H_2O_2-induced higher order chromatin degradation: A novel mechanism of oxidative genotoxicity

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The genotoxicity of reactive oxygen species (ROS) is well established. The underlying mechanism involves oxidation of DNA by ROS. However, we have recently shown that hydrogen peroxide (H_2O_2), the major mediator of oxidative stress, can also cause genomic damage indirectly. Thus, H_2O_2 at pathologically relevant concentrations rapidly induces higher order chromatin degradation (HOCD), i.e. enzymatic excision of chromatin loops and their oligomers at matrix-attachment regions. The activation of endonuclease that catalyzes HOCD is a signalling event triggered specifically by H_2O_2. The activation is not mediated by an influx of calcium ions, but resting concentrations of intracellular calcium ions are required for the maintenance of the endonuclease in an active form. Although H_2O_2-induced HOCD can efficiently dismantle the genome leading to cell death, under sublethal oxidative stress conditions H_2O_2-induced HOCD may be the major source of somatic mutations.

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1. Introduction

Oxidative stress, resulting from an unbalanced increase in the cellular levels of reactive oxygen species (ROS), is the causative factor in malignancy and aging (Li *et al* 1997; Klaunig *et al* 1998; Poulsen *et al* 1998; Baynes 2000; Yeldandi *et al* 2000; Kovacic and Jacintho 2001). The underlying mechanism entails genomic DNA damage leading to the generation of somatic mutations in genes that are necessary for maintaining genomic stability (Cutler 1992; Loft and Poulsen 1996). It is widely accepted that the primary damage occurs through direct interaction of ROS with genomic DNA, leading to oxidation of the bases and the deoxyribose phosphate backbone of the DNA (Halliwell and Aruoma 1991; Breen and Murphy 1995; Henle and Linn 1997). Hydrogen peroxide (H_2O_2) is the major mediator of oxidative stress, and a potent mutagen. Large amounts of H_2O_2 can be generated either endogenously by respiratory chain and several other metabolic pathways in target cells, or exogenously by activated inflammatory cells via oxidative burst mechanism. Although H_2O_2 is a week oxidant, it can be converted in the presence of reduced transition metals, such as ferrous and cuprous ions, to highly reactive hydroxyl radicals that are believed to mediate the genotoxicity of H_2O_2 (Halliwell and Gutteridge 1989).

We have recently discovered (Mouzannar *et al* 2001; Konat *et al* 2001) that H_2O_2 can also damage genomic DNA indirectly by inducing enzymatic DNA digestion in a pattern consistent with higher order chromatin degradation (HOCD), i.e. the excision of chromatin loops and their oligomers from the chromosomes. HOCD has been originally described as an integral part of programmed cell death that marks the commitment of cells to death (Brown *et al* 1993; Sun *et al* 1993; Oberhammer *et al* 1993; Cohen *et al* 1994; Zhivotovsky *et al* 1994; Beere

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Abbreviations used: HOCD, Higher order chromatin degradation; MAR, matrix attachment region.

et al 1995; Lagarkova *et al* 1995). The DNA backbone digestion occurs at matrix attachment regions (MARs), and proceeds in distinct, spatio-temporal stages (figure 1). The, as yet unidentified, MAR-associated endonuclease works through a single strand scission mechanism (Walker *et al* 1997). Thus, the enzyme initially cuts only one DNA strand; while a second, closely spaced cut in the opposite strand results in bifilar fragmentation of the DNA.

2. Results

H_2O_2-induced HOCD is a very rapid process (Mouzannar *et al* 2001; Bai and Konat 2002). A representative experiment depicted in figure 1 (lower panel) shows that practically total nuclear chromatin can be monofilarly digested within 30 min of exposure to 1 mM H_2O_2. Evident HOCD can be elicited by 5 μM H_2O_2, and HOCD rate plateaus at approximately 500 μM H_2O_2. Such H_2O_2 concentrations are featured *in vivo* under oxidative stress conditions. For example, the interstitial H_2O_2 concentration in rat brain tissue during ischemia/reperfusion reaches approximately 150 μM and lasts over an hour (Hyslop *et al* 1995), while even a 15 min-exposure of rat oligodendrocytes to such H_2O_2 concentration results in an extensive HOCD (Mouzannar *et al* 2001). H_2O_2-induced HOCD is mostly reversible within a few hours following the removal of H_2O_2 (Mouzannar *et al* 2001; Bai and Konat 2002). Moreover, HOCD represents a general response of cells to H_2O_2, as it can be induced in cells of different origin (Bai and Konat 2002).

