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Expression of the human DNA glycosylase hSMUG1 in *Trypanosoma brucei* causes DNA damage and interferes with J biosynthesis

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**ABSTRACT**

In kinetoplastid flagellates such as *Trypanosoma brucei*, a small percentage of the thymine residues in the nuclear DNA is replaced by the modified base β-D-glucosyl-hydroxymethyluracil (J), mostly in repetitive sequences like the telomeric GGGTTA repeats. In addition, traces of 5-hydroxymethyluracil (HOMeUra) are present. Previous work has suggested that J is synthesised in two steps via HOMedU as an intermediate, but as J synthesising enzymes have not yet been identified, the biosynthetic pathway remains unclear. To test a model in which HOMeUra functions as a precursor of J, we introduced an inducible gene for the human DNA glycosylase hSMUG1 into bloodstream form *T. brucei*. In higher eukaryotes SMUG1 excises HOMeUra as part of the base excision repair system. We show that expression of the gene in *T. brucei* leads to massive DNA damage in J-modified sequences and results in cell cycle arrest and, eventually, death. hSMUG1 also reduces the J content of the trypanosome DNA. This work supports the idea that HOMeUra is a precursor of J, freely accessible to a DNA glycosylase.

**INTRODUCTION**

The nuclear DNA of kinetoplastid flagellates such as *Trypanosoma* and *Leishmania* contains the hypermodified base β-D-glucosyl-hydroxymethyluracil, called J (1,2). About 1% of the thymine (Thy) residues are replaced by J. The modification is predominantly found in repetitive DNA (3), being most abundant in the telomeric GGGTTA repeats where ~50% of the total J is localised (4). J is absent from chromosome-internal and transcribed genes and the modification is a target for a specific binding protein (5). The function of J is not known.

In addition to J, trypanosome DNA contains a small amount of 5-hydroxymethyluracil (HOMeUra) (<0.02% of total DNA) (6,7). In our current model for J biosynthesis (Fig. 1), 5-hydroxymethyldeoxyuridine (HOMedU) is the precursor of J (7). At the DNA level, thymidine (dT) is first converted to HOMedU which is then glucosylated. Support for this two-step pathway comes from the finding that exogenous HOMedU is randomly incorporated into the DNA and converted into J (7). Despite major efforts, the enzymes catalysing J biosynthesis have not been identified thus far. Direct support for the two-step pathway is therefore still missing. It is also not known whether the two steps are normally separated or coupled events. In the latter case HOMedU might only be an intermediate bound to the modifying machinery, not a ‘normal’ component of the DNA.

To test the two-step model for J biosynthesis, we introduced an enzyme into trypanosomes that excises the base HOMeUra out of the DNA. The enzyme used was the human single-strand-selective monofunctional uracil-DNA glycosylase, hSMUG1 (8). hSMUG1 is a component of the base excision repair (BER) system (reviewed in 9,10). It excises HOMeUra (11) and uracil (12) from single- and double-stranded DNA. The excision results in an abasic site, which is then further processed by other BER factors. hSMUG1 homologs are found in several higher eukaryotes (12) and its presence might be evolutionarily linked to DNA methylation (11,13). We were unable to detect homologous sequences in the trypanosome databases (S.Ulbert and P.Borst, unpublished results). In this paper we present the devastating effects of hSMUG1 on *Trypanosoma brucei*.

**MATERIALS AND METHODS**

Trypanosomes, culture conditions and nucleoside feeding experiments

Bloodstream form trypanosomes of strain 427 of *T. brucei* (14) were cultured as described (15). For the nucleoside feeding experiments (7) thymidine was omitted from the medium. Tetracycline induction was performed with or without continuous nucleoside feeding for HOMedU and 5-bromodeoxyuridine (BrdU), respectively. Nucleosides were purchased from Sigma. Tetracycline (Roche) was used at 1 µg/ml or 5 ng/ml for high or low levels of induction, respectively. Tetracycline-free fetal calf serum was purchased from Clontech.
The oligonucleotides were 32P-labelled at the 5' end. The oligonucleotides used in the BER assays were a 24mer containing four HOMeUra residues at position 5, 11, 17 and 23. Subsequently the cells were disrupted by douncing for 10 strokes and three sonications using a Sonifier B-30 cell disruptor (output control 5, intensity 50%, five pulses). The crude cell lysate was frozen in small aliquots. Aliquots of the extracts were used for the in vitro assays. The oligonucleotides used in the BER assays were a 24mer containing four HOMeUra residues at position 5, 11, 17 and 23 (Sigma) and a 26mer containing one uracil residue at position 14 (Sigma). The oligonucleotides were 32P-labelled at the 5' end and annealed to a 5-fold molar excess of unlabelled complementary strand containing a guanine residue opposite the modified base. One unit of the PBS1 uracil glycosylase inhibitor (New England Biolabs) was used to inhibit the endogenous uracil-DNA glycosylase.

