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Nucleolar segregation as an early marker for DNA damage; an experimental study in rats treated with 4-hydroxyaminoquinoline 1-oxide

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Abstract Male 6-week-old Sprague Dawley rats were given a single intravenous injection of 4-hydroxyaminoquinoline 1-oxide (4HAQO) at a dose of 20 mg/kg in order to produce ultrastructural changes as possible morphological biomarkers for toxicity. Immunohistochemically demonstrated formation of 4HAQO-DNA adduct was correlated with the changes found. Nucleolar alteration, demonstrable by electron microscopy as segregation of nucleolar components into granular and fibrillar compartments, was evident in cells of the target organs, exocrine pancreas and adrenocortex, but not of the non-target liver parenchyma. Sequential observation clarified that such alteration was highest in frequency 6 h and 4 h after 4HAQO administration in pancreatic acinar cells and adrenocortical cells respectively. Electron microscopically, apoptotic changes of acinar cells were evident 2 h after injection of 4HAQO. DNA adduct formation was consistently demonstrated in the same target organs showing nucleolar segregation, the highest frequency being noted 4 h after 4HAQO treatment in both pancreatic acinar cells and adrenocortical cells. Our results thus indicate an identity of the target cells for nucleolar segregation and 4HAQO-DNA adduct formation which correlates with 4HAQO-toxicity. We suggest that nucleolar segregation occurs subsequent to the generation of DNA damage.

Key words Nucleolar segregation · 4-Hydroxyaminoquinoline 1-oxide · Rat · DNA adducts · Apoptosis

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Introduction

The potent carcinogen 4-nitroquinoline 1-oxide (4NQO) was first demonstrated in 1957 by Nakahara et al. [22] to induce skin tumours in mice, with the metabolite 4-hydroxyaminoquinoline 1-oxide (4HAQO) being later considered as the proximate active species [30]. Indeed, the reductive pathway which yields 4HAQO has been assumed to be essential for the carcinogenic mechanism of 4NQO [32, 33]. Several experiments have revealed that, in addition to sarcoma induction at the site of administration, 4HAQO exhibits clear organotropic and species specificity in its carcinogenesis [9, 13, 18, 31]. For example, with a single intravenous administration, 4HAQO selectively induces pancreatic tumours in rats [5, 7, 25, 29], while subcutaneous administration results in the development of pulmonary tumours in mice [17]. Konishi et al. [12] have reported pancreatic acinar cell damage in rats given a single injection of 20 mg/kg 4HAQO. It is generally assumed that covalent binding of ultimate carcinogens with DNA is of prime importance for tumour initiation [16] and it has been shown that 4HAQO reacts with DNA to yield guanine and adenine adducts, their structures identified to date are N^4-(guan-8-yl)-4-aminoquinoline 1-oxide, 3-(guan-N^2-yl)-4-aminoquinoline 1-oxide and 3-(aden-N^6-yl)-4-aminoquinoline 1-oxide [2, 35].

As an early morphological change, we have found that a single injection of 10 mg/kg 4HAQO induces segregation of nucleolar components in peripheral nerve cells [6] as does a single injection of 15 mg/kg 4NQO in pneumocytes of rats [4]. However, no report has hitherto been published on the comparative time course of appearance of nucleolar segregation in the target organs of 4HAQO-induced tumorigenicity. In order to obtain more information regarding nucleolar segregation as a possible biomarker for early toxic events caused by 4HAQO, an ultrastructural study of nucleolar alterations in target and non-target organs of rats intravenously injected with 4HAQO was conducted. A comparison with immunohistochemically demonstrated 4HAQO-DNA adduct formation was also included.

Materials and methods

Male 6-week-old Sprague-Dawley rats (Sankyo Laboratory, Tokyo, Japan), initially weighing approximately 160 g, were used in the experiment. They were housed, four per wire cage, in an air-conditioned room maintained at 23±2°C and 60±5% relative humidity under a daily cycle of alternating 12 h periods of light and darkness. The animals were given a commercial standard diet (CRF-1: Charles River, Tokyo) and tap water ad libitum.

