Review

In Vitro and In Vivo Genotoxicity Induced by Fullerene (C\textsubscript{60}) and Kaolin

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Nanomaterials are being utilized for many kinds of industrial products, and the assessment of genotoxicity and safety of nanomaterials is therefore of concern. In the present study, we examined the genotoxic effects of fullerene (C\textsubscript{60}) and kaolin using in vitro and in vivo genotoxicity systems. Both nanomaterials significantly induced micronuclei and enhanced frequency of sister chromatid exchange (SCE) in cultured mammalian cells. When ICR mice were intratracheally instilled with these nanomaterials, DNA damage of the lungs increased significantly that of the vehicle control. Formation of DNA adducts in the lungs of mice exposed to nanomaterials were also analyzed by stable isotope dilution LC-MS/MS. 8-Oxodeoxyguanosine and other lipid peroxide related adducts were increased by 2- to 5-fold in the nanomaterial-exposed mice. Moreover, multiple (four consecutive doses of 0.2 mg per animal per week) instillations of C\textsubscript{60} or kaolin, increased gpt mutant frequencies in the lungs of gpt delta transgenic mice. As the result of mutation spectrum analysis, G:C to C:G transversions were commonly increased in the lungs of mice exposed to both nanomaterials. In addition, G:C to A:T was increased in kaolin-exposed mice. In immunohistochemical analysis, many regions of the lungs that stained positively for nitrotyrosine (NT) were observed in mice exposed to nanomaterials. From these observations, it is suggested that oxidative stress and inflammatory responses are probably involved in the genotoxicity induced by C\textsubscript{60} and kaolin.

Key words: nanomaterials, genotoxicity, fullerene (C\textsubscript{60}), kaolin, DNA adducts

Introduction

Recently, nanomaterials are being utilized for cosmetics and industrial products, and applications in medicine are under consideration. The assessment of genotoxicity and safety of nanomaterials is therefore of concern. One reason behind this is the asbestos crisis (1). Some nanomaterials are not only nano-sized particles, but also asbestos shape-like fibers, and the carcinogenic potential of such nanomaterials has attracted much attention over the years. Moreover, it is thought that nano-sized particles can be taken up in cells and cause intracellular damage (2,3). With this background, we here investigated induction of in vitro and in vivo genotoxicity using fullerene (C\textsubscript{60}) and kaolin as examples. To clarify the mechanisms of mutations due to these nanomaterials, we analyzed the formation of DNA adducts in the lungs of mice after exposure. Here, we briefly summarize our data and also discuss mechanisms of genotoxicity induced by nanomaterials.

Size Distribution in Suspensions of Nanomaterials

The size distribution of nanomaterials used in the present study was analyzed by dynamic light scattering (DLS) as described previously (4). The most abundant sizes were at 234.1 ± 48.9 and 856.5 ± 119.2 nm for C\textsubscript{60} and 357.6 ± 199.4 nm for kaolin, respectively.

In Vitro Genotoxicity Test

Micronucleus test: The micronucleus genotoxicity/clastogenicity test is widely used for assessment of environmental substances and medicinal chemicals. Here, we investigated the micronucleus inducing activity of C\textsubscript{60} and kaolin using human lung carcinoma A549...
In Vitro and In Vivo Genotoxicity Induced by Fullerene (C_{60}) and Kaolin

Cells (4). Six-hours treatment with 200 μg/mL kaolin caused growth inhibition of 60% whereas, C_{60} at the same concentration was without effect. C_{60} and kaolin particles both increased the number of micronucleated cells. The background frequency of micronucleated cells was 0.7% to 1.0%, and this rose to 10% and 5% with 200 μg/mL of C_{60} and kaolin, respectively, the increase being statistically significant in both cases. To investigate the effects of an anti-oxidative agent on the micronucleus induction, we conducted tests with or without N-acetyl cysteine (NAC) using Chinese hamster ovary CHO-AA8 cells. As shown in Fig. 1, the frequency of micronucleated cells was decreased significantly in the presence of NAC. With 20 μg/mL of C_{60} and kaolin for 6 h without NAC the results were 3.8% and 8%, respectively, but in the presence of 10 mM NAC these decreased to 1.7% and 2.3%. From this observation, oxidative stress might be involved in the genotoxicity induced by nanoparticles. Furthermore, it is known that photoexcited C_{60} produces reactive oxygen species (5) and in the present experiments, the cells and C_{60} were not shielded from visible light completely. Therefore, reactive oxygen species might contribute to micronucleus-induction in C_{60}-treated cells.

On the other hand, biologically relevant features of kaolin are unclear and further studies will be required to elucidate genotoxic mechanisms.