HOCD is not a result of oxidative stress *per se*, but is rather specifically triggered by H_2O_2 as no HOCD can be induced by equitoxic concentrations of hypochlorite, nitric oxide (Bai and Konat 2002) or peroxynitrite (Konat G W, unpublished results). Also, no HOCD can be induced by H_2O_2 in isolated nuclei (Bai and Konat 2002), indicating that H_2O_2 exerts its effect indirectly by triggering cytoplasmic signalling cascades that activate MAR-associated endonuclease. The activating signalling cascades are not directly triggered by an increase in the concentration of intracellular Ca^{2+}, and the endonuclease itself is Ca^{2+}-independent and requires only Mg^{2+} for full activity (Konat *et al* 2001). Interestingly, the chelation of intracellular Ca^{2+} with ethylenedioxybis (o-phenylenenitrilo) tetraacetic acid (BAPTA) abrogates H_2O_2-induced HOCD and converts MAR-associated endonuclease into an inactive form. Also, a profound attenuation of H_2O_2-induced HOCD can be achieved by the chelation of iron with desferroxamine (Konat *et al* 2001), indicating that the activating cascades may involve hydroxyl radicals generated from H_2O_2 by Fenton reaction.

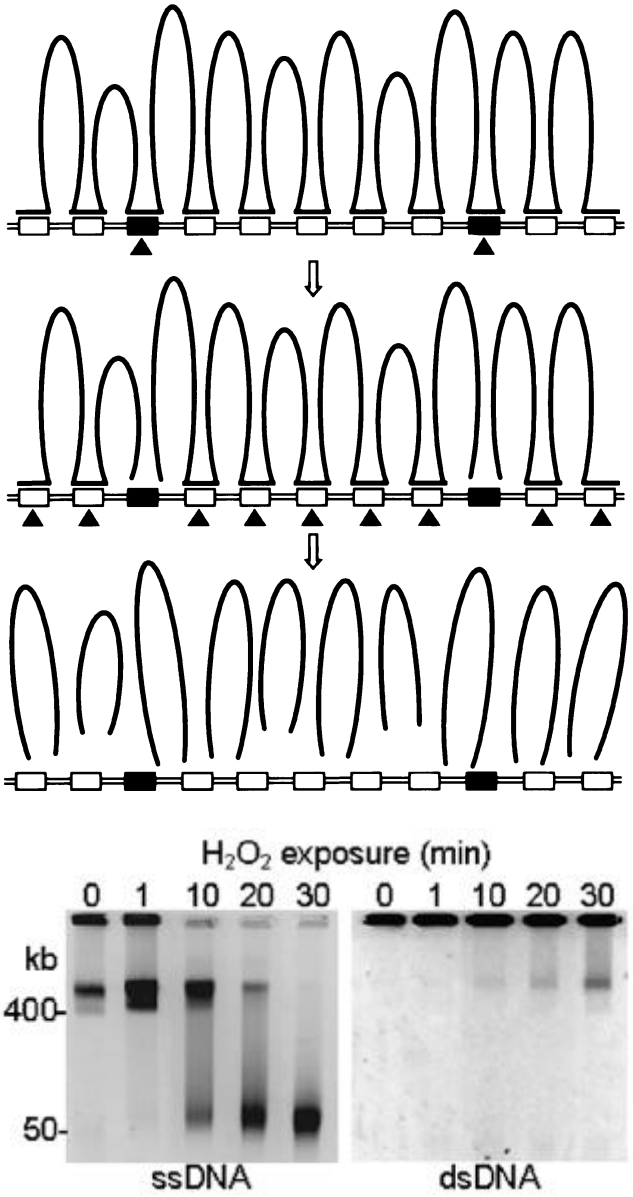

Figure 1. Higher order chromatin digestion (HOCD). Upper panel shows a flowchart of HOCD. Chromatin loops (thick line) are anchored to the nuclear matrix (double line) at matrix attachment regions (MAR). The sites of nuclear matrix where the chromatin fiber is anchored (rectangles) contain specific endonuclease that mediates HOCD. Chromatin is initially digested at AT-rich MARs (filled rectangles) generating oligomers of chromatin loops of > 400 kb. Subsequent scission at remaining MARs (open rectangles) results in the liberation of individual loops of ~ 50 kb. Lower panel shows time course of H_2O_2-induced HOCD in rat glioma C6 cells. The cells were exposed to 1 mM H_2O_2 for various period of time as indicated. Genomic DNA was isolated by agarose embedding followed by SDS-extraction (Mouzannar *et al* 2001). The embedded DNA was either directly analysed by field inversion gel electophoresis (FIGE) to detect double strand fragments (dsDNA gel), or was digested with S1 endonuclease prior to FIGE to detect single strand fragments (ssDNA gel). A negative of ethidium bromide image is shown.

3. Discussion

