Increased DNA damage sensitivity of Cornelia de Lange syndrome cells: evidence for impaired recombinational repair

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Corinella de Lange syndrome (CdLS) is a rare dominantly inherited multisystem disorder affecting both physical and mental development. Heterozygous mutations in the NIPBL gene were found in about half of CdLS cases. Scc2, the fungal ortholog of the NIPBL gene product, is essential for establishing sister chromatid cohesion. In yeast, the absence of cohesion leads to chromosome mis-segregation and defective repair of DNA double-strand breaks. To evaluate possible DNA repair defects in CdLS cells, we characterized the cellular responses to DNA-damaging agents. We show that cells derived from CdLS patients, both with and without detectable NIPBL mutations, have an increased sensitivity for mitomycin C (MMC). Exposure of CdLS fibroblast and B-lymphoblastoid cells to MMC leads to enhanced cell killing and reduced proliferation and, in the case of primary fibroblasts, an increased number of chromosomal aberrations. After X-ray exposure increased numbers of chromosomal aberrations were also detected, but only in cells irradiated in the G2-phase of the cell cycle when repair of double-strand breaks is dependent on the establishment of sister chromatid cohesion. Repair at the G1 stage is not affected in CdLS cells. Our studies indicate that CdLS cells have a reduced capacity to tolerate DNA damage, presumably as a result of reduced DNA repair through homologous recombination.

INTRODUCTION

Cornelia de Lange Syndrome (CdLS; OMIM 122470) is a rare multisystem developmental disorder with characteristic facial dysmorphism, growth and cognitive retardation, malformations of the upper limbs and a variety of other abnormalities affecting a wide range of tissues and organs (1–3). The prevalence of CdLS is estimated to be as high as 1/10,000 to 1/30,000 and most cases are sporadic. CdLS is genetically heterogeneous and at present three disease-causing genes have been identified, all of which are implicated in sister chromatid cohesion. Approximately, half of CdLS patients carry heterozygous mutations in the NIPBL gene (4–9). Recently, mutations in the X-linked SMCP gene have been identified in ~5% of the CdLS cases. One CdLS patient is currently known carrying a mutation in SMCP (10–12). Primarily truncation mutations and amino acid substitutions have been observed. Large rearrangements of NIPBL do occur in CdLS but are likely to be infrequent (13). The majority of affected individuals carry de novo mutations and only a very few familial cases of CdLS have been reported.

The NIPBL gene is predicted to code for two isoforms of 2804 and 2697 amino acids, termed delangin-A and delangin-B, respectively. The human delangin proteins share homology with Nipped-B from Drosophila melanogaster and Scc2 from Saccharomyces cerevisiae. Scc2 and its orthologs have an essential role in sister chromatid cohesion, which is crucial for proper chromosome segregation during mitosis (14).

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In fungi, the cohesin complex consists of two structural maintenance of chromosomes (SMC) proteins, Scc1 and Scc3 and two non-SMC proteins, Scc2 and Scc4. In vertebrates, Scc3 exists as two isoforms called SA1 and SA2. Live-cell imaging experiments in mammalian cells revealed that cohesin dynamically binds to DNA during most of the cell cycle, but it is during S-phase that cohesin becomes stably bound to DNA to mediate cohesion of sister chromatids until segregation (15). The Scc2 protein in *S. cerevisiae* is not a subunit of cohesin but functions in collaboration with Scc4 as a cohesin-loading complex (16). Analogous to the function of Scc2 and Scc4 in *S. cerevisiae*, the *NIPBL* gene product, in conjunction with human Scc4, was shown to facilitate the chromatin association of cohesin subunits (17,18). Loading of cohesin occurs on unreplicated DNA. Establishment of cohesion between sister chromatids occurs during S-phase and is dependent on the acetyltransferase protein Eco1/Ctf7/Eso1 in yeast. Although the cohesin complex can be loaded in its absence, Eco1, via its interaction with PCNA, facilitates cohesion at the replication fork (19,20). Recently, mutations in the *ESCO2* gene, one of the human *Eco1* orthologs, were shown to be associated with Roberts syndrome (OMIM 268300), a disorder with characteristics similar to CdLS (21).

