects the activation state of PARP.

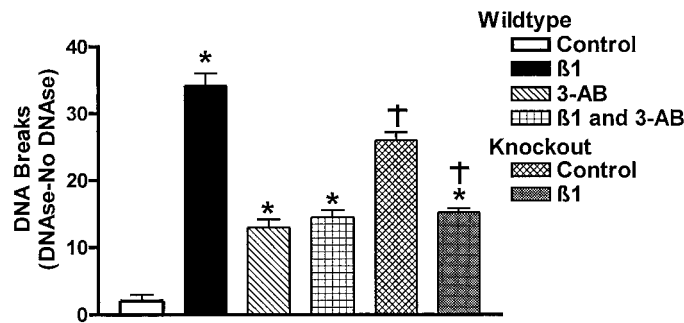Integrin clustering inhibited the increase in basal poly-(ADPR) and PARP activation by BLM in +/+ MLEC (Fig. 9).

Fig. 6. PARSIS assay of PARP activation in +/+ and −/− MLEC. +/+ (A, C, D, F, G) and −/− MLEC (B and E) were incubated without (A–C) or with 0.2 mg/ml BLM (D–G) for 45 min and fixed in acetone. MLEC were incubated in reaction buffer containing 0 (C and F) or 20 μM NAD (A, B, D, E, and G) for 10 min and then anti-poly(ADPR) antibody followed with Cy3-goat anti-rabbit IgG. In G, 2.5 mM 3AB was included in the reaction with 20 μM NAD. Images are negatives of Cy3 fluorescence. The bar in A represents 60 μm.

