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DIEPOXYBUTANE INDUCES THE EXPRESSION OF A NOVEL p53-TARGET GENE XCL1 THAT MEDIATES APOPTOSIS IN EXPOSED HUMAN LYMPHOBLASTS.

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Abstract

Diepoxybutane (DEB) is the most potent active metabolite of the environmental chemical 1, 3-butadiene (BD). BD is a human carcinogen that exhibits multi-organ systems toxicity. Our previous studies demonstrated that XCL1 (X-C motif chemokine ligand 1) gene expression was up-regulated 3.3-fold in a p53-dependent manner in TK6 lymphoblasts undergoing DEB-induced apoptosis. The tumor suppressor p53 protein is a transcription factor that regulates a wide variety of cellular processes, including apoptosis, through its various target genes. Thus, the objective of this study was to determine whether XCL1 is a novel direct p53 transcriptional target gene, and deduce its role in DEB-induced toxicity in human lymphoblasts. We utilized the bioinformatics tool *p53scan* to search for known p53 consensus sequences within the XCL1 promoter region. The XCL1 gene promoter region was found to contain the p53 consensus sequences 5'-AGACATGCCTAGACATGCCT-3' at three positions relative to the transcription start site. Furthermore, the XCL1 promoter region was found through reporter gene assays to be transactivated at least 3-fold by wild-type p53 promoter in DEB-exposed human lymphoblasts. Inactivation of the XCL1 promoter p53-binding motif located at −2.579 kb relative to transcription start site reduced the transactivation function of p53 on this promoter in DEB-exposed cells by 97%. Finally, knock-down of XCL1 mRNA with specific siRNA inhibited DEB-induced apoptosis in human lymphoblasts by 50%. These observations demonstrate for the first time that XCL1 is a novel DEB-induced direct p53 transcriptional target gene that mediates apoptosis in DEB-exposed human lymphoblasts.

Keywords

diepoxybutane; p53; XCL1; apoptosis; p53-target-gene

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

1 INTRODUCTION

1, 2:3, 4-Diepoxybutane (DEB) is the most potent active metabolite of the environmental chemical 1, 3 butadiene (BD).[1] BD is a regulated hazardous air pollutant that is genotoxic, mutagenic and carcinogenic.[1–4] Human exposure to BD occurs through inhalation of the compound found at industrial sites, in automobile exhaust, cigarette smoke, and forest fires. DEB is known to exhibit both inter-strand and intra-strand DNA cross-linking ability, react with proteins, and generate reactive oxygen species (ROS); all these insults may result in DNA damage and cell death.[5–7] Details of the molecular mechanisms of BD and DEB toxicity are not completely understood.

Apoptosis is a form of programmed cell death that plays a critical role in regulating physiological processes, and maintaining homeostasis in multicellular organisms.[8] Apoptosis is triggered in response to stress stimuli, such as DNA damage, growth factor deprivation and oxidative stress.[8, 9] DEB was reported to induce apoptosis.[6, 8, 10, 11] In human lymphoblasts, this process is caspase dependent, is mediated through the mitochondrial apoptotic pathway[8, 10–12], and is regulated by elevated p53 levels.[12] The molecular mechanisms of p53 action and signaling in DEB-induced apoptosis in human lymphoblasts remain to be elucidated.

The p53 protein is a sequence-specific transcription factor that binds the consensus sequence 5'-AGACATGCCT-AGACATGCCT-3'. It is activated in response to a variety of stresses to mediate various cellular responses, such as cell cycle arrest, DNA repair, and apoptosis.[13] The pleiotropy of p53 activity is due to its ability to transcriptionally activate different classes of p53 transcriptional target genes that play roles in different outcomes.[14] Thus, identification of p53 target genes is of paramount importance towards the understanding of pathways regulated by p53, such as those that control cell growth arrest and apoptosis.[14]

XCL1 (X-C motif chemokine ligand 1) is a C class chemokine that is also known as lymphotactin.[15] The XCL1 gene is predominantly expressed in various immune cells[15, 16], and its expression is associated with infections, inflammatory and immunological responses, as well as autoimmune diseases.[15,17–22] Compounds known to upregulate the expression of the XCL1 gene within cells include brefeldin A (BFA), polysaccharide K (PSK), cyclosporine, phorbol myristate acetate (PMA), and ionomycin.[17, 22–26] Exogenously expressed XCL1, as well as XCL1 upregulating chemical compounds, such as BFA and PSK, induce apoptosis in some systems.[31, 35–38] The occurrence of p53-dependent apoptosis triggered by BFA and LPS has also been reported.[27, 28] Although BFA, PSK, and LPS elevate p53 levels in some cell types, no relationship between p53 levels and XCL1 gene expression has been reported.[27–30] The role of XCL1 in mediating apoptosis can vary both by cell type, and by the nature of the stimuli.[31, 32] Thus, additional studies on the role of XCL1 in apoptosis, as well as its relationship to p53 within the same cells are needed.

