Accumulation of (5'S)-8,5'-cyclo-2'-deoxyadenosine in organs of Cockayne syndrome group B gene knockout mice

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ABSTRACT

Cockayne syndrome (CS) is a human genetic disorder characterized by sensitivity to UV radiation, neurodegeneration, premature aging among other phenotypes. CS complementary group B (CS-B) gene (*csb*) encodes the CSB protein (CSB) that is involved in base excision repair of a number of oxidatively induced lesions in genomic DNA *in vivo*. We hypothesized that CSB may also play a role in cellular repair of the helix-distorting tandem lesion (5'S)-8,5'-cyclo-2'-deoxyadenosine (S-cdA). Among many DNA lesions, S-cdA is unique in that it represents a concomitant damage to both the sugar and base moieties of the same nucleoside. Because of the presence of the C8–C5' covalent bond, S-cdA is repaired by nucleotide excision repair unlike most of other oxidatively induced lesions in DNA, which are subject to base excision repair. To test our hypothesis, we isolated genomic DNA samples from brain, kidney and liver of wild type and *csb* knockout ($csb^{-/-}$) mice. Animals were not exposed to any exogenous oxidative stress before the experiment. DNA samples were analyzed by liquid chromatography/mass spectrometry with isotope-dilution. Statistically greater background levels of S-cdA were observed in all three organs of $csb^{-/-}$ mice than in those of wild type mice. These results suggest *in vivo* accumulation of S-cdA in genomic DNA due to lack of its repair in $csb^{-/-}$ mice. Thus, this study provides, for the first time, the evidence that CSB plays a role in nucleotide excision repair of the DNA helix-distorting tandem lesion S-cdA. Accumulation of unrepaired S-cdA *in vivo* may contribute to the pathology associated with CS.

1. Introduction

Cockayne syndrome (CS) is an autosomal, recessive human genetic disease with severe growth failure, premature aging and death, hypersensitivity to sunlight, and microcephaly and other neurologic abnormalities (reviewed in [1, 2]). There are two complementation groups of CS, CS-A and CS-B. The latter is more prevalent than the former and linked to mutations in the CSB gene (*csb*), accounting for approximately 80% of CS patients [3]. CS also forms complexes with some forms of xeroderma pigmentosum groups [1]. *Csb* encodes the CSB protein (CSB) with a molecular mass of 168 kDa and 1493 amino acids [4, 5]. Hypersensitivity to UV radiation is one of the hallmarks of CS-B and results from a pronounced defect in the cellular repair of DNA damage caused by UV-radiation [6]. This defect is limited to active genes and transcription coupled repair (TCR), which removes bulky and helix-distorting DNA lesions resulting from UV-radiation exposure, and is a subpathway of nucleotide excision repair (NER) [7-9]. Inherited defects in TCR of NER is thought to be the molecular basis of some features of CS [10, 11]. It is unlikely, however, that UV-radiation sensitivity of CS-B accounts for other clinical features of CS such as progressive neurodegeneration, because UV- radiation can not penetrate inner organs. Therefore, CS-B cells may be deficient not only in TCR of UV-radiation-induced DNA damage, but also in other processes such as repairing and/or processing oxidatively induced DNA damage [12].

There is evidence that oxidatively induced damage to DNA is involved in the development of progeroid syndromes [1, 2, 13, 14]. This type of DNA damage is generated in aerobic cells by endogenous and exogenous agents that cause formation of free radicals [15]. Among free radicals, the highly reactive hydroxyl radical readily reacts with DNA components, resulting in a plethora of modifications in DNA including base and sugar lesions, single- and