Fig. 4. Effect of integrin clustering, PARP inhibition and PARP knockout on DNase I sensitivity. +/+ and −/− MLEC were treated with 0 or 2.5 mM 3AB for 18 h, then with 0 or 1.0 μg/ml anti-β1 integrin antibody at 4°C for 1 h and then with goat-anti rat IgG (2 μg/ml final concentration) at 37°C for 4 h. Cells were processed and digested with 0.75 U/ml DNase I for 25 min and breaks in DNA were labeled by ISNT. Bars represent the mean difference in nuclear fluorescence intensity between cells incubated with or without nuclease ± S.E. of the difference for 100 to 500 cells in each experimental group. *$p < 0.05$ for comparison between 0 and 0.2 mg/ml BLM. †$p < 0.05$ for comparison between +/+ and −/− MLEC treated in the same way.
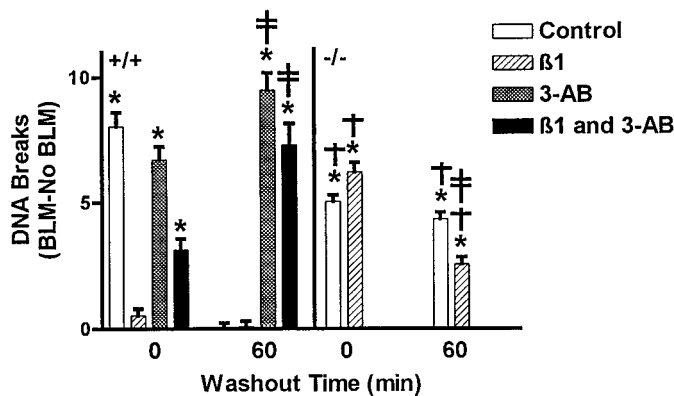
Fig. 5. Effect of integrin clustering, PARP inhibition and PARP knockout on BLM-induced DNA strand breakage. +/+ (A) and −/− (B) MLEC were treated with 0 or 2.5 mM 3AB for 18 h, then with 0 or 1.0 μg/ml anti-β1 integrin antibody at 4°C for 1 h, then with goat-anti rat IgG (2 μg/ml final concentration) at 37°C for 4 h and finally with 0 or 0.2 mg/ml BLM for 45 min. Cells were then fixed and processed for ISNT to measure DNA strand breaks. Bars represent the mean difference in nuclear fluorescence intensity between cells incubated with 0 or 0.2 mg/ml BLM ± S.E. of the difference for 100 to 500 cells in each experimental group. *$p < 0.05$ for comparison between 0 and 0.2 mg BLM/ml. †$p < 0.05$ for comparison between +/+ and −/− MLEC. ‡$p < 0.05$ for comparison between 0 and 60 min washout for each treatment.
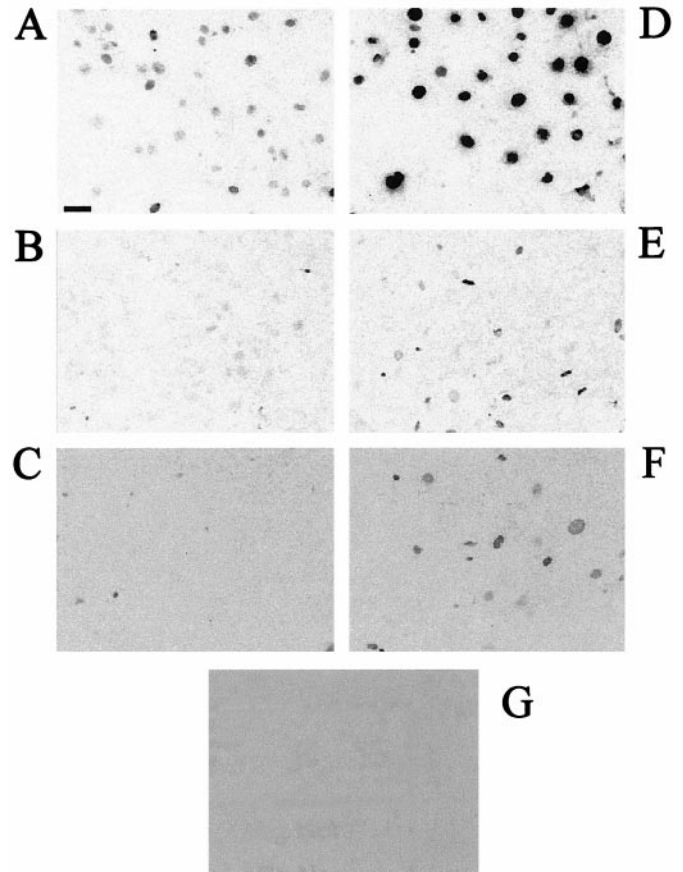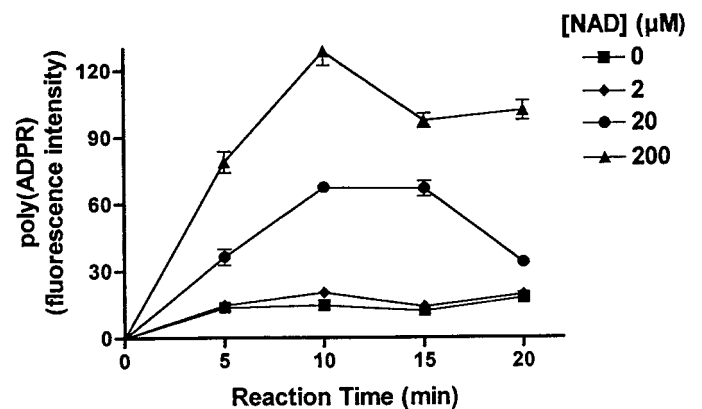
Fig. 7. Kinetics of PARSIS reaction. +/+ MLEC were treated with 0.2 mg/ml BLM for 45 min and processed as in Fig. 5 with additional PARSIS reaction times and NAD concentrations as indicated. Images of cells were analyzed for nuclear fluorescence intensity. Symbols represent the mean ± S.E. of 100 to 500 cells in each experimental group.

Inhibition of BLM-induced DNA breakage as seen in Fig. 5 may account for this, as PARP would not be activated in the absence of DNA breaks.

+/+ and −/− MLEC differed greatly in PARP activation by BLM. −/− cells were incapable of synthesizing the large amounts of poly(ADPR) seen in wild-type cells. Nevertheless, there was a small but significant signal in BLM-treated −/− MLEC incubated with the highest level of NAD (200 μM, Fig. 8). Other minor poly(ADP-ribose) polymerases that are activated by DNA-damaging agents have been discovered since the generation of PARP knockout mice. The majority of this residual PARP is sensitive to inhibition by benzamide (Shieh et al., 1998). These minor isozymes may account for the low-level PARP activation seen in these cells, in which only PARP-1 has been deleted. Although the activity of alternative poly(ADP-ribose) polymerases is low compared with the major enzyme, they could have unrecognized biological functions (Shieh et al., 1998; Smith et al., 1998; D'Amours et al., 1999).