We previously found, through microarray analysis, that the expression of the XCL1 gene was upregulated in a p53 dependent manner in DEB-exposed human lymphoblasts. In this study, we have confirmed these findings, and investigated for the first time whether the XCL1 gene is a direct p53 transcriptional target gene in DEB-exposed lymphoblasts. In

addition, we have investigated for the first time the apoptotic role of elevated XCL1 gene expression levels in DEB-exposed human lymphoblasts.

2 MATERIALS AND METHODS

2.1 Cell culture and exposure of cells to diepoxybutane

The human p53-proficient B-lymphoblastic TK6 cell line and the genetically homologous p53-deficient NH32 cell line were passaged and exposed to DEB as previously described.[11, 33]

2.2 Quantitative reverse-transcriptase polymerase chain reaction

Total RNA was isolated using the RNAqueous-4PCR kit, and 1μg was reverse transcribed using the QuantiTech Reverse Transcription Kit; subsequent PCR utilized a SYBR Green qPCR Master Mix (Qiagen, Inc). The human XCL1 (Cat# PPH00702B), p53 (Cat# PPH00213F), and GAPDH (Cat# PPH00150F) PCR primers utilized were obtained from Qiagen, Inc. The nanoluciferase PCR primers (Forward: 5'-AAGGATTGTCCTGAGCGGTG-3'; Reverse: 5'-AACACGGCGATGCCTTCAT-A-3') utilized were custom synthesized (Integrated DNA Technologies, Inc). Data were normalized to GAPDH mRNA levels in each sample. All reactions were carried out in triplicates.

2.3 Construction of plasmids containing XCL1 promoter fragments

Two sets of three XCL1 promoter fragments were PCR amplified from human TK6 lymphoblasts genomic DNA (see Table 1). Amplifications were carried out utilizing the GoTaq® Long PCR Master Mix. The cleaned XCL1 promoter fragments were subsequently cloned into the Sac 1 and Xho1 sites of the promoter less vector pNL1.2 [NlucP] encoding the nanoluciferase coding region.

2.4 Nucleofection with XCL1 promoter plasmids and Nano-Glo luciferase assay

Cell nucleofection utilized the DS 137 program on the Amaxa Nucleofector II 96-well Shuttle System (Lonza). TK6 cells at 1 x 10^6 cells/20μl of supplemented solution SF were nucleofected with 0.4μg of either empty vector control or XCL1 promoter fragment plasmids. NH32 cells received 0.2μg of XCL1 promoter fragment plasmids or empty vector; 0.2μg of the control vector pCMVneoBam or wild-type p53 expressing plasmid pCMV-p53 (Clontech, Cat # 631922) or mutant p53 expressing plasmid pCMV-p53mt135 (Clontech Inc, Cat# 631922) were also added. Cells were then seeded at 5X10^5 cells/ml for 14h, and exposed to 10 μM DEB at 2X10^5 cells/ml; control un-exposed cells received vehicle (RPMI media). Cloned XCL1 promoter region activity, and its transactivation by p53 were determined by utilizing the Promega Nano-Glo Luciferase assay. Assays were performed in triplicates, utilizing the Spectramax M5 microplate reader at 21°C.

2.5 Site-specific mutagenesis of the XCL1 promoter p53 binding motif

The p53 binding motif around −2.579kb within the cloned XCL1 promoter region was mutagenized according to the protocol within the QuikChange II XL site-directed mutagenesis kit (Cat # 200522, Agilent Technologies, Inc.). The forward and reverse

mutagenesis primers were 5’ATATACCTGATTGCTATTTGTGTGTTTTATTTTGAGAAAT**T** TCTATT**A**AA**T**TCTTTTACCCATTTTTAAATTGTGTTGTTTTC-3’ and 5’CAAGAAAACAA CACAATTTAAAAATGGGTAAAAGA**A**TT**T**AATAGA**A**ATTTCTCATCAAAATAAAAC ACA CAAATAGCAATCAGGTATATGAA-3’, respectively. The changed residues within the mutagenesis primer sequences are highlighted in color and underlined. Control mutagenesis experiments performed in parallel utilized the wild type sequence (see Figure 4A). The purified mutant and wild-type plasmid DNAs obtained were subjected to XCL1 promoter reporter gene assays.