**Sister chromatid exchange (SCE) test:** SCE is also used for mutagenic testing of many products. While the mechanisms responsible for SCE are not completely understood, they involve breakage of both DNA strands, followed by exchange of whole DNA duplexes. This occurs during the S phase and is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication. To investigate SCE inducing activity of nanoparticles, we examined CHO-AA8 cells following 1 h treatment with C_{60} and kaolin (Fig. 2). The SCE frequencies in cells treated with 2.0 μg/mL of C_{60} and kaolin were approximately 11 and 7 times higher than the control level, respectively (P<0.01 at 0.1 μg/mL or higher concentrations). C_{60} demonstrated stronger genotoxic/clastogenic potency than kaolin. Cozzi et al. earlier reported that H_{2}O_{2}-treatment produced reactive oxygen species and induced SCE in CHO cells, and antioxidants, such as ascorbic acid and β-carotene, reduced the frequency (6). In the present study, the results of the micronucleus test indicated involvement of reactive oxygen species so that they might contribute to SCE induction as well.

**In Vivo Genotoxicity Test**

**Comet assay:** The comet assay is known as a standard simple and sensitive technique for evaluation of
DNA damage. The types of damage usually detected are single and double strand breaks. The pH (usually between neutral and alkaline pH) of the lysis condition can be adjusted depending upon the type of damage. Under alkaline conditions, AP sites and others where excision repair takes place are detected as DNA damage. We here evaluated DNA damage induced by particles using the comet assay under alkaline conditions. The values for DNA tail moment in the lungs with single-particle treatment at 0.2 mg/body for 3 h were measured, and DNA damage was significantly increased, around 2-fold, as compared with the vehicle control, and its intensity was C60 > kaolin. When we examined the effects of oxidation of purines, DNA damage was analyzed by formamidopyrimidin-glycosylase (FPG)-modified comet assay. DNA damage induced by kaolin was not changed, whereas DNA damage caused by C60 was elevated up to 1.7 fold compared with the vehicle control (Fig. 3). In addition, Jacobsen et al. also reported that C60 significantly increased the level of FPG sensitive sites/oxidized purines determined by the comet assay using the E1-Mutatrade markMouse lung epithelial cell line (7). From these findings, it seems that oxidative damage would be partly involved in the induction of DNA damage by C60, although other changes responsible for DNA damage might be induced by kaolin.

**Oxidative and lipid peroxide related DNA adduct formation:** DNA adducts, formed by reactions with exogenous or endogenous agents, are known to induce gene mutations. Reactive oxygen species (ROS) are one type of endogenous agent that can produce oxidative DNA adducts such as 8-oxo-2'-deoxyguanosine (8-oxodG), a widely recognized and utilized biomarker of oxidative stress, and a major mutagenic lesion producing predominately G to T transversion mutations (8). In addition, ROS generate lipid hydroperoxides to yield heptanone-etheno (He)–adducts, such as HeC and HeA and HeC via 4-oxo-2-nonenal (4-ONE) (9). These adducts can lead to mutations, if not repaired. We examined whether these oxidative and lipid peroxide related DNA adducts were induced in the lungs of mice by intratracheally instilled nanoparticles. 8-OxodG and three kinds of He-adducts were analyzed in the lungs of ICR mice 3, 24, 72 and 168 h after intratracheal instillation of 0.2 mg/body of C60 or kaolin, and quantified by the stable isotope dilution LC-MS/MS method described by Chou et al. (10). Compared with a vehicle control, DNA adduct levels were increased by about 2- to 5-fold in the lungs of mice 24 h after injection of nanoparticles (Fig. 4). The increases were time dependent until 72 h then gradually decreased within 168 h of injection (data not shown). Related to this, oxidative DNA damage was induced by intratracheal instillation of C60 or kaolin in the comet assay with FPG treatment, as described above. In addition, Folkmann et al. reported that oral gavage of C60 increased the levels of 8-oxodG in the liver and the lungs of F344 rats (11). Moreover, Tsurudome et al. described increased 8-oxodG levels induced by intratracheally instilled diesel exhaust particles in the lungs of F344 rats, and 8-oxoguanine DNA glycosylase 1 (OGG1) mRNA was also overexpressed (12). The decreased DNA adducts in the present study at 168 h may have been a result of a repair enzyme such as OGG1. This is the first observation that He-lipid peroxide related DNA adducts are increased by nanoparticles. Such adducts could clearly contribute to nanomaterial-induced DNA damage and mutation. Our findings suggest involvement of ROS generation, although differences between C60 and kaolin still require clarification.

**gpt Mutations in the lungs of gpt transgenic mice:** Transgenic gpt delta mice are a useful model system for detecting both point mutations and large deletions (<10 kb) (13). λE1G10 transgenes carrying gpt (detection of point mutations) and red, gam (detection of deletion) genes have been integrated into mouse chromosome 17, and point mutations and deletions observed in any tissues can be detected as 6-thioguanine (6-TG) resistant colonies and Spi plaque, respectively. To examine in vivo mutagenicity of nanoparticles, gpt delta transgenic mice were exposed to C60 and kaolin at four different doses by intratracheal instillation, and gpt mutations were analyzed. The background gpt mutant frequency (MF) in lungs was 10.3 ± 0.53 × 10⁻⁶. MFs were significantly increased by 2 to 3-fold to 30.75 ± 3.32 × 10⁻⁶ (p = 0.019) for C60 and 19.30 ± 4.82 × 10⁻⁶ (p = 0.002) for kaolin (4).

