Evidence for Radical Species as Intermediates in Cadmium/Zinc-Metallothionein-dependent DNA Damage *In Vitro*

Thomas Müller,1 Rainer Schuckelt,2 and Lothar Jaenicke2

1INBIFO Institut für biologische Forschung, Köln, Federal Republic of Germany; 2Institut für Biochemie, Universität Köln, Köln, Federal Republic of Germany

Toxicologic data on cadmium (Cd) indicate that intracellular metallothionein (MT) is protective for Cd exposure, whereas extracellular Cd-containing MT might be toxic. Moreover, Cd is suspected to be a carcinogen though the underlying mechanism is not known. Here we report on the genotoxic activity of cadmium/zinc-metallothionein (Cd/Zn-MT) in a cell-free test system: a concentration-dependent increase in DNA strand breaks was detected with increasing doses of Cd/Zn-MT, whereas no DNA strand breaks were observed in the presence of heat-denatured MT or Cd or Zn ions alone. Modifications of native Cd/Zn-MT by the metal ion-chelating agent EDTA or the sulfhydryl group alkylating agents N-ethylmaleimide and iodoacetamide suggest that the various cysteine residues of MT, together with the attached heavy metal ions, may be involved in the DNA cleavage reaction. Furthermore, DNA strand breaks caused by Cd/Zn-MT seem more likely to be random than sequence- or base-specific. Results from experiments with radical scavengers and electron spin resonance spectroscopy point to radical species formed by Cd/Zn-MT as mediators of the DNA damage. Thus, the actual activity of Cd/Zn-MT—whether protective or damaging—appears to depend on various parameters governed by the extra- and intracellular environment.—*Environ Health Perspect* 102(Suppl 3):27–29 (1994).

Key words: cadmium/zinc-metallothionein, DNA strand breaks, radicals

Introduction

Data based on animal as well as epidemiologic studies provide strong evidence that cadmium (Cd) compounds are potential carcinogens in humans (1,2). However, the mechanism of Cd-induced carcinogen-<ref>esis is only poorly understood. Using *in vitro* cellular systems, investigations on the genotoxicity of Cd revealed evidence for the intermediate appearance of active oxygen species in Cd-dependent DNA damage (3). However, no DNA strand breaks were observed when naked DNA was incubated with Cd salts (4) although there is a strong affinity of Cd ions to DNA (5). Since DNA strand-breaking activity of Cd ions can thus be excluded, other—indirect—mechanisms, including possible proteinaceous mediators of Cd-related cellular damage, may be involved. One working hypothesis suggests that transactivating zinc (Zn)-finger proteins (6) which play a crucial role in the regulation of genes involved in development and differentiation, may become disordered when Cd instead of Zn ions are introduced into the Zn-finger domains (7). In addition, the binding of Cd ions by these proteins may also lead to the release of free radicals. This hypothesis (7)—though not yet proven—may explain how Cd may be genotoxic via a free radical mechanism. Other possible candidates mediating Cd-dependent DNA damage are, for example, the Zn-dependent DNA and RNA polymerases which showed impaired function in the presence of Cd ions (8).

Other cellular components also exhibiting a strong affinity to Cd ions are the metallothioneins (MT) (9,10) whose native conformation is stabilized by the tight binding of metal ions exhibiting a d10 electron configuration. These conserved cysteine-rich small proteins have been identified in the cytosol as well as in the nucleus of higher eukaryotic cells (11). The expression of the MT genes is strongly enhanced by a battery of physiologic as well as stress-related inducers including heavy metal ions. With respect to the cellular function of MT, the aforementioned features have been interpreted differently. On the one hand, a storage function for zinc ions as the main purpose of intracellular MT is favored. According to this point of view, the ability of MT to detoxify the cell of heavy metal ions is a secondary effect related to the chemical nature of the protein (12). On the other hand, it has been suggested that the MTs have evolved and are strongly conserved just because of their ability to protect the cell from heavy metal ion- and free radical-mediated damage (9). With respect to a primary protective function, it is difficult to understand that Cd-containing MT is itself toxic, at least in an extracellular environment. For instance, it has been shown that Cd-MT is a more potent nephrotoxin than Cd ions themselves (13). Thus, a general interpretation of data from the literature leads to the assumption (10) that intracellular MT is protective for Cd exposure, whereas extracellular MT might be toxic. In this article, we report on the potential DNA-damaging activity of Cd/Zn-MT as observed in a cell-free assay. Evidence will be presented that the Cd/Zn-MT-dependent DNA damage is based on the intermediate formation of free radicals.

The results referred to here have already been described in full detail (4). Here, they are summarized placing special emphasis on key experiments.

Discussion

Initial results from incubations of Cd/Zn-MT—obtained from a commercial source...
without defined purity (MT1, MT2, isolated from rabbit liver) and as an electrophoretically pure sample [MT2, isolated from rat liver, (14)]—together with a supercoiled DNA target showed a Cd/Zn-MT concentration-dependent increase of DNA strand breaks (Figure 1). However, no DNA strand scission was observed with Cd (Figure 1) or Zn ions (data not shown) alone. Figure 1 may give the impression that there is preferential formation of the linear plasmid conformation in the presence of rat liver MT2. This would be indicative of a preferred appearance of DNA double-strand breaks rather than DNA single-strand breaks (open circular conformation). However, this observation was not made in most of the experiments performed.