2.6 siRNA knockdown of XCL1 gene expression and apoptosis quantification

TK6 lymphoblasts were transfected with either 2.5 nM *Silencer Select* XCL1 siRNA or *Silencer Select* scrambled negative control siRNA using Lipofectamine® RNAiMAX reagent. Cells were then exposed to control or 10 μM DEB at 10h post-transfection. Apoptosis was quantified utilizing the Caspase-Glo®3/7 assay, and by assessing nuclear morphology[11, 33]. Experiments were repeated three times.

2.7 Statistical analysis

All statistical analyses were performed using Graph Pad Prism version 7.1. Data are shown as means ± standard error (SE). The significance of difference was determined by using a t-test to compare means of values between groups. Values of $p < 0.05$ were considered statistically significant.

3 RESULTS

3.1 Diepoxybutane up-regulates XCL1 gene expression in human lymphoblasts in a p53-dependent manner

The p53-dependent up-regulation of XCL1 mRNA levels in DEB-exposed lymphoblasts was investigated (Figure 1). XCL1 mRNA levels were up-regulated 2.85 and 3.35-fold compared to controls in the p53-proficient TK6 cells exposed to DEB for 24h to 36h, respectively (Figure 1, left panel). XCL1 mRNA were down-regulated by 40% and 30% compared to control in the homologously-paired p53-deficient NH32 cells to exposed to DEB for 24h and 36h, respectively (Figure 1, right panel). These results raised the possibility that XCL1 might serve as a p53 target gene in DEB-exposed p53-proficient TK6 lymphoblasts.

3.2 The human XCL1 promoter region contains p53 binding motifs and is transactivated by wild-type p53 in diepoxybutane-exposed human lymphoblasts

The possibility that the XCL1 gene serves as a potential p53 target gene in DEB-exposed cells was investigated (Figure 2). *In silico* p53 binding sites were found within the human XCL1 promoter region (Figure 2A). XCL1 promoter nanoluciferase activity assays in TK6 cells indicated that the −2.930 to +0.031kb and the −4.888 to +0.031kb promoter constructs had the most basal activity (Figure 2B). DEB exposure increased the activity of these promoter regions to within a similar range; this was also reflected in nanoluciferase and endogenous XCL1 mRNA levels in the same cells (Figure 2C). These results indicate that

the XCL1 promoter is trans-activated by endogenous p53 in DEB-exposed TK6 lymphoblasts, primarily through the −2.579 kb p53-motif.

The direct transactivation effects of exogenous wild-type p53 (wt-p53) on the XCL1 promoter region in DEB-exposed p53-deficient NH32 cells were investigated (Figure 3A). Expression of wt-p53 protein in DEB-exposed cells significantly ($p<0.05$) increased promoter activity of the −2.930 to +0.031kb and the −4.888 to +0.031kb constructs as compared to the corresponding control un-exposed cells. The transactivation of the XCL1 promoter region was reduced by 35% in DEB-exposed cells expressing mutant p53 (mt-p53), as compared to control wt-p53 expressing cells (Figure 3B). Collectively, the results of Figures 2 and 3 imply that functional wt-p53 transactivates the XCL1 promoter in DEB-exposed human lymphoblasts, primarily through the −2.579kb p53 binding motif.

3.3 The XCL1 gene is a diepoxybutane-induced novel direct p53 target gene in human lymphoblasts

The effect of inactivating residues essential for p53-binding on the activity of the XCL1 promoter in DEB-exposed TK6 and NH32 cells was investigated (Figure 4). The p53 binding motif around −2.579kb within the XCL1 promoter was subjected to site-specific mutagenesis (Figure 4A). The activity of the mutant XCL1 promoter obtained was reduced by 75.9% (panel B) and by 97.2% (panel C) in DEB-exposed TK6 cells and NH32 cells expressing exogenous wt-p53, respectively, as compared to the activity of the wild-type promoter in the corresponding control exposed cells. These results confirm that the XCL1 gene is a novel DEB-induced direct target of the p53 protein in DEB-exposed cells.