After a 1 week acclimatization period, 24 rats were given a single injection of 4HAQO (Iwai Chemicals, Tokyo) dissolved in 0.005 M hydrochloric acid (Ha) into the saphenous vein at a dose of 20 mg/kg body weight in 0.3 ml of solution, and 4 rats each were killed under ether anaesthesia at 2, 4, 6, 8, 24 and 48 h after injection. Six control rats received a single intravenous injection of acidic aqueous solution alone, and two each were killed at 4, 24 and 48 h thereafter.

At autopsy, samples of the pancreas, adrenal and liver were immediately excised from each rat. The pancreas was investigated as a target organ for 4HAQO-toxicity or tumorigenicity, and the liver as a non-target organ. The adrenal was chosen as a possible target organ for 4HAQO-toxicity because we previously observed that adrenocortical peliosis appears at high incidence 20 weeks after injection of 4HAQO in rats (unpublished data). Small specimens of all tissues were fixed in phosphate-buffered 2.5% glutaraldehyde for 2 h at 4°C, post-fixed in phosphate-buffered 1.5% osmium tetroxide for 1 h, dehydrated in a series of alcohol concentrations and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under an electron microscope (JEM 1000CXS, JEOL, Tokyo). For counting the numbers of cells exhibiting nucleolar segregation, electron microphotopgraphs (× 8,000) were taken for samples of 30–60 randomly selected pancreatic acinar cells, adrenocortical cells and hepatocytes from each of the 4 animals per sub-group.

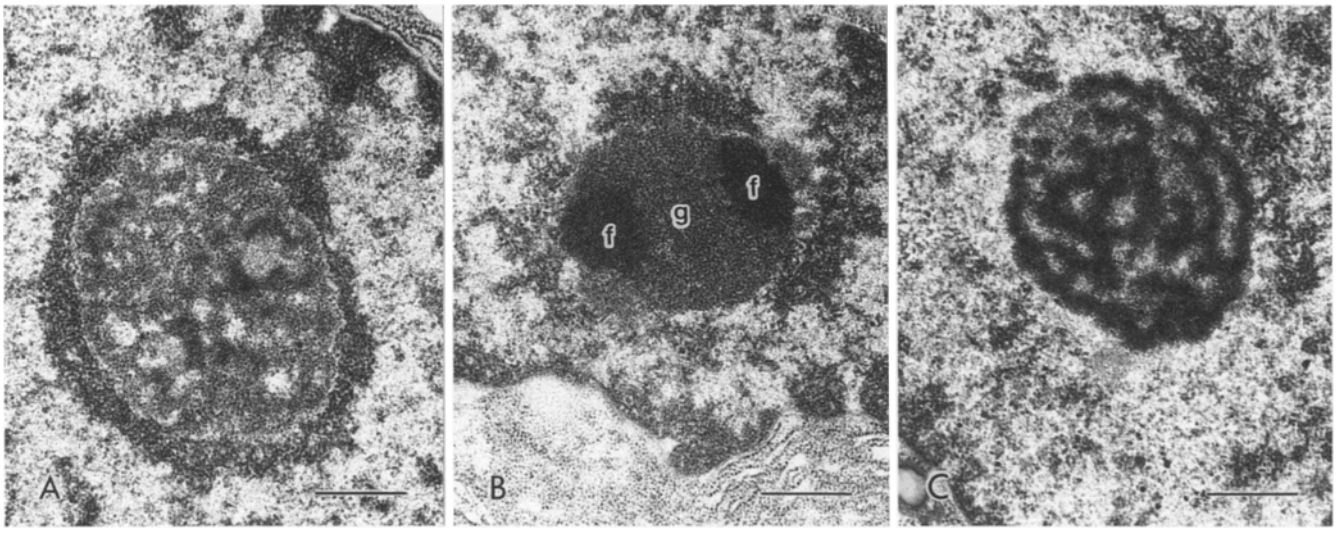
Tissue specimens were fixed in 10% buffered formalin solution for 24 h at room temperature and embedded in paraffin. Sections were cut at 4 μm and stained with haematoxylin and eosin, and immunohistochemical staining procedures were carried out according to the method described by Nakagawa et al. [20]. For all tissues, expression and localization of 4HAQO-DNA adduct was examined with polyclonal antibody against 4HAQO-DNA adduct using a labelled streptavidin biotin (LSAB) method. To avoid any cross-reaction with RNA, the sections were first treated with RNase A (200 μg/ml, pretreated at 70°C for 10 min to inactivate DNase) and RNase T_1 (50 U/ml) at 37°C for 1 h. The sections then were treated with 2 M HCl at room temperature for 5 h. Masking was conducted with 3% normal goat serum in phosphate-buffered saline containing 0.5% casein for 30 min. Incubation with the following primary antibody was performed at 4°C for 16 h and binding visualized by the labelled LSAB kit (Dako), with 0.05% 3,3′-diaminobenzidinetetrachloride (Dojindo Laboratories, Kumamoto, Japan) plus 0.01% hydrogen peroxide in TRIS-buffered saline containing 10 mM sodium azide. The sections were then washed in deionized water, dehydrated through graded alcohol and xylene, and mounted for microscopic examination. For counting the numbers of DNA adduct-positive cells, 200–300 cells were randomly selected from four areas in each section and examined under the light microscope at a magnification of × 400.