**In vitro BER assays**

To prepare crude cell lysates, 3 × 10^8 trypanosomes were harvested from in vitro cultures and washed in phosphate-buffered saline. The pellet was resuspended in 500 μl of lysis buffer (10 mM HEPES, pH 7.6, 1 mM EDTA, 50% glycerol, supplemented with the complete protease inhibitor mixture, EDTA-free, from Roche) and left on ice for 30 min. Subsequently the cells were disrupted by douncing for 10 strokes and three sonications using a Sonifier B-30 cell disruptor (output control 5, intensity 50%, five pulses). The crude cell lysate was frozen in small aliquots. Aliquots of 0.1–1 μl of the extracts were used for the in vitro assays. The oligonucleotides used in the BER assays were a 24mer containing four HOMeUra residues at position 5, 11, 17 and 23 (Sigma) and a 26mer containing one uracil residue at position 14 (Sigma). The oligonucleotides were 32P-labelled at the 5' end and then HOMedU is converted into J by a putative thymidine 7-hydroxylase and then HOMedU is converted into J by a putative β-glucosyltransferase. From van Leeuwen et al. (7).

**Figure 1.** Speculative two-step model for J biosynthesis. At the DNA level, thymidine is first converted to HOMedU by a putative thymidine 7-hydroxylase and then HOMedU is converted into J by a putative β-glucosyltransferase. From van Leeuwen et al. (7).

**Insertion of the hSMUG1 gene into T.brucei**

The coding sequence for hSMUG1 was amplified using PCR on plasmid pGEX6P1, which contains the hSMUG1 cDNA as a GST fusion (11). The primers used contained a HA epitope tag at either the 5' or 3' end. The gene was cloned into the vector pH615 PAC that is based on the plasmid pH615 (16), but contains a puromycin acetyltransferase resistance gene instead of a hygromycin resistance gene. The inducible promoter of this construct contains a tetracycline operator (Fig. 2). The cell line used for transfection of the final construct (pHDhSMUG1) was HN TET. These cells have the tetracycline repressor gene from the construct pHD 449 (16) in the α-tubulin gene array and a hygromycin phosphotransferase resistance gene in the 221 VSG gene expression site as well as a neomycin resistance gene in the VO2 VSG gene expression site (17). HN TET cells were continuously grown in phleomycin at 2 μg/ml to select for the tetracycline repressor gene. Bloodstream form transfection was carried out as described (18). Drug selection of the hSMUG1 transfectants was done using puromycin (Sigma) at 0.1 μg/ml and phleomycin at 2 μg/ml to select for the hSMUG1 construct and for the tetracycline repressor gene, respectively. The HA tag was situated at either the N- or the C-terminus of the hSMUG1 gene. Transfectants for both constructs were cloned out and further investigated. As the position of the HA tag did not seem to have any influence on the behaviour of the cells upon tetracycline induction, all the experiments reported here were done using trypanosomes expressing the N-terminally HA tagged hSMUG1.

**Figure 2.** Schematic drawing of the construct pHDhSMUG1 used to yield inducible expression of hSMUG1. The construct is based on pHD615 (16). T-P, tetracycline-inducible promoter; V, constitutive VSG promoter; hSMUG1, human single-strand-selective monofunctional uracil-DNA glycosylase; PAC, puromycin acetyltransferase; black boxes, trypanosome-specific RNA processing signals (for details see 16); hatched boxes, flanks for integration into the non-transcribed rRNA spacer in opposite orientation to the direction of endogenous transcription. The solid black line represents pGEM vector sequence. The two open circles are tetracycline repressor molecules derived from transcription of the repressor gene in the αβ-tubulin arrays (data not shown). Binding of the repressor is inhibited by tetracycline.