3.4 The XCL1 gene mediates DEB-induced apoptosis in human TK6 lymphoblasts

To determine the role of the XCL1 gene in DEB-induced apoptosis, XCL1 siRNA knockdown experiments were performed (Figure 5). The presence of XCL1 siRNA reduced apoptosis levels by 50% (Figure 5A,B), and prevented the upregulation of XCL1 mRNA levels (Figure 5C), in DEB-exposed cells as compared to control cells treated with scrambled siRNA. These findings demonstrate that the XCL1 gene is involved in mediating DEB-induced apoptosis in TK6 cells.

4 DISCUSSION

In this study, we investigated whether the XCL1 gene served as a p53-target gene in DEB-exposed lymphoblasts, and deduced its role in apoptosis in the same cells. Wild-type p53 transactivated activity of the XCL1 promoter region in these cells, primarily through the p53 binding motif located around −2.579kb relative to the transcription start site (TSS). Inactivation of this p53 binding motif drastically reduced the transactivation function of p53 on this promoter in DEB-exposed cells. Finally, knock-down of the upregulated XCL1 mRNA levels inhibited DEB-induced apoptosis by 50%. These observations demonstrate for the first time that XCL1 is a novel DEB-induced direct p53 target gene mediating apoptosis in DEB-exposed human lymphoblasts.

Brefeldin A (BFA), polysaccharide K (PSK), cyclosporine, phorbol myristate acetate (PMA), and ionomycin upregulate XCL1 gene expression in exposed cells.[17, 22–26] On the

other hand, BFA, PSK, and LPS elevate p53 levels in other cell systems.[27–42] No association, however, was investigated between XCL1 and p53 status in these systems. Thus, our finding that DEB regulated the expression of XCL1 in a p53-dependent manner is novel, and raised the possibility of XCL1 being a novel p53 target gene.

Genome-wide p53 binding and transactivation landscapes are differentially affected by diverse stresses, including DNA damage.[34, 35] Thus, these reports explain why exogenously expressed wild type p53 transactivated the XCL1 promoter in DEB-exposed cells but not in un-exposed cells, and why novel p53 target genes are continuously being discovered.[36, 37] The experimental strategies utilized to determine that XCL1 is a novel direct p53 transcriptional target gene have been utilized by others to identify novel p53 target genes PinX1, fractalkine, NCF2 , and CSF1.[14,15,48–51] Dutta *et al* (2010) utilized bioinformatics to predict, but did not localize, the existence of a consensus p53 binding site in the XCL1 gene.[38] Since these authors did not determine whether XCL1 is transactivated by p53 or serves as a p53 target gene, our findings are the first to provide such evidence.

We report for the first time that the XCL1 gene mediated DEB-induced apoptosis in exposed human lymphoblasts. Exogeneous XCL1, and XCL1 up-regulating BFA and PSK, were reported to induce apoptosis in various cell systems.[31, 35–42, 59] The relationship between XCL1 up-regulation and the p53 status in these systems was not investigated. It is possible, however, that the apoptosis induced by these XCL1 up-regulating compounds in these systems is partly mediated by XCL1; this will align with our observations on the apoptosis mediating role of XCL1 in DEB-exposed cells. The role of XCL1 in mediating apoptosis, however, can vary by cell type, and according to the stimuli.[31, 32] The exact mechanism by which XCL1 mediates apoptosis in DEB-exposed lymphoblasts is currently under investigation.

In summary, we have demonstrated for the first time that XCL1 is a novel direct p53 target gene mediating apoptosis in DEB exposed human lymphoblasts. These findings contribute towards understanding p53 signaling in DEB-induced apoptosis and toxicity.[37, 39] These findings also expand the current knowledge base surrounding the relationship between p53 function and diverse stresses, as expressed through its target genes.[39] Collectively, this knowledge is useful in DEB and BD risk assessment, as well as in design of novel therapeutic protocols in management of DEB and BD exposure.