Results

Ultrastructural observations

Nuclei of normal cells in control rats without 4HAQO treatment generally contained a single, irregularly contoured, nucleolus consisting of electron-dense granules, fibrils and amorphous material. These components were observed to be intimately intermingled forming a meshwork of nucleolonemal strands (Figs. 1A, 2A, 3A). In contrast, as the most prominent ultrastructural change caused by administration of 4HAQO, the nucleoli were clearly segregated into separate granular and fibrillar zones in cells of the exocrine and endocrine pancreas (Fig. 1B) and adrenocortex (Fig. 2B) but not of the liver parenchyma and pancreatic ducts (Fig. 3B). The results of sequential investigation of the percentages of cells exhibiting nucleolar segregation in response to 4HAQO are summarized in Figure 6A. The nucleolar alteration was already evident 2 h after injection of 4HAQO in cells of the acinar pancreas and adrenocortex, the former show-

Fig. 1 **A** A pancreatic acinar cell nucleus from a control rat, showing an irregularly contoured nucleolus with a mesh-work of nucleolonema. **B** A pancreatic acinar cell nucleus from a rat 2 h after 4-hydroxyaminoquinolone 1-oxide (4HAQO) administration. The nucleolus is compact and smaller than normal and exhibits segregation into granular (*g*) and fibrillar (*f*) components. **C** A pancreatic acinar cell nucleus from a rat 48 h after 4HAQO administration, showing a nucleolus with thickened nucleolonema. *Bar* = 500 nm, ×30,000