**Cell cycle analysis**

Trypanosomes were fixed in formaldehyde (19) and transferred to a microscope slide. The slides were dehydrated using 70, 90 and 100% ethanol and the DNA was stained with DAPI. The nuclei and karyoplasts of the trypanosomes were counted under a fluorescence microscope. Between three and five counts were performed per cell type and at least 100 cells were analysed per count. The DNA measurement by flow cytometry was performed as described (20).

**DNA analysis**

Trypanosomes were incorporated into low melting point agarose blocks (2.5 × 10^7 cells/block) and digested with proteinase K (Merck) for 48 h. The blocks were loaded on a
standard 1% agarose gel that was run overnight at 10 V. Alkaline gel electrophoresis, blotting and hybridisation were done according to Sambrook et al. (21). Alkaline gels were run under the same conditions as neutral gels. The probe for the telomeric repeats was an oligonucleotide with the sequence (TAGGGT)₄ and the α/β-tubulin probe has been described (22). The anti-J-DNA immunoblot was carried out as described (23). After stripping, the blot was rehybridised with a probe for the α/β-tubulin genes and the J content of the cells was calculated based on DNA loading.

**RESULTS**

**Construction of trypanosomes containing an inducible hSMUG1 gene**

To test whether HOMedU is an accessible intermediate in the biosynthesis of J we introduced a DNA glycosylase that is able to remove HOMeUra from DNA (11) into bloodstream form T. brucei. As the expression of a DNA repair enzyme without HOMeUra could be harmful to a trypanosome, we used the tetracycline system (24) to control the transcription of hSMUG1. The trypanosome line HN TET contains the gene for the tetracycline repressor (see Materials and Methods). This cell line was used to insert a copy of the human hSMUG1 under the control of a tetracycline-inducible promoter (Fig. 2). The site of integration was the rDNA array (16). HN TET trypanosomes are referred to as wild-type cells.

**Functional hSMUG1 is made in the transfectant upon tetracycline induction**

To investigate the functionality of our constructs, we determined the hSMUG1 activity in lysates prepared from trypanosomes cultured with or without tetracycline. In vitro assays were performed using radiolabelled oligonucleotides with HOMedU at defined positions. Incubation with functional hSMUG1 is made in the transfectant upon tetracycline induction on a protein blot using antibodies directed against the HA tag. As shown in Figure 3C, a band of the correct size (11) could be detected after 3 h and a maximum signal was reached after 9 h.

**hSMUG1 causes cell cycle arrest and cell death in T. brucei, dependent on the level of HOMedU in the DNA**

The hSMUG1 transfectants showed no significant difference in growth compared to wild-type trypanosomes (data not shown). However, addition of tetracycline to the medium killed the cells. After maximally two cell divisions the culture stopped growing and the cells started to die (Fig. 4). The cells did not survive in tetracycline concentrations >5 ng/ml (data not shown). This indicates that even slight induction above the leaky expression of hSMUG1 is lethal to the trypanosome.
To assess how the trypanosomes died, we investigated the influence of hSMUG1 on the cell cycle pattern. In *T. brucei*, progression through the cell cycle can be determined by counting the number of kinetoplasts (a structure containing mitochondrial DNA networks) and nuclei within one cell. As the kinetoplast initiates the S and G2 phases earlier than the nucleus (26), a cell with two kinetoplasts and one nucleus (2K/1N) is in the G2 phase of the cell cycle. In wild-type cells and *hSMUG1* transfectants without tetracycline the number of 2K/1N cells was ~10% (Fig. 5A), whereas cultures expressing high levels of hSMUG1 showed an increase in 2K/1N cells to ~50%. Thus, a large part of the culture was stalled in the G2 phase of the cell cycle. To verify these results, we measured DNA content by flow cytometry. Figure 5B shows that the number of cells in the G2/M fraction increased relative to the G1 fraction. In addition, cells with less than the diploid amount of DNA (dying or dead) were present.

To test whether the lethal effect of hSMUG1 could be increased by raising the level of HOMedU in the DNA, we grew the trypanosomes in medium containing the nucleoside HOMedU for about six generations. This HOMedU is incorporated into the DNA and converted into J (7). Without tetracycline, the *hSMUG1* transfectants showed no significant difference in growth in medium containing HOMedU at 200 μM compared to wild-type cells (Fig. 5C). This indicates that the endogenous HOMedU is already in excess to the low amounts of hSMUG1 produced by leaky transcription. However, when tetracycline was added, hSMUG1 cells that were HOMedU fed showed a stronger growth defect (Fig. 5C). They hardly doubled in number and started to die earlier than the control (non-fed) *hSMUG1* trypanosomes.