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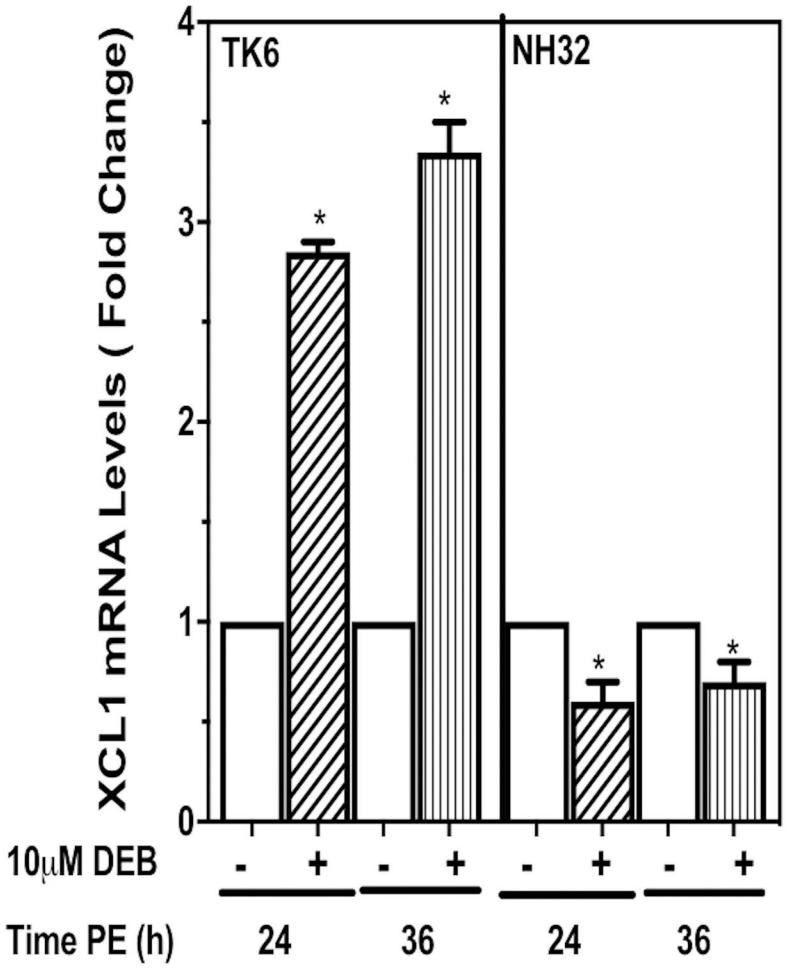

FIGURE 1. XCL1 gene expression is up-regulated in a p53 dependent manner in diepoxybutane-exposed lymphoblasts.

TK6 (p53 proficient) and NH32 (p53 deficient) cells were exposed to vehicle or 10μM DEB. The extracted RNA was then subjected to reverse-transcriptase polymerase chain reaction in duplicates. The fold change values were obtained by comparing the relative ratio of XCL1 to GAPDH for each exposed sample over that of the corresponding un-exposed sample. Experiments were performed in duplicated and were repeated three times. PE=post exposure. (*: *p*-value <0.05). ***Significant difference between DEB exposed and unexposed cells.**