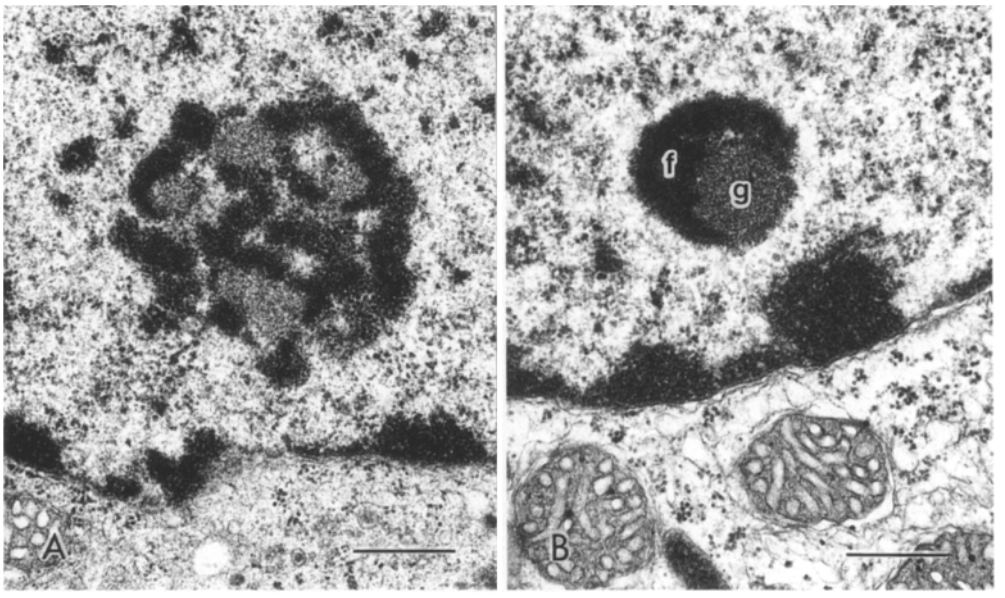


Fig. 2 **A** An adrenocortical cell nucleus from a control rat having a nucleolus with a nucleolonemal structure. **B** An adrenocortical cell nucleus from a rat 4 h after 4HAQO administration. The nucleolus exhibits segregation into granular (*g*) and fibrillar (*f*) components. *Bar* = 500 nm, ×30,000

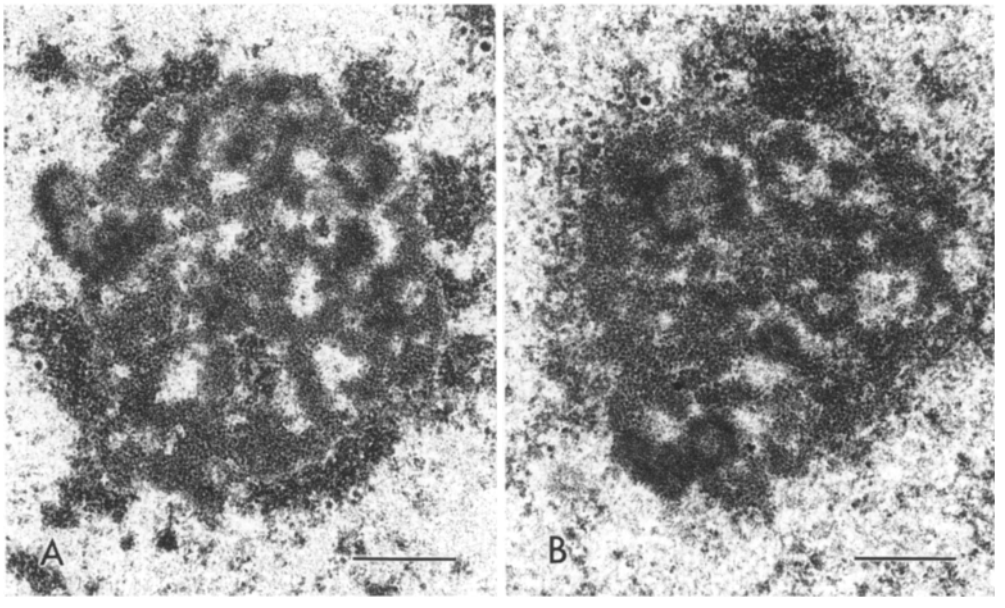


Fig. 3 Nuclei of hepatocytes from a control rat (**A**) and from a rat 6 h after 4HAQO administration (**B**), showing irregularly contoured nucleoli with meshwork nucleolonema. *Bar* = 500 nm, ×30,000