We also analysed the effect of BrdU feeding on trypanosome death by *hSMUG1* induction. Growing trypanosomes for eight to nine generations in BrdU at 100 μM leads to a replacement of 15–20% of the Thy residues. BrdU cannot be converted into HOMedU and growth in BrdU decreases the J level about 3-fold (7). Furthermore, BrdU is not removed from DNA by repair (G.W.Teebor, unpublished observation). Figure 5D shows that induction of hSMUG1 in these cells had much less effect on cell growth than induction in trypanosomes that had not been pre-grown in BrdU. The BrdU-fed trypanosomes also reacted to hSMUG1, but the damage caused by the enzyme was less, allowing the cells to survive. The culture even continued growing at a low rate. Taken together these results indicate that the growth inhibitory effect of hSMUG1 on trypanosomes is strictly dependent on the level of HOMedU in the trypanosome DNA.

**Expression of hSMUG1 causes DNA damage in *T. brucei***

The rapid decrease in trypanosome multiplication after hSMUG1 induction suggested that the cells were overwhelmed by DNA damage. To verify this, cells were incorporated into agarose blocks, digested with proteinase K and loaded on a standard agarose gel run at low voltage. The gel was transferred to a nitrocellulose membrane and probed for the telomeric GGGTTA repeats, which contain high amounts of J. As can be seen in Figure 6, the DNA of wild-type cells and uninduced *hSMUG1* transfectants remained mostly in the slot. After tetracycline induction the DNA of the *hSMUG1* transfectants was fragmented and migrated to a substantial extent into the gel. The fraction of DNA migrating into the gel was further increased when an alkaline gel was used (data not shown). As abasic sites in the DNA are alkali-labile, DNA fragmentation increases under alkaline conditions. We also investigated the chromosome-internal α/β-†ubulin gene arrays, which do not contain J (23), and found that the †ubulin genes migrated only marginally into the gel compared to the telomeric repeats (Fig. 6), even when an alkaline gel was used (data not shown).

It was shown previously that excessive BER in mammalian cells leads to double-strand breaks (27). As the number of abasic sites arising from the action of hSMUG1 exceeds the capacity of the endogenous repair machinery, a fragmentation of sequences with a high HOMedU level is the consequence. The massive abundance of double-strand breaks and alkali-labile sites in *hSMUG1* cells after tetracycline induction correlates with the effect of hSMUG1 on growth. Our interpretation of these results is that high expression of hSMUG1 leads to excessive DNA repair. The result is a DNA damage response of the cells that leads to cell cycle arrest and eventually to death. Our results also confirm that HOMedU is absent from transcribed, chromosome-internal genes as there is no DNA damage detectable in the α/β-†ubulin array.

Interestingly, there was no significant difference detectable between wild-type cells and *hSMUG1* transfectants without tetracycline (Fig. 6), although these trypanosomes show a low level of functional hSMUG1 (Fig. 3A). This means that the endogenous BER system of the trypanosomes can cope with the ‘extra repair’ due to hSMUG1 (confirming the growth curve in Fig. 4), until it exceeds a certain limit. This limit is presumably set by the endogenous BER factors of the trypanosome (such as AP endonucleases, DNA polymerase β, etc.) that become limiting after tetracycline induction.

**hSMUG1 interferes with J biosynthesis**

As HOMedU has been shown to be involved in J biosynthesis (7), we analysed the influence of hSMUG1 on the J level. The J content of the cells was determined by a dot-blot analysis using a polyclonal anti-J antibody. Twenty-four hours after tetracycline addition the hSMUG1 trypanosomes had about four times less J than non-induced or wild-type cells (Fig. 7). As the trypanosomes can maximally complete two cell divisions in that period (Fig. 4) we conclude that hSMUG1...
is removing most of the available HOMedU made after DNA replication, thereby inhibiting de novo J synthesis.

It is unlikely that hSMUG1 excises J directly, as we have shown by in vitro assays with purified, recombinant hSMUG1 that the enzyme does not excise J from duplex or single-stranded oligonucleotides (S.Ulbert, L.Eide, E.Seeberg and P.Borst, manuscript in preparation). We also investigated the effect of a low tetracycline induction over a longer period of time on J levels. Incubation of the hSMUG1 transfectants in tetracycline (1 µg/ml) for 5 days resulted in a 90% decrease in the J level and a slower growth of the cells (data not shown). We have not succeeded in decreasing the J level further for technical reasons. Tetracycline is unstable in culture systems and we were unable to maintain lower tetracycline levels than 5 ng/ml over a long timespan. Even with a daily refreshment of tetracycline the cells started to pick up normal growth after ~5 days and the J content was approximating wild-type levels again (data not shown).