Physical linkage of sister chromatids by the cohesin complex is essential for correct chromosome segregation, but is also vital for DNA double-strand break (DSB) repair by homologous recombination (HR) during the S- and G2-phase of the cell cycle (22). Inactivation of either Scc2/Scc4 or one of the cohesin subunits results in a reduced efficiency of postreplicative DSB repair in G2/M cells. Recently, it became evident that cohesin is specifically recruited to sites of DSBs. Evidence presented by Ström et al. (23) and Ünal et al. (24) showed that the local enrichment of cohesin depends on the Scc2/Scc4 complex. This damage specific recruitment of the cohesin complex is however distinct from its normal chromatin binding because of the dependence on γH2AX and Mre11 proteins which are required for DSB repair (24). Based on these data, it can be concluded that tethering of the broken DNA ends to the sister chromatid is required for efficient repair through HR. Also in higher organisms, evidence has been obtained for a role of cohesin in DSB repair. Depletion of SCC1 in chicken DT40 cells leads to a marked increase in the formation of chromosomal aberrations after exposure to ionizing radiation and reduced levels of sister chromatid exchanges (SCE) after treatment with 4NQO (25). Local irradiation of HeLa cells showed the recruitment of cohesin to the site of damage (26). Additionally, in a recent genome wide screen in *Caenorhabditis elegans* for genes required for resistance to ionizing radiation, a homologue of *NIPBL*, *pgn-85*, was identified. RNAi mediated ablation of this gene resulted in increased sensitivity to radiation and cisplatin (27).

The implication of cohesin and delangin homologues in the DNA damage responses in yeast and higher eukaryotes raises the question if cells derived from CdLS patients display increased sensitivity to DNA-damaging agents and defects in DSB-repair. Evidence presented here shows a drastic reduced survival after exposure to the DNA interstrand cross-link inducing agent mitomycin C (MMC) as well as an increased frequency of chromosomal aberrations in response to ionizing radiation at the G2-phase of the cell cycle.

**RESULTS**

**Cornelia de Lange Syndrome is associated with increased sensitivity to DNA-damaging agents**

To determine whether CdLS is associated with increased sensitivity to DNA-damaging agents at the cellular level, we obtained two fibroblast and five B-lymphoblastoid cell lines from CdLS patients. To screen for the presence of *NIPBL* mutations in these lines, exon sequences, including exon–intron junctions, were amplified and PCR products were analyzed by denaturing high performance liquid chromatography (DHPLC). Heterozygous mutations were detected in two B-cell lymphoblastoid lines. Cell line CdLS11165 harbors a three base-pair deletion in exon 16 (c.3813delGAA), leading to a lysine (p.Lys1271del) deletion in a conserved part of the protein. Line CdLS11167 contains a single nucleotide insertion (c.3940_3941ins A) causing a premature stop codon. Using multiplex ligation-dependent probe amplification (MLPA) analysis a large genomic rearrangement, resulting in a duplication of exon 11—22, was identified in cell line CdLS45. In the other four CdLS lines, no mutations could be detected in the coding region of the *NIPBL* gene by DHPLC and MLPA. Screening for mutations in the *SMC1A* gene by DHPLC also did not reveal any causative genetic alterations in these four patients.

In budding yeast, the absence of the Scc2/Scc4 cohesin loading complex compromises the repair capacity for X-ray-induced DNA breaks (22). However, exposure of CdLS45 and CdLS3478 fibroblast lines to increasing doses of ionizing radiation did not result in a robust increase in radiation sensitivity as was observed for radiation-sensitive cells derived from ataxia telangiectasia (AT5BIVA) and severe combined immunodeficiency (SCID) (Artemis-6) patients (Fig. 1A). Only at the highest dose tested both CdLS fibroblasts lines displayed a decrease in survival in comparison to the control fibroblasts. At lower doses, only the CdLS3478 line reproducibly showed a marginal increase in radiation sensitivity in comparison with the three fibroblast lines derived from normal individuals. Growth inhibition assays for the five lymphoblast CdLS lines also did not reveal a distinct hypersensitivity to ionizing radiation (results not shown).