ing the highest frequency 6 h after the carcinogen injection and the latter at 4 h. The frequency of nucleolar segregation was much higher in pancreatic acinar cells than in adrenocortical cells at the 2–24 h time points. The highest percentages of cells with nucleolar segregation were 47% and 12% respectively, for pancreatic acinar and adrenocortical tissues. The nucleolar alteration was no longer evident in pancreatic acinar cells at 48 h, while adrenocortical cells continued to show a few nucleolar alterations even at 48 h. At 48 h, nucleoli of pancreatic acinar cells, but not adrenocortical cells, appeared enlarged and associated with a distinct rearrangement of nucleolonemal strands (Fig. 1C). 4HAQO treatment thus caused an increase and/or accumulation of interchromatin and perichromatin granules in pancreatic acinar cells. At later time points cytoplasmic organelles of cells with nucleolar segregation also appeared altered. In particular, the rough surfaced endoplasmic reticulum of pancreatic acinar cells became markedly dilated with depletion of membrane-bound and free ribosomes 8 h after injection of 4HAQO. At 24 h, the number of zymogen granules in the affected cells was markedly decreased and at 48 h, degenerative and apoptotic changes of acinar cells were evident in addition to necrosis. Starting at 2 h after injection of 4HAQO, acinar cells showing ultrastructural features of apoptosis characterized by condensation and margination of chromatin against nuclear membrane [26] increased consistently. Some of these changes represent a possible conglomeration of interchromatin particles suggesting karyorrhexis or apoptosis (Fig. 4). Such nucleolar and nuclear alterations were not seen in hepatocytes of rats at any time after 4HAQO treatment.

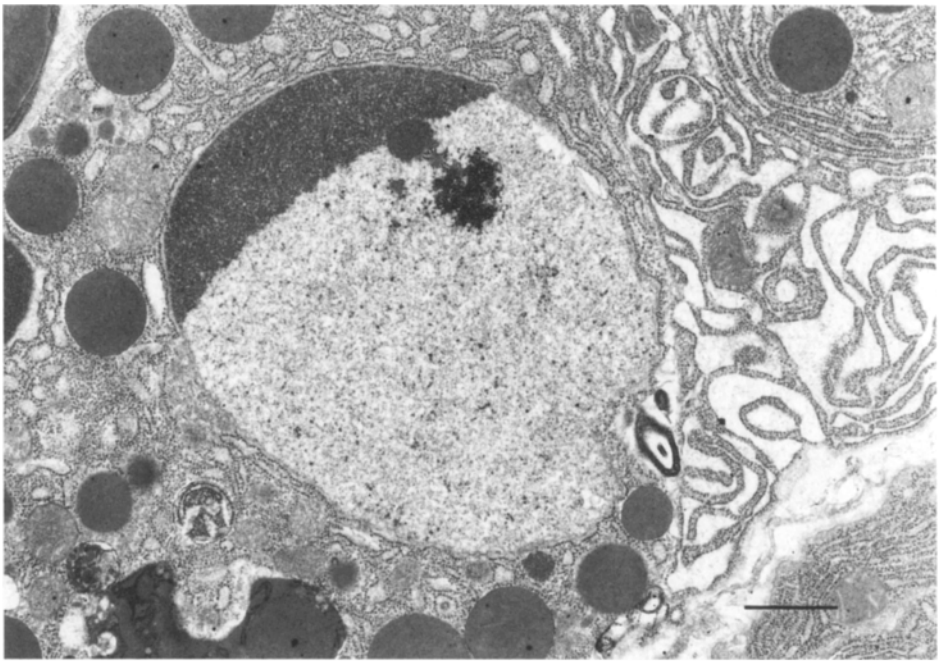


Fig. 4 Condensation of chromatin in a pancreatic acinar cell nucleus, indicative of the first stage of apoptosis, in a rat 8 h after 4HAQO administration. Note sharply segregated dense and lucent areas. *Bar* = 1 μm ×12,400