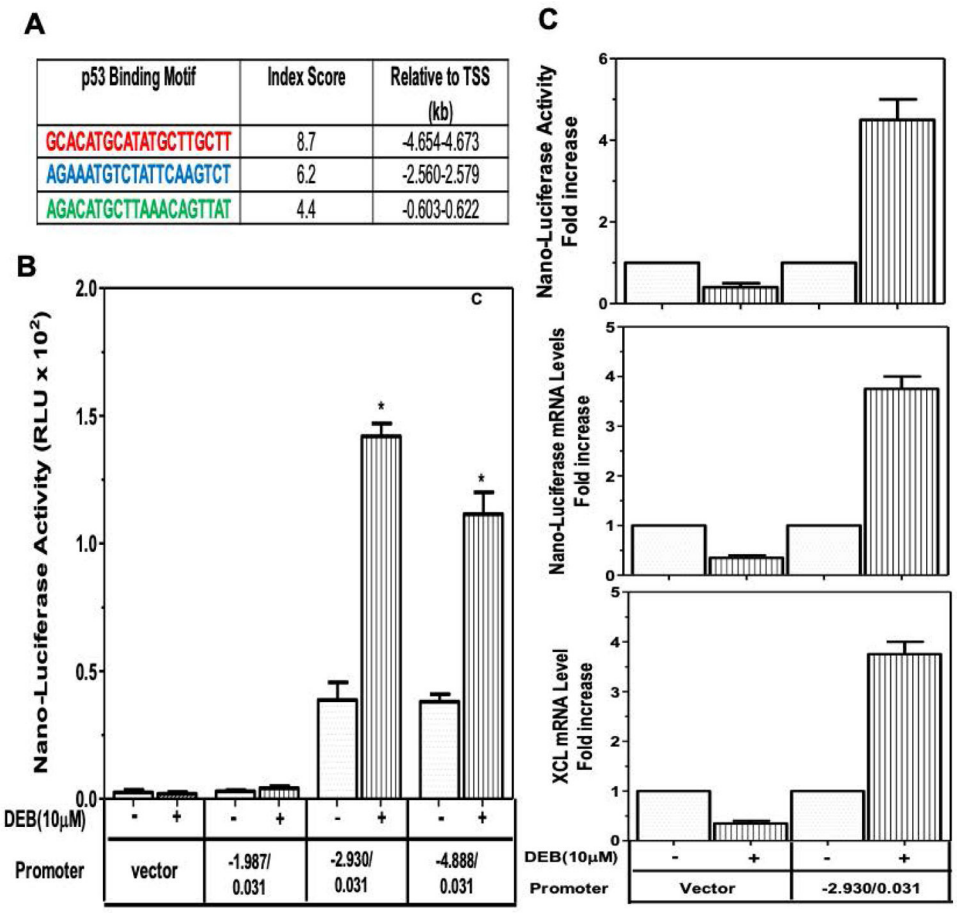

Figure 2. Diepoxybutane up-regulates the activity of the XCL1 promoter region in exposed p53-proficient TK6 human lymphoblasts.

The various XCL1 promoter fragments (designed to segregate the three p53 binding sites within the promoter region and terminating 3 bases before the XCL1 translation start site) were cloned into the promoter-less vector pNL1.2 [NlucP], encoding the nanoluciferase coding region. The following plasmids used here were generated: pNL1.2/XCL1(−1.987/+0.031), pNL1.2/XCL1 (−2.930/+0.031); and pNL1.2/XCL1(−4.888/+0.031); all given insert coordinates refer to distances in kb, with respect to XCL1 transcription start site located at genomic location 168,576,605 of the reference sequence NC_000001.11, GRCh38.p13 primary assembly. Cells were subsequently nucleofected with the pNL1.2 vector or various XCL1 promoter fragment nanoluciferase constructs at 14h prior to exposure to 10μM DEB. All assays were conducted at 36h post DEB-exposure. **A:** The location and p53 binding indexes of the three p53 binding motifs within the XCL1 promoter region is shown; this was determined by using the bioinformatics tool *p53scan*. **B.** Reporter gene activity of the various XCL1 promoter constructs in exposed TK6 cells: Equal number of cells (1 x 10^5) were then subjected to nanoluciferase assay. The relative luminescence units (RLU) of the various XCL1 promoter constructs and the control vector are shown. **C.** Expression of the nanoluciferase reporter gene activity is proportional to endogenous XCL1 levels in DEB-exposed TK6 cells: The pNL1.2 vector or pNL1.2/XCL1(−2.930/+0.031) were utilized. The relative levels of nanoluciferase activity (upper panel), nanoluciferase

mRNA (middle panel) and endogenous XCL1 mRNA (bottom panel) levels were determined in DEB-exposed cells compared to control unexposed cells. All mRNA expression levels were determined by quantitative reverse-transcriptase polymerase chain reaction and normalized to GAPDH mRNA levels. A representative of three experiments is shown. (*: *p*-value <0.05). ***Significant difference between DEB exposed and unexposed cells.**

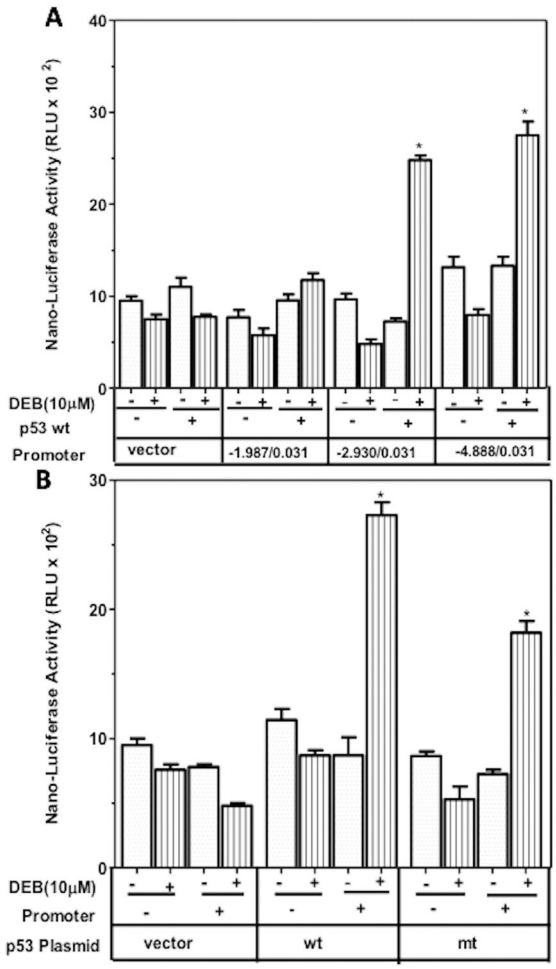

FIGURE 3. Diepoxybutane Up-Regulates the XCL1 Promoter Region in a p53-dependent manner in NH32 human lymphoblasts.