It was interesting that in wild-type MLEC 3AB prevented the activation of PARP even though DNA breaks were present, and repair was blocked (Fig. 5). We observed a similar action of 3AB on PARP in nuclei isolated from BLM-treated lung slices using radiolabeled NAD as the reaction substrate (Hoyt and Lazo, 1992). 3AB is a competitive inhibitor of PARP that presumably blocks poly(ADPR) synthesis in living MLEC during exposure to BLM (Purnell and Whish,
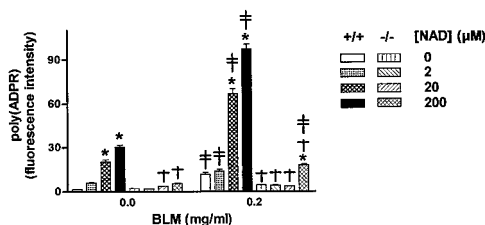
1980). Prevention of enzyme activation by the competitive inhibitor cannot be explained if 3AB were actually washed out before the start of the in vitro PARSIS reaction. However, 3AB may have altered nuclei during the incubation with live cells such that PARP could not be activated by BLM, despite the presence of DNA breaks (Fig. 5). 3AB did increase the sensitivity of wild-type nuclei to DNase I, indicating that there was a change in chromatin structure (Fig. 4). We speculate that PARP probably bound to DNA breaks in BLM-treated cells, although polymerase activity was blocked by the competitive action of 3AB. This could have affected other systems regulated by PARP, such as DNA repair complexes, topoisomerases, and p53, that would otherwise operate in an environment of massive poly(ADP-ribosyl)ation after DNA breakage. This situation may have limited the activation of PARP detected in vitro in fixed cells despite the presence of DNA breaks.

Integrin clustering affected chromatin differently in wild-type and knockout MLEC. In contrast to +/+ cells, nuclei from −/− cells initially had a high level of DNase sensitivity, which was lowered by integrin clustering (Fig. 4). These data suggest that integrins acted in knockout cells by a PARP-independent mechanism to restrict the accessibility of DNA to nucleases (Fig. 10), whereas PARP-dependent actions (i.e., those seen in wild-type cells) had the opposite effect, opening chromatin to digestion with nucleases. PARP-independent actions of integrins likely involve other chromatin regulatory processes. Phosphorylation or acetylation of nuclear proteins or the methylation of DNA may be regulated by integrins, for example.

We propose that a balance exists between integrin-induced, PARP-dependent elevation, and PARP-independent suppression of nuclease sensitivity (Fig. 10). In the absence of PARP, clustering cannot further enhance digestion, leaving actions that lower nuclease sensitivity unopposed. The ability of integrin clustering to inhibit damage by BLM was PARP-dependent (Fig. 5). Our model proposes that PARP-dependent opening of chromatin structure by β1-integrin activation could contribute to this inhibition of damage. The

Fig. 8. Activation of PARP by BLM in +/+ and −/− MLEC. Cells were treated with 0.0 or 0.2 mg/ml BLM for 45 min and analyzed as in Figs. 6 and 7 after a 10-min PARSIS reaction with the indicated NAD concentrations. Bars represent the mean ± S.E. fluorescence intensity of 100 to 500 cells in each experimental group. *$p < 0.05$ for comparison with 0 μM NAD within each genotype; †$p < 0.05$ for comparison with +/+ cells; ‡$p < 0.05$ for comparison with 0 mg/ml BLM.
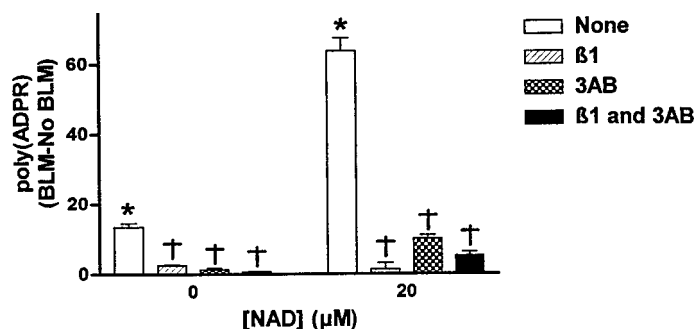