However, exposure to the DNA interstrand cross-link-inducing agent MMC revealed a strong increase in sensitivity of all fibroblast and lymphoblast CdLS lines (Fig. 1B and C). In comparison with VH25 and FN1 normal cells, the D10 values (dose of MMC leading to 10% survival) for both fibroblast CdLS lines are approximately 3-fold lower. Surprisingly, the increased MMC-sensitivity of CdLS45 and CdLS3478 is in the range of the MMC hypersensitivity of Fanconi’s anemia (FA) (Fig. 1B). Likewise, all five CdLS B-lymphoblastoid cell lines exhibited enhanced sensitivity for MMC when compared with control cells (Fig. 1C). Growth inhibition experiments indicate a 2-fold reduction of the IC50 values (dose of MMC leading to a growth reduction of 50%) of the CdLS lines in comparison with both normal human B-lymphoblastoid lines. In contrast to MMC, exposure...
Figure 1. Survival of normal and CdLS cells after genotoxic treatment. CdLS cell lines are represented by open symbols. Data are the average of at least two independent experiments. Error bars represent the standard deviation. (A) Clonogenic survival of primary normal (VH10, VH25, FN1) and CdLS (CdLS3478, CdLS45) fibroblasts after X-ray exposure. The Artemis-6 (SCID) and AT5BIVA (AT) X-ray sensitive cell lines are shown for comparison. (B) Clonogenic survival of primary fibroblasts after MMC exposure. The MMC hypersensitive EUFA423 (FA-D1/BRCA2) cell line is shown for comparison. (C) Growth inhibition assay of normal (JVM, Ramos) and CdLS (CdLS11165, 11166, 11167, 11168, 13976) B-lymphoblastoid cells exposed to MMC. The MMC hypersensitive EUFA696 (FA-J) cell line is shown for comparison.
of CdLS cells to UV-C light does not cause increased sensitivity (data not shown).

CdLS cells have increased levels of chromosomal aberrations after exposure to ionizing radiation

The formation of DSBs after exposure to DNA-damaging agents is counteracted by either non-homologous end joining (NHEJ) or HR. Whereas NHEJ is believed to function throughout the cell cycle, HR occurs predominantly during the S- and G2-phase, when sister chromatids are available as a template for repair synthesis (28,29). The role of delangin and the cohesin complex in DNA repair is most likely to enhance linkage between damaged and undamaged chromatids, to facilitate efficient repair of the lesion, thereby allowing HR to occur. To investigate the role of cohesion in repair of DSBs at different stages of the cell cycle, we first analyzed the formation of chromosomal aberrations after exposure to X-rays at the G1-stage of the cell cycle. Confluent normal VH10 and CdLS fibroblasts were irradiated with different doses of X-rays, and the frequency of dicentric chromosomes and acentric fragments was determined. Exposure to X-rays resulted in a dose-dependent increase in dicentric and acentric fragments in both VH10 and CdLS3478 cells (Table 1). Both normal and CdLS fibroblast showed a very similar dose-response relation for the formation of chromosome aberrations after irradiation with different doses of X-rays (0.25–1 Gy).

To determine the induction of chromosome aberrations after irradiation of cells in the G2-phase of the cell cycle, metaphase preparations were made 3 h after exposure to X-rays. In this experimental set-up, only G2 cells were analyzed (see Materials and Methods for details). In mock treated cells, the frequency of chromatid breaks was similar in VH10 and CdLS3478 cells. After exposure to low doses of X-rays (0.1 and 0.25 Gy), the frequency of chromatid exchanges in VH10 and CdLS3478 fibroblasts was comparable. However, a significant increase was found in CdLS cells following exposure to doses of 0.5 and 1 Gy (Table 2). Exposure to increasing doses of X-rays (0.1–1 Gy) also caused a strong increase in the level of residual chromatid breaks in CdLS fibroblasts when compared with control cells. At the highest dose tested (1 Gy), a 4-fold difference was seen in the number of residual breaks between CdLS and normal cells. In two B-lymphoblastoid cell lines derived from CdLS patients, a similar increase in radiosensitivity of G2 cells was observed. The level of chromatid-type breaks was drastically enhanced (3–4.5-fold) in both lines tested in comparison with normal B-lymphoblastoid cells (Table 2). In addition to ionizing radiation, we also analyzed chromatid-type aberrations after treatment with MMC. In CdLS fibroblasts, the level of chromatid breaks and exchanges was found to be approximately 3-fold higher than in normal cells (Table 3). In CdLS B-lymphoblastoid cells, no increase in chromatid-type aberrations was observed when compared with normal cells (data not shown) despite the strong MMC induced growth inhibition. It is known that B-lymphoblastoid cells readily go into apoptosis after inflicting DNA damage (30). Therefore, severely damaged B-lymphoblastoid cells may not reach the next metaphase.