A. NH32 cells (p53-deficient) were nucleofected with the pNL1.2 plasmid vector, or the various XCL1 promoter fragment nanoluciferase constructs at 14h prior to exposure to 10μM DEB. The cells were also co-nucleofected with either pCMVneoBam vector as a control (p53 wt, −) or pCMV-p53 wild type p53 expressing plasmid (p53 wt, +). **B.** NH32 cells were nucleofected with the pNL1.2 vector (promoter, −) or the pNL1.2/XCL1(−4.888/+0.031) promoter-nanoluciferase construct (promoter, +). The cells were also co-nucleofected with either pCMVneoBam vector as control (vector), pCMV-p53 wild type p53 expressing plasmid (wt), or mutant p53 plasmid (pCMV-p53mt135, mt) as shown. In both experiments, equal number of cells (1 x 10^5) were then subjected to nanoluciferase assay at 36h post-DEB exposure, as described in Methods. The relative luminescence units (RLU) of the various XCL1 promoter constructs, as well as the control vector are shown. Experiments were repeated three times (*: *p*-value <0.05). ***Significant difference between DEB exposed and unexposed cells.**

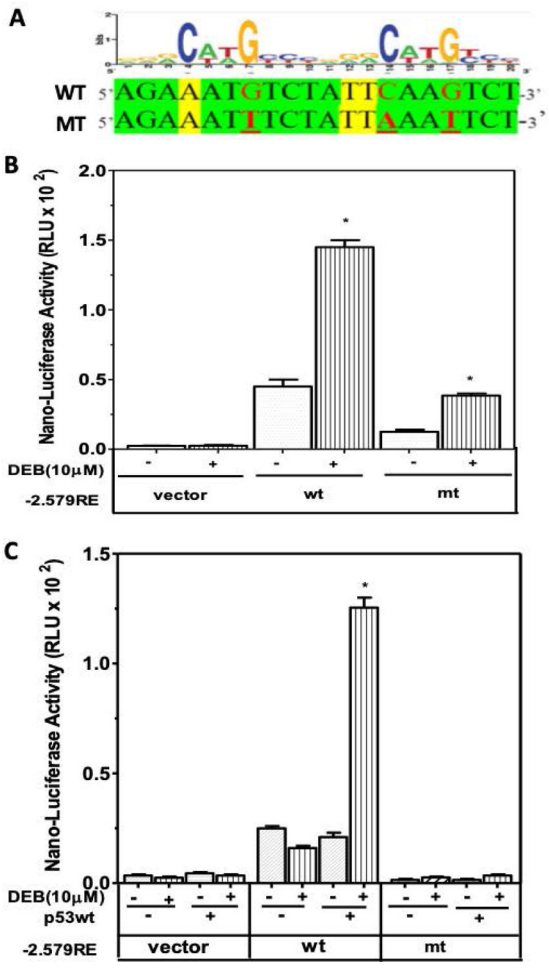

FIGURE 4. Effect of site-specific mutagenesis of the XCL1 promoter p53 binding motif located around −2.579kb on the activity of the promoter in DEB-exposed cells.

A. Site specific mutagenesis of the −2.579 kb XCL1 promoter p53 binding motif within the plasmid pNL1.2/XCL1(−2.930/0.031): The Quick ChangeXL site-specific mutagenesis kit (Stratagene, Inc.) was utilized, as described in methods. Residues essential for p53-binding (represented by elevated residues within the shown p53-binding consensus sequence[40] were mutagenized. Green and yellow shading represents XCL1 promoter residues similar and different to consensus sequence, respectively. Mutagenized residues are shown in red, and the mutant residues are underlined. The mutant plasmid created is pNL1.2/XCL1 (−2.930 /+ 0.031/p53RE-2.579mt); this is labeled as −2.579RE in Panels B, C below. **B.** Activity of the mutant XCL1 promoter in DEB-exposed p53-proficient TK6 cells. The empty vector pNL1.2, the wild-type plasmid pNL1.2/XCL1(−2.930/0.031) (−2.579RE wt), and the mutant plasmid pNL1.2/XCL1(−2.9300.031/p53RE-2.579mt) (−2.579RE mt), were transfected into TK6 cells. The experiment was carried out essentially as described in Methods, and nanoluciferase activity was determined at 36h post-DEB exposure. **C.** Transactivation of the mutant XCL1 promoter by p53 in DEB-exposed p53-deficient NH32 cells. The experiment was conducted in NH32 cells as described for panel B, but cells were also co-transfected with either the control plasmid (pCMVneoBam, p53 wt −) or the wild-type p53-expressing plasmid pCMV-p53 (p53wt,+). All experiments were conducted in duplicates, and were

repeated three times. (*: p-value <0.05). ***Significant difference between DEB exposed and unexposed cells.**