Moreover, we examined the mutational characteris-
Fig. 4. Oxidative and lipid peroxide related DNA adduct formation in the lungs of ICR mice induced by nanoparticle exposure. DNA was extracted from lungs of mice 24 h after intratracheal instillation of 0.2 mg/body of C60 or kaolin, and digested enzymatically. Control animals were exposed to saline containing 0.05% Tween80. The 8-oxodG and 3 kinds of H2-adducts were quantified by the stable isotope dilution LC-MS/MS method described by Chou et al. (10).

Fig. 5. Classification of gpt mutations from the lungs of control and nanoparticle treated mice.

In Vitro and In Vivo Genotoxicity Induced by Fullerene (C60) and Kaolin

tics induced by particles by PCR and DNA sequencing analysis of 6-TG resistant mutants. Classes of mutations found in the gpt gene are shown in Fig. 5. Interestingly, G:C to C:G transversions were increased in common with both particle treatments. Since these mutations were commonly increased regardless of the constituents (i.e., C60 is graphite and kaolin is aluminum silicate), the mechanisms might be the same. It has been reported that various oxidative stresses caused by sunlight, UV radiation, hydrogen peroxide and peroxy radicals frequently induce G:C to C:G transversions in various in vitro assay systems (14–17). Moreover, a variety of ox-
idative lesion products of guanine other than 8-oxodG, including imidazolone (Iz), oxazolone (Oz), spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh), have been reported (18–24). Three such molecules, Oz, Sp and Gh are now thought to be key causes G to C transversions with translesion synthesis systems (22–25). Therefore, it is suggested that G:C to C:G transversions induced by C60 and kaolin could involve Oz, Sp and Gh formation. In addition, G:C to A:T transitions were also significantly increased by instillation of kaolin but not C60. In general, G to A (or C to T) transitions have commonly been observed in spontaneous and chemically-induced mutants, and deamination of guanine or 5-methylcytosine might be involved. Burney et al. reported that nitric oxide induces DNA damage. NO can form N2O3, and direct by this agent can lead to DNA deamination via diazonium ion formation (26). Moreover, nitric oxide is produced by activated macrophages in inflamed organs. In fact, test substance-phagocytized macrophages and granulomas were frequently observed in the lungs of mice (4).

Immunohistochemical Analysis of Inflammation Factors

In order to confirm enhancement of nitric oxide production by C60 and kaolin, we examined immunohistochemical staining of an inflammation factor, nitrotyrosine (NT), in the lungs of gpt delta mice treated with these nanoparticles using the same procedure reported previously (27) with minor modification. As shown in Fig. 6, the pattern of NT staining corresponded to the areas of inflammation within lung parenchyma. In the case of C60 exposure, many regions of the lungs stained positively (data not shown), and intense NT staining was localized in test substance-phagocytized macrophages and granulomas. Similarly, staining with NT antibodies was observed in macrophages and alveolar epithelial cells in the lungs of mice exposed to kaolin, although to a lesser extent as compared with C60.

Conclusion

Our results clearly demonstrated that both in vitro and in vivo genotoxicity are induced by C60 and kaolin. However, the mechanisms have yet to be fully clarified, and oxidative stress might be at least partly involved. There are a number of ways in which reactive oxygen species (ROS) could be generated: i) nanoparticles might trigger ROS production by iron-catalysed Fenton reactions; ii) nanoparticles could accumulate in cells due to phagocytosis, then enhance the production of ROS by NADPH oxidase (28,29). Recently, innate immune activation through Nalp3 inflammasomes has been suggested to play an important role in pulmonary fibrotic disorders of silicosis and asbestosis (30,31). It has been reported that proinflammatory cytokines, such as interleukin 1β are key molecules for pneumoconiosis. At

Fig. 6. Immunohistochemical localization of nitrotyrosine (NT). Since C60 is brown in color, we used an SG substrate kit (Vector Laboratories, USA) for peroxidase, with positive cells stained dark blue-gray. A: alveolar region in a control mouse, with no significant staining for NT. B: alveolar region in a mouse exposed to C60, with positive macrophages phagocytizing test substance and epithelial cells. The brown colored material is C60. C: alveolar region in a mouse exposed to kaolin. Note intense staining for NT in the granulomatous region.
present, no data are available for activation of the Nalp3 inflammasome pathway by C60 and kaolin. However, it is likely that both nanoparticles can activate in the same way as asbestos and silica, because oxidative stress was increased in the lungs of treated mice. Further studies of the mechanisms of genotoxicity are needed.

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