The formation of SCE reflects the occurrence of HR between sister chromatids (31,32). As repair of DSBs through HR is

<table>
<thead>
<tr>
<th>Cell line</th>
<th>X-ray dose (Gy)</th>
<th>Abnormal cells (%)</th>
<th>Dicentrics</th>
<th>Excess of acentric fragments</th>
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<tr>
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<tr>
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<td>1</td>
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<td></td>
<td>2</td>
<td>26</td>
<td>18</td>
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Confluent primary fibroblasts were exposed to X-rays. Chromosomal aberrations are indicated per 100 cells.

### Table 1. X-ray induced chromosomal aberrations in G1 fibroblasts

<table>
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<tr>
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<th>X-ray dose (Gy)</th>
<th>Abnormal cells (%)</th>
<th>Chromatid Breaks</th>
<th>Exchanges</th>
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<td>0</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>0.1</td>
<td>0</td>
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<td>1</td>
<td>0</td>
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</table>

Chromosomal aberrations are indicated per 100 cells.  

### Table 2. X-ray induced chromosomal aberrations

<table>
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<tr>
<th>Cell line</th>
<th>MMC dose (ng/ml)</th>
<th>Abnormal cells (%)</th>
<th>Chromatid Breaks</th>
<th>Exchanges</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH10</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>45</td>
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<td>16</td>
<td>2</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>34</td>
<td>44</td>
<td>6</td>
</tr>
</tbody>
</table>

Chromosomal aberrations are indicated per 100 cells.
dependent on cohesion between sister chromatids, we reasoned that the level of SCE induction may be reduced in CdLS cells. To induce SCEs, we treated B-lymphoblastoid cells with MMC and fibroblast cells with MMC or UV-C light. In contrast to X-rays, both agents efficiently induce SCEs (33). As can be seen in Figure 2, SCEs were induced with equal efficiency in normal and CdLS B-lymphoblastoid cells after treatment with MMC. Similar results were obtained in fibroblasts after exposure to MMC and UV-C light (data not shown).

Normal Rad51 and γH2AX foci formation in CdLS cells

A central player in HR is the Rad51 molecule, a protein that promotes pairing and strand exchange reactions (34). Upon treatment with DNA-damaging agents, Rad51 relocates and forms nuclear foci, which most probably represent centers of DNA repair (35,36). To determine whether the reduced cohesin loading affects the ability to form nuclear Rad51 foci, we exposed normal and CdLS cells to MMC and X-rays. In untreated cells between 2 and 5% of the nuclei contained five Rad51 foci or more. After treatment with X-rays or MMC between 6 and 31% of the normal and CdLS B-lymphoblastoid cells contained over five Rad51 foci (Fig. 3A). This indicates that all CdLS cell lines tested are proficient for DNA damage induced foci formation although the level of induction varied between different cell lines. A similar analysis using primary fibroblasts derived from CdLS patients also showed normal induction of Rad51 foci after MMC treatment (data not shown).

Exposure to ionizing radiation leads to phosphorylation of histone H2AX (γH2AX) near sites of DSBs which can be visualized as nuclear foci. Because the number of γH2AX foci is thought to correlate with the number of DSBs, the analysis of γH2AX foci can be used to evaluate the repair capacity of a cell (37). Here we quantified the number of Rad51 and γH2AX foci per nucleus after X-ray exposure of exponentially growing CdLS and normal fibroblasts. Analysis of γH2AX foci was limited to cells that were also positive for Rad51 and presumably represent S- or G2-phase cells. No significant difference in the number of Rad51 or γH2AX foci was observed between normal and CdLS fibroblasts either 12 or 24 h after irradiation (Fig. 3B).

DISCUSSION

The CdLS is a dominantly inherited multisystem congenital disorder and has been associated with heterozygous mutations in NIPBL, SMC1A or SMC3 (4–13). Although the clinical manifestations of CdLS have been described in great detail, little is known about the characteristics at the cellular level. Studies in budding yeast have revealed that a complex consisting of Scc2 and Scc4 is required for loading of cohesin onto chromosomes before replication starts. Sister chromatid cohesion is established at the replication fork and involves additional proteins, including Eco1, Ctf4 and Ctf18 (20,38,39). Establishing cohesion between sister chromatids is essential not only for correct chromosome segregation, but also for post-replicative DNA repair. Experiments in yeast have shown that repair of DSBs in S- and G2-phase cells requires de novo formation of cohesion at the site of the damage (23,24). To determine if CdLS is associated with impaired repair of DSBs, we studied the survival of CdLS cell lines, the induction of chromosomal aberrations and the levels of γH2AX and Rad51 nuclear foci after treatment with DNA-damaging agents.