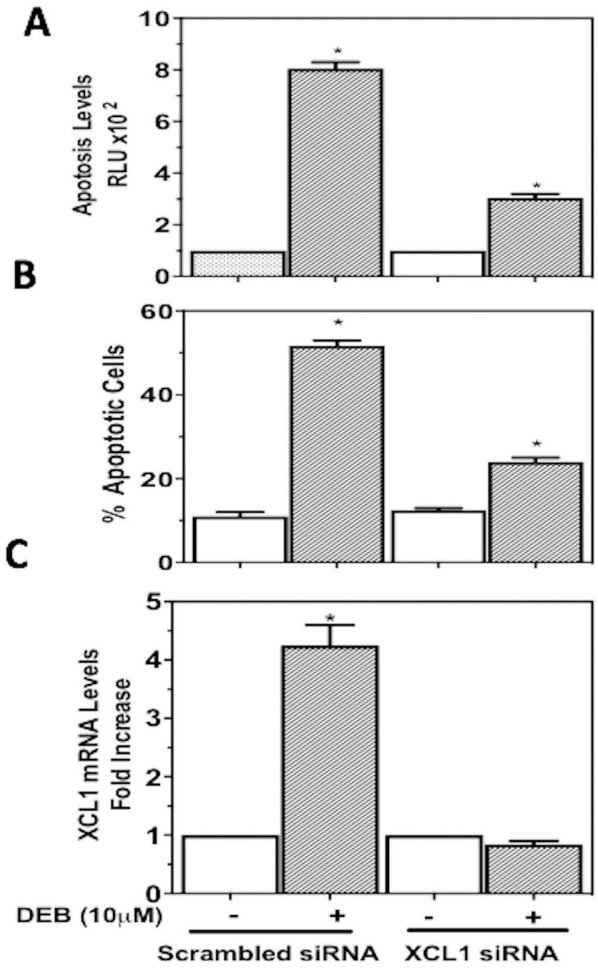

FIGURE 5. XCL1 gene mediates diepoxybutane-induced apoptosis in human TK6 cells.

Cells were transfected with 2.5nM *Silencer-Select* XCL1 siRNA using LipofectamineRNAimax® transfection reagent; control cells were transfected with 2.5 nM *Silencer Select* control scrambled siRNA under identical conditions. At 10h post-transfection, control and XCL1 siRNA transfected cells were exposed to vehicle or 10μM DEB. **A.** Apoptosis levels were quantified at 14h post-DEB exposure utilizing the Caspase-Glo® 3/7 assay. Luminescence values (RLU) were determined by utilizing the Molecular Devices SpectraMax M5 microplate reader. **B.** Apoptosis levels were quantified at 36h post-DEB exposure by assessing nuclear morphology using dual staining with acridine-orange and ethidium bromide, as described in Methods. The percentage of apoptotic cells for each sample is shown. **C**. XCL1 mRNA levels were determined at 36 h post-DEB exposure using quantitative reverse-transcriptase transcriptase polymerase chain reaction, as described in Methods. All expression levels were normalized to GAPDH. Experiments were conducted in duplicates and repeated three times. (* p-value <0.05). ***Significant difference between DEB exposed and unexposed cells.**

Table 1.

PCR Primers and Conditions for Generation of XCL1 Promoter Fragments

Fragment Name/ Annealing Temp.	***Sac1 Linker*- Forward Primer (5'→3')**	***Xho1-Linker*-Reverse Primer (5'→3')**
XCL1 (−1.987 /+0.031) 65°C	*TATGAGCTC*GAGGAGGAGTTTACAAGT	5' AATCTCGAGCTGAGGTCCTGCTGAG 3'
XCL1 (−2.930 /+0.031) 55°C	*TATGAGCTC*CATGGGAGTGCACATATCTCTT	
XCL1 (−4.888/+0.031) 65°C	*TATGAGCTC*GTCAGATGAATTAAGATATTCAGACCA	