The dismantling of the genome through H_2O_2-induced HOCD under severe oxidative stress inevitably leads to cell death. In fact, H_2O_2 may provide the signal that elicits HOCD in cells undergoing programmed cell death (PCD), because various death stimuli ultimately induce oxidative stress instigated by degenerating mitochondria that produce vast amounts of H_2O_2 (Quillet-Mary *et al* 1995; Bredesen 1995; Um *et al* 1996; Kroemer *et al* 1997). On the other hand, sublethal oxidative stress may lead to only a partial HOCD. Such a partial HOCD poses a great mutagenic risk to the surviving cells, because ensuing strand-break repair process may generate a variety of genomic rearrangements (Winegar *et al* 1992; Richardson and Jasin 2000; Lin and Waldman 2001; Khanna and Jackson 2001). Even single strand fragmentation is highly detrimental to the genomic stability (Thompson *et al* 1982; Domingues *et al* 1998; Veld *et al* 1998) plausibly via the conversion of single strand breaks into double strand breaks during DNA replication (Kuzminov 2001). Thus, unfaithful religation of HOCD-generated breaks may lead to the loss of chromatin loops resulting in the deletion or truncation of genes as genes are either contained within chromatin loops or span multiple loops (Reynolds and Gottesfeld 1985; Wang and Hogan 1985; Ruiz-Carrilo 1984). Moreover, free DNA ends can be trimmed by exonucleases before being religated (Winegar *et al* 1992), and consequently, generate small intragenic deletions that may profoundly alter structure/function of the encoded proteins through changing splicing pattern, through shifting translational frame, and/or through loss of strategically positioned amino acids. Also, because MARs often contain gene regulatory regions (Nickol and Felsenfeld 1983; Boles and Hogan 1987), such short deletions may profoundly alter transcriptional activity of genes. The analysis of H_2O_2-induced mutations revealing a plethora of short and large deletions (Turker *et al* 1999) validates the HOCD-based mechanisms. In addition, the mis-religation of chromatin loops may dramatically change gene expression through swapping of gene promoters and/or of regulatory regions, leading to neoplastic transformation.

In conclusion, H_2O_2 triggers cytoplasmic signalling pathways that activate MAR-associated endonuclease resulting in HOCD. H_2O_2-induced HOCD is a rapid process by which oxidatively stressed cells can efficiently dismantle their genomes, and thus, commit themselves to death. This process may also generate somatic mutations with neoplastic potential in surviving cells. Moreover, H_2O_2-induced HOCD may play the key role in the etiopathology of neurodegeneration, and in nonneural degenerative conditions that feature oxidative stress as the causative factor.

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