Pathogenic NIPBL mutations were identified in three (CdLS11165, CdLS11167 and CdLS45) out of seven cell lines examined. In the remaining four CdLS lines, no mutations were identified in NIPBL or SMC1A, which has recently been implicated in CdLS (10–13). However, the screening methods used cannot exclude the presence of mutations in intronic sequences or in the promoter region of the genes. As sister
chromatid cohesion involves various factors, the presence of disease-causing mutations in additional genes can also not be ruled out.

The induction of chromosomal aberrations (dicentric and acentric fragments) after exposure of G1 CdLS fibroblast cells to X-rays was similar to normal cells. However, both CdLS fibroblast and B-lymphoblastoid cells showed a strong dose dependent increase in the formation of chromatid exchanges and chromatid breaks when exposed to X-rays in the G2-stage of the cell cycle. Our observations are consistent with a defect in DSB repair through HR, resulting in delayed and/or aberrant repair of DSBs. In G1 cells, X-ray-induced DSBs are repaired primarily through NHEJ, while repair of DSBs in S- and G2-phase proceeds via NHEJ as well as via HR (28,29). A G2 specific increase in the induction of chromosomal aberrations has previously also been reported for cells derived from Bloom syndrome patients (40). BLM, the protein affected in these cells, is known to function in HR (41), which would suggest that a bias for ionizing radiation induced chromosomal aberrations in G2 is a general feature of cells deficient in HR. However, in spite of the clear increase in the formation of chromosomal aberrations during G2, clonogenic survival of CdLS cells after X-ray exposure did not differ significantly from that of control cells. The absence of

Figure 3. Rad51 and γH2AX nuclear foci formation is normal in CdLS cells. (A) Rad51 foci in normal and CdLS B-lymphoblastoid cells after X-rays (12 Gy) and MMC (2.4 μg/ml for 1 h). Cells containing more than five nuclear foci were considered as positive. (B) γH2AX and Rad51 nuclear foci after X-ray exposure (5 Gy). Only cells positive for Rad51 foci were analyzed. Data are the means of at least two experiments. Error bars represent the standard error of the mean.
a hypersensitivity to X-rays in this assay might reflect the relative contributions of NHEJ and HR during the cell cycle. In an asynchronous population of primary fibroblasts, a large fraction of cells will be in G1 or early S and hence the role of HR in the repair of DSBs is expected to be less important. This is opposed to the situation in G2 where the contribution of HR is significant and cannot be fully compensated by NHEJ (42–44). It should, however, be noted that an increased sensitivity was observed in hTERT immortalized human fibroblasts after RNAi mediated knockdown of NIPBL (27). Consistent with our data is the observation that mammalian cell lines containing hypomorphic mutations in RAD51C or BRCAl2 are hypersensitive to cross-linking agents as a consequence of impaired HR but are not or only mildly sensitive for X-rays (42–44). Although clonogenic survival assays did not reveal a drastic increase in X-ray sensitivity of CdLS cells, a distinct MMC hypersensitivity was observed. All seven CdLS cell lines tested displayed an increased sensitivity to MMC. Repair of cross-links is dependent on several DNA repair pathways, including nucleotide excision repair, HR and post-replication/translesion synthesis repair (45). DSBs, which are presumably formed as intermediates in the repair of interstrand cross-links, are processed by HR. No difference in sensitivity was observed between CdLS lines containing pathogenic NIPBL mutations (CdLS11165, CdLS11167 and CdLS45) and the other four CdLS lymphoblast lines without detectable NIPBL mutation. This suggests that these four cell lines also have a defect in the establishment of cohesion and that MMC hypersensitivity is a general feature of CdLS at the cellular level. The reduced ability to process DNA cross-links is further demonstrated by the increased frequency of chromatid exchanges and chromatid breaks that is observed in CdLS fibroblasts after MMC exposure.