double-strand breaks, DNA-protein cross-links and 8,5'-cyclopurine-2'-deoxynucleosides (reviewed in [16-19]). There is evidence that unrepaired and accumulated DNA damage plays an important role in mutagenesis, carcinogenesis and aging (reviewed in [1]). Most of the oxidatively induced DNA lesions are repaired by base excision repair (BER) pathway [1]. Recent studies suggested that CSB participates directly in repair of oxidatively induced DNA damage [2, 20-25]. Furthermore, CSB interacts with BER enzymes [26, 27]. Most of the studies dealt with the involvement of CSB in repair of 8-hydroxyguanine (8-OH-Gua), the most commonly investigated DNA lesion. However, there are numerous other DNA lesions in oxidatively damaged DNA. Among these, helix-distorting tandem lesions 8,5'-cyclopurine-2'-deoxynucleosides are exclusively repaired by NER because of the presence of a 8,5'-covalent bond between the sugar moiety and the base within the same purine-2'-deoxynucleoside [19, 28, 29]. However, it is not known whether CSB plays a role in NER of these lesions. A recent study showed the accumulation of (5'S)-8,5'-cyclo-2'-deoxyadenosine (S-cdA) in cultured keratinocytes from CS-A patients upon exposure to a low dose of ionizing radiation followed by a repair period, suggesting that CSA participates in NER of S-cdA [30]. In the present study, we wished to test whether CSB plays a role in repair of S-cdA using a mouse knockout model devoid of *csb*.

2. Materials and Methods

2.1. Materials

Nuclease P1 (from *Penicillium citrinum*) was purchased from United States Biological (Swampscott, MA). Snake venom phosphodiesterase was obtained from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase was purchased from Roche Applied Science

(Indianapolis, IN). Acetonitrile (HPLC grade) was from Burdick and Jackson (Muskegon, MI). Biomax5 ultrafiltration membranes (5 kDa molecular mass cutoff) from Millipore (Bedford, MA) were used to filter hydrolyzed DNA samples. Water (HPLC-grade) for analysis by liquid chromatography/mass spectrometry (LC/MS) was from J. T. Baker (Phillipsburg, NJ).

2.2. Isolation of DNA from mouse brain, kidney and liver of mice

CSB knockout (*csb*$^{-/-}$) mice were kindly provided by Dr. Jan Hoeijmakers (Erasmus University Medical Center, Rotterdam, Netherlands). Knockout and wild type (wt) animals were bred at the Gerontology Research Center (GRC) Animal Facility of the National Institute on Aging under standard conditions. Mice were sacrificed by cervical dislocation, and the brain, liver and kidney were immediately removed and processed. All experiments were approved by the GRC Animal Care and Use Committee and performed in accordance with "National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*, National Institutes of Health Publication 85-23, National Institutes of Health, Bethesda, MD". Total genomic DNA was isolated from brain, liver and kidney of *csb*$^{-/-}$ and wt animals using a modification of the salting-out method [31], as described previously [32]. Isolated DNA samples were precipitated with ethanol and then centrifuged to remove ethanol. DNA pellets were washed several times using 70% ethanol followed by centrifugation, and were subsequently air-dried at room temperature.

2.3. ***Analysis by liquid chromatography/mass spectrometry***

DNA samples were blinded prior to analysis. The measurement of S-cdA was performed using LC/MS with isotope-dilution [33]. DNA samples were dissolved in water for 24 h at 4 ºC. The UV spectrum of each DNA sample was recorded between the wavelengths of 200 nm and 350

nm with an absorption spectrophotometer to measure the DNA concentration (absorbance of 1 at 260 nm = 50 μg of DNA/mL) and to judge the quality of DNA. Aliquots (50 μg) of DNA samples were dried in a SpeedVac under vacuum. The stable isotope-labeled analog of S-cdA, i.e., S-cdA-$^{15}N_5$ was prepared as described [34]. Aliquots of S-cdA-$^{15}N_5$ were added as an internal standard to 50 μg aliquots of DNA samples. The samples were hydrolyzed with nuclease P1, snake venom phosphodiesterase and alkaline phosphatase for 24 h, and then filtered and analyzed by LC/MS as described [35]. A Synergi 4 μ Fusion-RP column (25 cm x 2 mm i.d., 4 μm particle size) (Phenomenex, Torrance, CA) with a guard column (1 cm x 2.1 mm i.d.) was used. The solvent A was acetonitrile plus water (98:2, v/v) and the solvent B was 100% acetonitrile. A gradient of 1%/min of the solvent B was used. The flow rate was 0.25 mL/min. The column was kept at 40 ºC. An aliquot of 25 μL of filtered samples was injected onto the LC column. For identification and quantification, selected-ion monitoring was used to monitor the characteristic ions of S-cdA (m/z 164 and 250) and S-cdA-$^{15}N_5$, (m/z 169 and 255) at the appropriate retention time period during LC/MS analysis [33, 35].