Fig. 9. Effect of BLM, integrin clustering, and 3AB on poly ADP-ribosylation. +/+ MLEC were treated with 0 or 2.5 mM 3AB for 18 h, then with 0 or 1.0 μg/ml anti-β1 integrin antibody at 4°C for 1 h, then with goat-anti rat IgG (2 μg/ml final concentration) at 37°C for 4 h, and finally with 0 or 0.2 mg/ml BLM for 45 min. The PARSIS assay was then performed with 0 or 20 μM NAD included in a 10-min reaction. Bars represent the mean difference in nuclear fluorescence intensity between cells incubated with 0.2 mg/ml BLM and 0 mg/ml BLM ± SE of the difference for 100 to 500 cells in each experimental group. *$p < 0.05$ for comparison between 0 and 0.2 mg/ml BLM. †$p < 0.05$ for comparison with BLM alone (open bar).
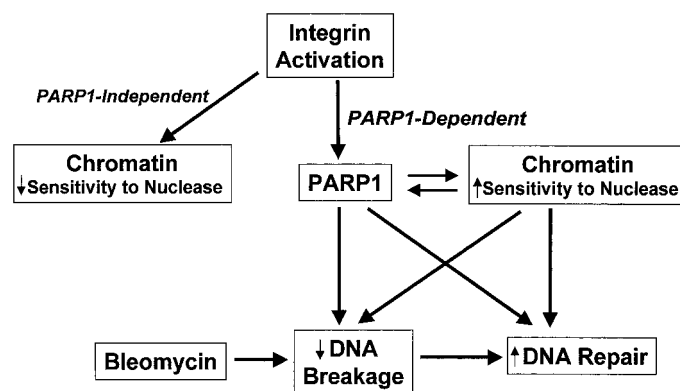

Fig. 10. Model for PARP-dependent and -independent effect of integrins. Integrin clustering alters chromatin structure by PARP-dependent and -independent mechanisms. The PARP-dependent mechanism is indicated by integrin-induced alteration of nuclease sensitivity that is reduced by the PARP inhibitor 3AB. A PARP-independent mechanism is indicated by reduction of nuclease sensitivity from a high level in −/− MLEC. Alteration of chromatin structure in turn affects DNA repair in a PARP-dependent manner, as evidenced by lack of DNA repair in +/+ MLEC in the presence of 3AB. Note that all effects of integrin clustering on BLM-induced DNA damage are PARP-dependent.

opening of chromatin by integrin clustering in wild-type cells could inhibit cleavage of DNA by BLM, although access of the drug to DNA would be expected to increase, as for DNase. It is more likely that DNA repair was increased in wild-type MLEC, limiting the breakage detected after BLM exposure.

An apparent paradox is that integrin clustering did not inhibit BLM-induced DNA breakage in PARP knockout cells, despite reducing nuclease sensitivity from its initial high level. Because BLM is a much smaller molecule than DNase, this PARP-independent chromatin closing action may not have been be sufficient to limit cleavage by the drug. Integrin clustering may be less effective at reducing damage by BLM in PARP knockouts if integrin engagement normally enhances PARP-dependent repair, which is absent in those cells (Fig. 5). We are currently examining this possibility. Although the mechanism remains to be determined, it is clear that integrin clustering altered chromatin structure and that PARP modulated the nuclear response.

In conclusion, integrin activation inhibited BLM-induced DNA breakage and PARP activation in wild-type MLEC, despite increasing the access of DNA to nucleases. 3AB and PARP knockout reduced the protection from BLM afforded by integrin clustering. Integrin clustering caused a PARP-independent suppression of nuclease sensitivity in the knockout cells. The results demonstrate that the antigenotoxic and chromatin-modulating actions of integrins are sensitive to PARP.

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