The formation of SCEs signifies HR between sister chromatids and consequently defects in HR affect the induction of SCEs after exposure to DNA-damaging agents (31,32). Abolition of the cohesion factors Smc3, Sccl or the delangin ortholog Scc2 in chicken DT40 cells or budding yeast was found to impair the formation of SCEs (25,46). However, in contrast to these observations, we observed efficient formation of SCEs in CdLS cells after exposure to UV light or MMC. One explanation for this apparent contradiction would be that the capacity to repair DNA lesions via HR is still substantial in CdLS cells. In the former studies, protein levels are likely to be reduced to very low levels, whereas in CdLS cells the residual levels of delangin protein could be sufficient to induce near normal levels of SCEs. In support of this would be the recent observation that FA-D1/BRCAl2 patient derived cells, despite being highly sensitive for MMC, are also proficient in SCE formation (44).

In the current study, we observed efficient induction of Rad51 foci in all CdLS B-lymphoblastoid cells upon MMC or X-ray treatment (Fig. 3A). Apparently, the redistribution of Rad51 protein to the site of the damage is not, or hardly affected in CdLS cells. The variation in the induction of Rad51 foci in the various cell lines is not the result of differences in cell cycle distribution, as was shown by FACS analysis, but most likely reflects cell line specific differences (data not shown). Analysis of the number of Rad51 and γH2AX foci after X-ray exposure of fibroblasts also did not reveal a difference between CdLS478 and control fibroblasts. The decline in the number of γH2AX foci after exposure to X-rays suggests efficient processing of DSBs in CdLS cells and is consistent with the unperturbed clonal survival observed after X-rays. The reduction of Rad51 foci in time suggests that HR is still possible in CdLS cells. However, due to reduced levels of delangin protein, small differences in the recombination efficiency between normal and CdLS cells might exist, as implied by the G2 specific formation of chromatidal aberrations.

In man, multiple diseases are known which negatively affect the capacity of cells to repair or process DNA lesions, often resulting in a predisposition for developing tumors. The occurrence of neoplasms in CdLS individuals however appears infrequent, with only four cases being described in the literature (47–49). Although our study clearly shows there is an increased susceptibility for CdLS cells to form chromatidal aberrations after genotoxic stress, there were no signs of chromatidal instability in untreated samples thus corroborating the low incidence of tumor development observed in CdLS individuals. Neither did we observe precocious sister chromatid separation (PSCS) in CdLS cells as was reported by Kaur et al. (50). The most striking feature of delangin deficiency in higher eukaryotes, like Drosophila and X. tropicalis, is its impact on development. Similarly, mutations in genes involved in the establishment of sister chromatid cohesion in man (e.g. ESCO2, NIPBL, SMCA1 and SMC3) result in Roberts syndrome or CdLS, disorders which are manifested by congenital malformations. Despite the recent identification of causal genes associated with these syndromes, the underlying mechanisms for the observed clinical features remain obscure. Drosophila Nipped-B is involved in the transcriptional regulation of cut and ultrabithorax genes, which are involved in embryonic development, in addition to its role in cohesion of sister chromatids after replication (51–53). It is possible that human delangin, like in Drosophila, is also involved in regulation of developmental genes, although at present no target genes have been identified. In this study, we have shown that CdLS, in addition to the established clinical phenotype, is characterized at the cellular level by an increased sensitivity to ionizing radiation and MMC. This hypersensitivity to DNA cross-linking agents may help improve diagnosis of CdLS and other human disorders associated with defects in sister chromatid cohesion.

MATERIALS AND METHODS

Cell culture

Primary fibroblasts were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (Bodinco), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). B-lymphoblastoid cells were grown in RPMI 1640 medium Dutch modification (Gibco) supplemented with glutamax (Gibco), 20 mM sodium-pyruvate (Gibco), 10% fetal calf serum (Bodinco), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The primary fibroblast lines used were normal (VH10, VH25, FN1), CdLS (GM00045 and GM03478; obtained from the Coriell Institute), ataxia telangiectasia (AT5BIVA), SCID (Artemis-6), Fanconi anemia-D1 (EUF423). B-lymphoblastoid cells used were
normal (JVM, Ramos), CdLS (GM11165, GM11166, GM11167, GM11168 and GM13976; obtained from the Coriell Institute) and Fanconi anemia-J (EUFA696). Both Fanconi anemia cell lines were kindly provided by Dr H. Joenje (VUMC, Amsterdam).