2.4. Statistical analysis

Statistical analyses of the data were performed using the software SPSS for Microsoft Windows 11.0 statistical program (SPSS Inc, Chicago, IL). The statistical analysis of the significance between groups of DNA base lesions was carried out using the nonparametric Kruskal-Wallis one-way Analysis of Variance (ANOVA) by ranks and Mann-Whitney *U* tests. A two-tailed *p*-value ≤ 0.05 was considered statistically significant.

3. Results

A number of progeroid syndromes are caused by DNA repair defects in genomic maintenance, suggesting that accumulation of unrepaired DNA damage might be a driving force in the pathology of these diseases [36, 37]. CS cells are genetically unstable, hypersensitive to DNA-damaging agents and accumulate oxidatively induced DNA damage (reviewed in ref [2]). This suggests that CSB may play a role in repair of oxidatively induced DNA lesions. However, most studies dealt with 8-OH-Gua that is repaired by BER, and presented evidence that CSB may be involved in BER of 8-OH-Gua. On the other hand, DNA-distorting unique tandem lesions in DNA, 8,5'-cyclopurine-2'-deoxynucleosides are repaired by NER [19, 28, 29]. 8,5'-Cyclopurine-2'-deoxynucleosides are formed in DNA by hydroxyl radical attack on purine 2′-deoxynucleosides followed by intramolecular cyclization between C5′ of the sugar moiety and C8 of the purine. The presence of the C8–C5′ covalent bond prevents the repair by BER. Thus, we hypothesized that CSB as a part of NER pathway may be involved in repair of these lesions. In the present work, we wished to test whether 8,5'-cyclopurine-2'-deoxynucleosides accumulate in organs of *csb*$^{-/-}$ mice due to lack of CSB and without exposure to exogenous oxidative stress. DNA was isolated from three different organs of *csb*$^{-/-}$ and wt mice, hydrolyzed to nucleosides using three enzymes and then analyzed by LC/MS with isotope dilution for the identification and quantification of S-cdA. For each data point, five animals were used, and DNA was isolated independently in each case. The structure of S-cdA is shown in Figure 1. Figure 2 illustrates the background levels of S-cdA in genomic DNA of brain, kidney and liver from wt and *csb*$^{-/-}$ mice. The results of the statistical analyses with *p* values are also shown. There were statistically significant differences between the background level of S-cdA in wt mice and that in *csb*$^{-/-}$ mice in all three organs with *p* values of 0.028, 0.009 and 0.027, respectively. These results

unequivocally showed the accumulation of S-cdA in *csb*$^{-/-}$ mice when compared to wt mice. The highest accumulation appeared to be in the brain.

4. Discussion

It has been known for some time that CSB-deficient cultured cells accumulate oxidatively induced lesions such as 8-OH-Gua and 8-hydroxyadenine (8-OH-Ade) when exposed to oxidative stress followed by a repair period, indicating the defective repair of these lesions [22, 23, 25]. The role of CSB in the repair of 8-OH-Gua has also been inferred from the analysis of DNA sites sensitive to *E. coli* formamidopyrimidine DNA glycosylase (Fpg) using different types of cultured cells isolated from *ogg1*$^{-/-}$/*csb*$^{-/-}$ mice, although cells from *csb*$^{-/-}$ mice have not exhibited the same effect [24]. However, Fpg recognizes and excises from DNA not only 8-OH-Gua, but also formamidopyrimidines (Fapys), i.e., 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) with similar excision kinetics [38, 39]. Therefore, the results obtained using the analysis of Fpg-sensitive sites may also be applicable to Fapys. Indeed, we recently showed significant accumulation of FapyGua and FapyAde in brain, kidney and liver DNA of *csb*$^{-/-}$ mice when compared to wt mice, supporting a role of CSB in the repair of these lesions (Muftuoglu et al., submitted). Similarly to CSB, CSA is involved in the repair of oxidatively induced DNA damage. Thus, using primary keratinocytes and fibroblasts from CS-A patients, the involvement of CSA in the repair of 8-OH-Gua has been demonstrated [30]. Furthermore, the same study showed accumulation of S-cdA in primary keratinocytes, which had been exposed to a low dose (5 Gy) of ionizing radiation and subsequently allowed to repair the DNA damage for 2 h. The results provided the first evidence that CS-A is involved in the repair of S-cdA. Thus far,