A closer characterization of the DNA strand-breaking reaction induced by Cd/Zn-MT showed a distinct dependence on physiological conditions: thus, the amount of rabbit MT1-induced DNA strand breaks peaked around 40°C, while the pH optimum was between pH 6.5 and 7.0. These findings, together with the observation that a heat-denatured as well as chemically hydrolyzed sample of rabbit MT1 lack any DNA strand-breaking activity, point to a protein (MT)-mediated reaction. Apart from this, the latter results represent important controls since they demonstrate that the solutions used in the incubations were sufficiently free of contaminants, e.g., radical-producing substances.

Further investigations on the mechanism of the MT-mediated DNA strand breakage yielded strong evidence that the metal thiolate clusters harbored by MT, i.e., structures forming coordinated bindings between suitable cations and the free thiolate groups of the various cysteine residues (15), were involved in the reaction. This was deduced from experiments that showed (Figure 2) that the DNA fragmentation by MT was inhibited by both the thiol group-alkylating agents N-ethylmaleimide (NEM) or iodoacetamide and by the divalent cation-scavenging agent EDTA. These compounds actually target the main components involved in the formation of the metal thiolate clusters, i.e., the free thiol groups of cysteine residues and the attached heavy metal ions. In contrast to NEM, which at increasing concentrations led to a complete inhibition of MT1-induced DNA strand breaks, no such complete inhibition was observed in the presence of even high concentrations of EDTA. The biphasic incomplete inhibition characteristic of EDTA can be explained by the fact that some cation binding sites harbored by the MT protein show a different resistance to cation capture by EDTA due to their higher affinity to Cd ions (12,16).

Investigation of a base or site specificity in the MT-mediated DNA cleavage using a linear DNA target molecule in combination with DNA sequencing techniques resulted in a concentration-dependent appearance of fragment ladders with no significant differences in the intensity of the bands generated.
As a next step, the intermediate formation of free radicals by MT was investigated. However, no clear-cut indications of the nature of the DNA strand-breaking species were obtained from standard incubations, i.e., incubations of MT and target DNA under physiological conditions, performed in the presence of more or less specific radical scavengers. For example, no reduction in DNA strand breaks was detected when either catalase or superoxide dismutase were used. Both enzymes are involved in the cellular detoxification of active oxygen species. However, benzoate, a known hydroxy radical scavenger, was able to inhibit MT-dependent DNA strand breaks, though at rather high concentrations. A more pronounced inhibition of MT-related DNA damage was observed in the presence of the unspecific radical scavenger α-tocopherol, while the effectiveness of carnosine, the most active inhibitor in this system, may result from its radical scavenging as well as cation-chelating properties (17).

More evidence that radicals may in fact be involved in the appearance of Cd/Zn-MT-mediated DNA strand breaks was obtained using ESR (Figure 3). Whereas no ESR-specific signals were observed in the presence of the spin trap reagent or in the presence of the spin trap reagent plus Zn or Cd ions alone (Figure 3a,c), small but typical ESR signals were detected in incubations of the spin-trapping agent together with Cd/Zn-MT1 from rabbit liver (Figure 3d). Since the MT sample used in these investigations was calculated to be not completely saturated with Cd ions, ESR signals were intensified by the exogenous addition of Cd ions to the incubation mixture (Figure 3e). Although these data provide evidence for the generation of radicals by Cd/Zn-MT, rather high MT concentrations as well as low concentrations of SDS, to solubilize the spin-trapping agent, and prolonged incubation times had to be employed. Also, the identity of the radical species generated by MT is by no means clear from these investigations.

Control experiments clearly showed that the effects of Cd/Zn-MT described here are not due to a DNase contamination of the MT samples used, since the enzyme activity of DNase I was not affected by NEM or benzoate at concentrations leading to a complete inhibition of the Cd/Zn-MT-dependent DNA strand-breaking activity.

On the basis of these results, the question arises how these data fit into the general scheme of a protective property of MT to heavy metal ion-induced cellular damage. Although these data provide evidence for a radical-generating property of Cd/Zn-MT, it should be kept in mind that our results were mainly derived from a cell-free assay, and rather high Cd/Zn-MT concentrations are obviously necessary to detect DNA strand-breaking activity. That the cellular environment may clearly modulate the potential properties of a certain substance, even reversing them, is shown by ascorbate (18). This substance has been shown to exhibit strong prooxidant activity in the presence of free transition metal cations, but is an "outstanding" antioxidant in their absence (18).

Nevertheless, with respect to Cd-induced carcinogenesis, one should keep in mind that MT—beyond its activities to protect the cell from heavy metal ion-induced side effects—may also damage DNA. According to these considerations, the actual activity of MT—whether protective or damaging—may be a function of the proximity of MT molecules to DNA and their concentration within the nucleus as recently suggested (19).

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