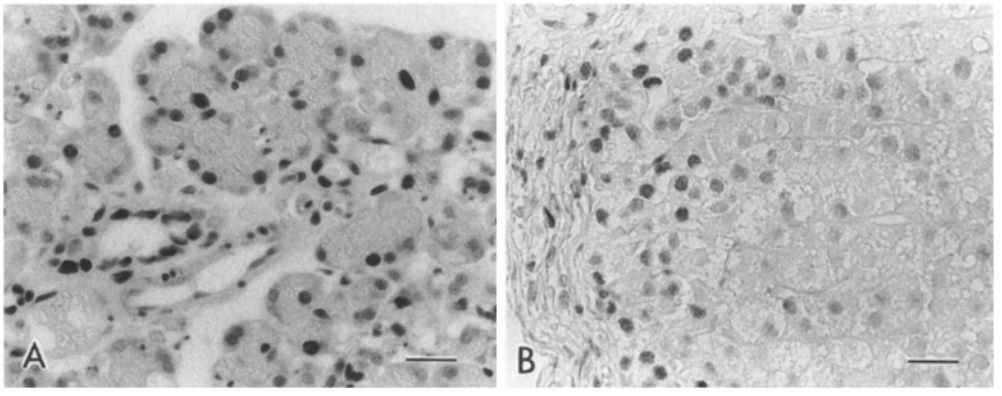


Fig. 5A,B Immunohistochemical staining of 4HAQO-DNA adducts. Demonstration of 4HAQO-DNA adducts in the pancreas in which acinar and ductal cells show positive reactivity (**A**) and adrenal gland in which cortical cells are positive (**B**) 8 h and 4 h, respectively, after 4HAQO administration. *Bar* = 20 μm, ×380

Immunohistochemical demonstration of DNA adducts

4HAQO-DNA adduct formation was evident in the nuclei of pancreatic acinar cells (Fig. 5A) and adrenocortical cells (Fig. 5B). In clear contrast, the non-target hepatocytes and cells from all tissues of control rats did not exhibit positive staining for DNA adducts. Thus, the target organ cells for 4HAQO-toxicity consistently showed both nucleolar segregation and DNA adduct formation. Serial observation revealed a gradual increase and then decrease in the intensity of immunohistochemical staining between 2 h and 24 h in DNA adduct-positive tissues, the highest intensity being observed at 4 h in both pancreatic acinar cells and adrenocortical cells (Fig. 6B). DNA adduct formation thus somewhat preceded nucleolar segregation in the exocrine pancreas. The staining pattern for 4HAQO-DNA adducts differed according to anatomical site and cell type. For example, in the pancreas of 4HAQO-treated rats, nuclei of acinar and ductal cells were clearly positive, whereas those of islet cells were much weaker. In the adrenal gland, nuclei of cells in the zona glomerulosa demonstrated more adduct formation than those in the zona fasciculata and zona reticularis.

Discussion

Our investigation of sequential ultrastructural changes between 2 h and 48 h after injection of 4HAQO to rats revealed nucleolar segregation of granular and fibrillar components as the most prominent alteration in the target pancreatic acinar and adrenocortical cells. This was followed by depletion of free ribosomes and marked dilation of endoplasmic reticulum in pancreatic acinar cells. Nucleolar alterations are known to occur in a variety of in vitro and in vivo cell systems following exposure to various compounds such as actinomycin D [28], 4-nitroquinoline 1-oxide [4], aflatoxin B1 [34], amanitin [11], proflavin [27] and adriamycin [8, 15]. These compounds possess in common the biochemical property of forming complexes with DNA either by nucleophilic substitution of nucleoside residues or by intercalation between adjacent base pairs, thereby interfering with DNA-