however, the role of CSB in the repair of this tandem lesion has not been demonstrated. In the present study, we found a significantly greater accumulation of S-cdA in three different organs of *csb*$^{-/-}$ mice than in those of wt mice. Thus, this study provides, for the first time, the evidence that CSB participates in the repair of the DNA helix-distorting tandem lesion S-cdA besides being involved in the repair of DNA base lesions such as 8-OH-Gua, 8-OH-Ade and Fapys. Our data are also the first to show the *in vivo* accumulation of S-cdA in *csb*$^{-/-}$ mice rather than in cultured cells and without exposure of animals to any exogenous oxidative stress.

CSB is involved in the repair of UV-radiation-induced DNA lesions by NER-TCR pathway [7-9]. However, CSB may also participate in the repair of 8-OH-Gua and other lesions in a different pathway independent of transcription [24]. S-cdA is repaired by NER, because the C8–C5′ covalent bond prevents the repair by BER (reviewed in ref [19]). S-cdA is a chemically very stable compound with a strong C8–C5′ covalent bond, which stabilizes the glycosidic bond against hydrolysis under even harsh acidic conditions [40]. These facts and the accumulation of S-cdA in *csb*$^{-/-}$ mice strongly suggest the involvement of CSB in NER of S-cdA *in vivo*. However, it is not known whether this repair depends on transcription and/or it is a part of the global genome repair (reviewed in ref [41]). In this context, there is evidence that CSA plays a role in the repair of S-cdA by NER from the genome overall [30]. By inference, CSB is likely to participate in the repair of S-cdA as a part of the global genome repair. On the other hand, it has been hypothesized that some oxidatively induced DNA lesions may also be repaired by NER-TCR (reviewed in [2]). This means that the NER-TCR pathway may be involved in repair of S-cdA as well. This is likely because S-cdA is capable of blocking transcription by RNA polymerase II [28, 42]. It has been argued that deficiency in TCR of oxidatively induced DNA damage may contribute to neurodegeneration in CS patients [20, 24, 43, 44]. At present,

however, it is not known whether S-cdA is repaired by NER-TCR and whether CSB plays a role in this type of repair of S-cdA.

The *in vivo* accumulation of S-cdA has also been observed in relation to other diseases including cancer, further evidencing a role of this lesion in disease processes [45-51]. Biological consequences of S-cdA include blocking transcription and DNA polymerases such as polymerases δ and η, and reducing gene expression [28, 29, 52, 53]. Furthermore, S-cdA causes transcriptional mutagenesis, neurodegeneration and neuronal death [42, 54, 55]. Thus, unrepaired and accumulated S-cdA, and other 8,5'-cyclopurine-2'-deoxynucleosides *in vivo* may have deleterious consequences for organisms. Greater endogenous DNA damage in CS cells during development might impair gene expression, explaining the clinical outcome of CS. More specifically, the high accumulation of S-cdA in the brain of *csb*$^{-/-}$ mice as shown in the present study may suggest a particular role for this lesion in generating neurodegeneration observed in CS patients.

Taken together, our data show for the first time that S-cdA accumulates *in vivo* in genomic DNA of *csb*$^{-/-}$ mice, suggesting a role for CSB in NER of this DNA helix-distorting tandem lesion. This observation also supports the hypothesis that unrepaired S-cdA may accumulate in CS-B patients *in vivo* and may contribute to the phenotype of CSB.

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Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Figure Legends

Fig. 1. Structure of S-cdA.

Fig. 2. Levels of S-cdA in genomic DNA of brain, kidney and liver from wt and *csb*$^{-/-}$ mice. DNA was isolated from animals aged from 20 to 24 months. For each data point, independently isolated DNA samples from five animals were analyzed. Each column represents the average of five measurements. The uncertainties are standard deviations.