**DISCUSSION**

*Trypanosoma brucei* and related kinetoplastid flagellates contain the modified base J in their nuclear DNA. Whereas the amount and distribution of J has been determined in detail, little is known as yet about its biosynthesis. We have previously shown by nucleoside-feeding experiments that exogenous HOMedU is incorporated in trypanosome DNA and converted into J (7). This suggests but does not prove that free HOMedU is an obligatory intermediate in J biosynthesis, and led to the model shown in Figure 1. However, all attempts...
to find the glucosyltransferase mediating the conversion of HOMedU into J have failed thus far and direct support for the scheme in Figure 1 is therefore still lacking. To test whether HOMedU is an intermediate that is freely accessible to a DNA glycosylase able to take out the base we have introduced such a glycosylase, hSMUG1, into T. brucei.

Expression of hSMUG1 in trypanosomes led to a dramatic loss of viability. This effect was dependent on the level of HOMedU in the DNA. Raising the HOMedU content led to a stronger effect, i.e. the cells died much earlier; lowering it by feeding BrdU reduced the sensitivity of the transfectants. We conclude from these results that hSMUG1 killed the cells by excising HOMeUra.

Analysis of the DNA in the trypanosomes expressing hSMUG1 revealed severe DNA damage due to an accumulation of double-strand breaks and abasic sites. It has been shown previously that overexpression of a DNA glycosylase can lead to DNA damage rather than increased, complete BER (28). An increase in the initial step of BER, the excision of a base, needs to be accompanied by an increase in the levels of the other BER factors. Otherwise the BER system becomes imbalanced (29), leading to genome instability and decreased viability. In our case we introduce a DNA glycosylase which attacks a genome in which one of its substrates is present in excess, as has been done by Kavli et al. (30) for Escherichia coli and Mi et al. (27) for mammalian cells. This should result in the accumulation of abasic sites that lead to single strand breaks. Double strand breaks are then the consequence of two adjacent single-strand breaks on both strands, as we detected in the telomeres of T. brucei.

It is unlikely that the trypanosomes died because of the decrease in J level caused by hSMUG1, as we know that bloodstream form T. brucei can tolerate much lower levels of J, as low as 5% of the wild-type (31). We rather think that hSMUG1 kills the trypanosomes by overwhelming DNA damage resulting in cell cycle arrest and death. The cells can deal with the damage as long as hSMUG1 is limiting and not induced. This shows that the endogenous BER system can process the abasic sites produced by low level excision of HOMedU.

It is remarkable that a 3-fold reduction in J in trypanosomes grown in BrdU prevents cell death by hSMUG1 induction.
Under these conditions, <20% of all dT in the DNA is replaced by BrdU, suggesting that the putative thymidine hydroxylase, which converts dT into HOMedU, could be inhibited by BrdU in DNA, resulting in a substantially decreased rate of HOMedU synthesis and, hence, much less HOMeUra excision by hSMUG1.

The excision of HOMeUra by hSMUG1 resulted in a substantial decrease in the level of J in trypanosome DNA. In a parallel study we investigated the role of J as a potential target for different DNA glycosylases (S. Ulbert, L. Eide, E. Seeberg and P. Borst, manuscript in preparation). We found that J is not recognised by hSMUG1 and, therefore, conclude that the J level of the hSMUG1 transfectants decreased as a consequence of the excision of HOMeUra, supporting the role of HOMeUra as a precursor of J.

Because all our attempts to detect a glucosyltransferase in trypanosome extracts have failed thus far, alternative routes for the biosynthesis of J have been considered. Glucosylation might be coupled to hydroxylation in a concerted reaction and the possibility has even been raised that hydroxylation is energetically driven by the glucosylation reaction (S. Beverley, personal communication). These schemes have become less attractive, as we have shown that the HOMedU used for J synthesis appears to be freely accessible to hSMUG1. The two-step synthesis of J presented in Figure 1 remains therefore a plausible working hypothesis and we are continuing our efforts to identify the enzymes involved.

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