NIPBL and SMC1A mutation analysis
Genomic DNA of all CdLS cell lines was isolated and screened for mutations in the NIPBL coding region [exons 2–47, for primer sequences and PCR conditions (8)]. Mutational analysis of the amplimers was performed by DHPLC (Transgenomic Wave). PCR products with altered DHPLC peaks were purified using a QiaQuick PCR purification kit (Qiagen) and sequenced bi-directionally on an ABI 377 sequencer. The NIPBL sequence in the NCBI nucleotide database (NM_015384) was used as a reference to identify mutations. MLPA analyses were performed using MLPA kits P141 and P142 (MRC-Holland) according to the manufacturer’s instructions. All probands negative for mutations in NIPBL were screened for the presence of mutations in SMCA (GenBank accession number NM_006306). The complete SMCA coding region was amplified in 22 fragments and analyzed by DHPLC (primer sequences and PCR conditions are available on request). Amplification products with altered chromatographic peaks were purified and sequenced bi-directionally.

Clonogenic survival and growth inhibition assays
Exponentially growing cells were trypsinized and 500–2000 cells were plated in 9 cm dishes in duplicate (controls in triplicate), and irradiated or exposed continuously to MMC, in complete medium. After 14–17 days, the dishes were rinsed with 0.9% NaCl, dried, stained with methylene blue (0.25%) and colonies were counted using a light microscope. In all experiments, normal fibroblasts were treated in an identical manner to serve as controls. For growth inhibition assays, B-lymphoblastoid cell cultures were seeded at a density of 5 × 10^4 cells/ml and exposed to X-rays or MMC. Cells were cultured for 3 to 10 days until unexposed controls had undergone three population doublings, at which point all parallel cultures were counted using a Z2 Coulter counter (Beckman Coulter).

Chromosomal aberrations and SCEs
For G1 chromosome aberration analysis, primary fibroblasts were grown until confluency and kept confluent for 1 week before irradiation. After exposure to 0, 0.25, 0.5, 1 and 2 Gy of X-rays, fibroblasts were sub-cultured and allowed to grow in the presence of BrdU (5 μM) for 52 h. Colcemid (25 μg/ml) was added to all cultures 4 h before harvesting. Air-dried preparations were made and stained with FPG (54). For G2, chromosome aberration analysis, exponentially growing cells were exposed to 0, 0.1, 0.25, 0.5 and 1 Gy of X-rays followed by 3 h incubation in the presence of BrdU and colcemid before harvesting. For MMC induced chromosome aberration analysis, exponentially growing normal and CdLS fibroblasts and B-lymphoblastoid cells were either mock-treated or treated with MMC (45 or 60 ng/ml) continuously during culturing. Following treatment BrdU was added to the medium. Cells were harvested for the analysis of chromosome aberrations at 28 h and for SCE analysis at 54 h after MMC treatment, including a 4 h incubation with colcemid. For chromosomal aberrations, 100 mitotic cells were analyzed for each dose, for SCE analysis 25 mitotic cells were scored for each dose.

Immunofluorescence labeling and microscopy
To examine γH2AX or Rad51 foci formation primary fibroblasts were grown on sterile glass slides, resulting in sub-confluent cultures at time of fixation. B-lymphoblastoid cells were grown in tissue culture flasks and transferred to 9 cm culture dishes prior to treatment at a density of 0.5 × 10^6 cells/ml. For Rad51 foci analysis, cells were either mock-treated or treated with MMC (2.4 μg/ml for 1 h) or X-ray irradiation (5 or 12 Gy). After an 8, 12 or 24 h recovery period, primary fibroblasts were fixed immediately using 2% formaldehyde in PBS, and permeabilized for antibody staining with PBS/0.1% Triton X-100. The (mock)-treated B-lymphoblastoid cells (1 × 10^6) were seeded on poly-D-lysine (Sigma) coated glass slides after an 8 or 24 h recovery period and left to attach for 15 min prior to fixation and permeabilization. Subsequently, the slides were blocked for 30 min in PBS/BSA (0.5%)/glycin (0.15%) and incubated with rabbit anti-Rad51 antisera (FBE2, kindly provided by Dr F.E. Benson) or mouse anti-γH2AX (Upstate Biotechnology) for 90 min in a humidified atmosphere. The slides were washed three times in PBS/0.1% Triton X-100 and incubated with AlexaTM 488-conjugated goat anti-rabbit or goat anti-mouse IgG (Molecular Probes) or Cy3 conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) for 1 h at 37°C in a humidified atmosphere. After three washes with PBS/0.1% Triton X-100, the cells were counterstained with 4,6-diamino-2-phenylindole (DAPI; 0.1 μg/ml) in Vectashield mounting medium (Vector Laboratories).

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