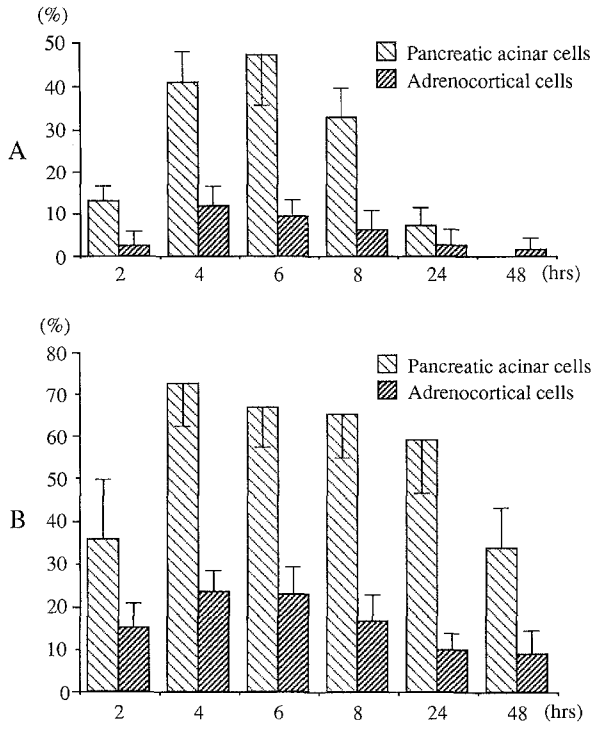


Fig. 6 Summary of sequential changes in nucleolar segregation (**A**) and 4HAQO-DNA adduct formation (**B**) for the exocrine pancreas and adrenocortex of rats treated with 4HAQO. Data represent mean ± standard deviation

dependent RNA-synthesis. In vivo interaction of 4HAQO with DNA was previously demonstrated by means of fluorescence spectroscopy [14] and autoradiography using tritium-labelled 4HAQO [1, 24]. Reynolds et al. [27] put forward the hypothesis that nucleolar segregation is an early morphological marker for a special type of cell injury involving DNA.

Previous studies have indicated the usefulness of carcinogen-DNA adduct-specific antibodies as tools to probe the relevance of DNA damage to carcinogenesis in vitro and in vivo [3, 10, 19, 20, 21, 23, 36]. The precise localization of 4HAQO-DNA adducts at the individual cell level found in the present experiments supports the specificity of the anti-4HAQO-DNA adduct antibody raised. Enzyme-linked immunosorbent assay [19] and immunohistochemical tests [21] have revealed a very high affinity of the antibody for 4HAQO-DNA adducts and no cross-reactivity with unmodified DNA or other carcinogen-modified DNA adducts. Thus, the immunohistochemical approach allows comparative morphological analysis as exemplified by the presently documented clear positive correlation between 4HAQO-DNA adduct formation and nucleolar alterations, consistently observed only in the target organs for 4HAQO-toxicity. The absence of staining for 4HAQO-DNA adducts and of remarkable nucleolar changes in hepatocytes, are in line with a non-target organ status. The reason for the essentially negative 4HAQO-DNA binding in the liver is enigmatic, since the liver is considered to be an active site for metabolism of this carcinogen [33]. However, it has been suggested that excessively high activities of hepatic enzymes reducing 4NQO to 4HAQO and further to 4-aminoquinoline 1-oxide, an inactive metabolite, may contribute to lack of DNA adduction of 4HAQO in the liver [32, 33]. Organ difference in repair of 4HAQO-DNA adduct formation could also play a role in negative 4HAQO-DNA binding in the liver as reported previously [1, 24]. Some differences in ultrastructural findings in adrenocortical cells from pancreatic acinar cells showing 4HAQO-tumorigenicity such as prolongation of nucleolar segregation may be related to the fact that 4HAQO caused adrenocortical peliosis as a toxic event but not tumorigenicity in the adrenal. Nucleolar segregation appears to be particularly indicative of toxic effects, but not directly of carcinogenic effects.

The fate of cells exhibiting nucleolar segregation remains to be investigated. In the present study, however, it appears that some of these cells readily undergo apoptosis although others may recover reversibly from such alterations, or progress to necrosis. The present study shows that 4HAQO induces DNA adduct formation and almost simultaneously (or a little later) brings about nucleolar segregation in the target organs for 4HAQO-toxicity in rats. Nucleolar segregation may find application as a very early biomarker for predicting cellular injury involving DNA damage.

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