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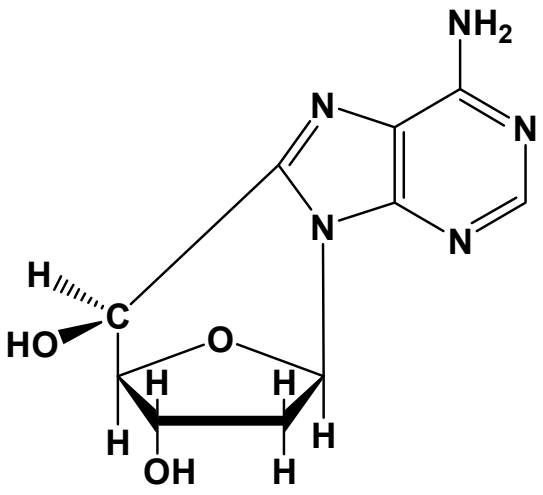

(5S′)-8,5′-cyclo-2′-deoxyadenosine
(S-cdA)

Figure 1

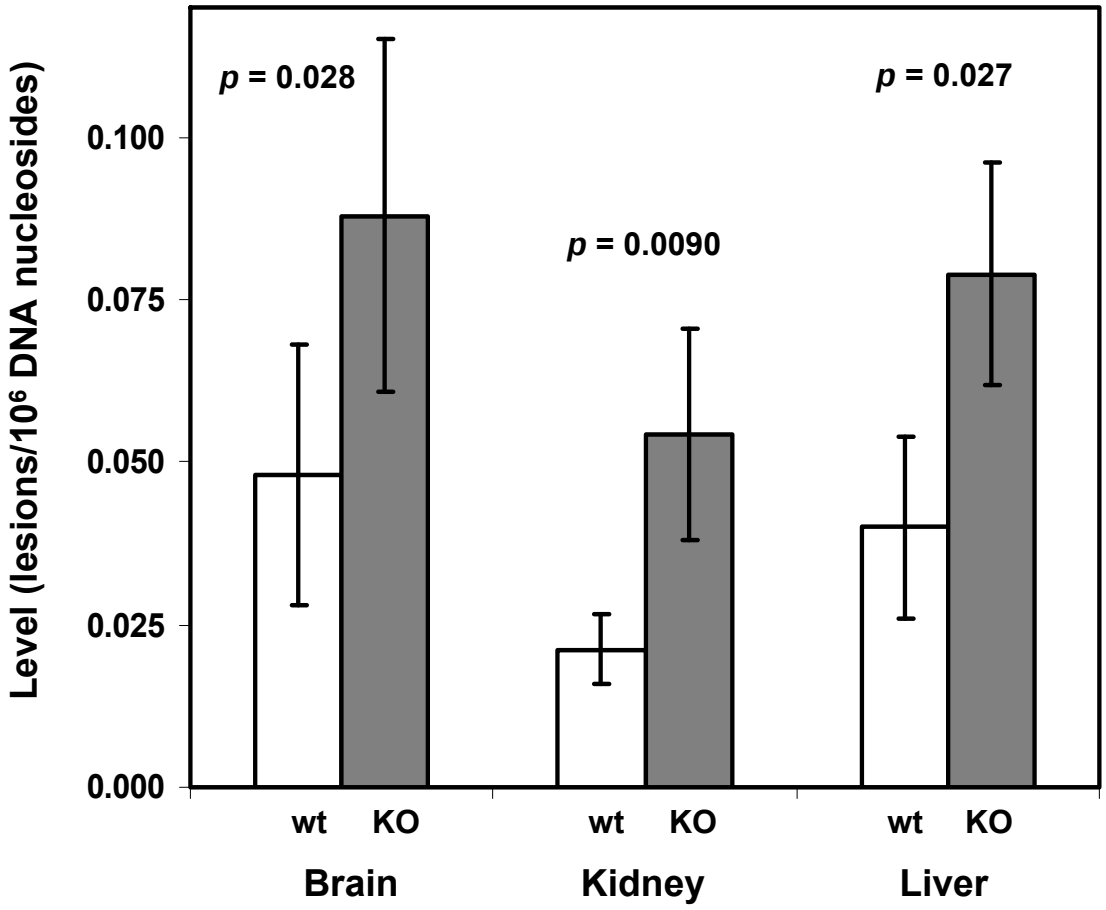
p = 0.028
p = 0.027
p = 0.0090
Level (lesions/10^6 DNA nucleosides)
0.100
0.075
0.050
0.025
0.000
wt
KO
wt
KO
wt
KO
Brain
Kidney
Liver

Figure 2