Increased 8-Hydroxyguanine in DNA and Its Repair Activity in Hamster and Rat Lung after Intratracheal Instillation of Crocidolite Asbestos

Raizo Yamaguchi, Takeshi Hirano, Yuko Ootsuyama, Shinya Asami, Yosuke Tsurudome, Shoko Fukada, Hiroshi Yamato, Toru Tsuda, Isamu Tanaka and Hiroshi Kasai

Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyusyu 807-8555

Asbestos and man-made-mineral fibers are known to increase one type of oxidative DNA damage, 8-hydroxyguanine (8-OH-Gua), in vitro. In this study, we analyzed the 8-OH-Gua level in DNA and its repair activity after a single intratracheal instillation of fibers (crocidolite or glass) or saline to Syrian hamsters or Wistar rats. The 8-OH-Gua level was measured with a high-performance liquid chromatography-electrochemical detector (HPLC-ECD) system. The 8-OH-Gua repair enzyme activity was determined with an endonuclease nicking assay using a 32P-labeled or fluorescently labeled 22mer DNA that contains 8-OH-Gua at a specific position. A significant increase in the 8-OH-Gua level in the lung DNA was observed 1 day after the exposure to crocidolite, as compared to the saline control. The repair activity was increased significantly at 7 days. On the other hand, after exposure to glass fibers, little or no increase of these carcinogenicity indicators was detected. These assays of 8-OH-Gua and its repair activity in short-term animal experiments will be useful for evaluating the carcinogenicity of fibers. This is the first report of the increase of 8-OH-Gua and its repair activity in the animal lung after the instillation of asbestos fibers.

Key words: 8-Hydroxyguanine — DNA repair — Endonuclease nicking assay — Asbestos — Carcinogenicity

It has been suggested that asbestos and man-made-mineral fibers with a specific diameter and length are associated with the induction of pulmonary fibrosis and lung cancer. The mechanism of the carcinogenic process is not clear, but reactive oxygen species produced during phagocytosis may play an important role.1–3)

One of the major types of oxidative DNA damage is 8-OH-Gua.4) The formation of 8-OH-Gua in nuclear DNA may play an important role in carcinogenesis, because it is known to cause GC→TA transversions.5) Many researchers have reported that 8-OH-Gua is a useful marker of oxidative DNA damage. However, using previous methods of DNA isolation for measurements of 8-OH-Gua, the background levels of 8-OH-Gua were rather high, and the deviations of the data even in the same experimental group were also high. Recently, the background levels of 8-OH-Gua were found to be reduced by using the NaI desferal method,6) and more reliable data on the 8-OH-Gua levels in cellular DNA can now be obtained. In addition, it has been reported that the 8-OHGua repair activity in mammalian systems is induced after cells are exposed to oxidative stress by ionizing radiation.7) We reported that the induction of the 8-OH-Gua repair activity could be another biological marker of cellular oxidative stress, and in addition to analyzing 8-OH-Gua, more reliable data for evaluating the carcinogenicity can be obtained.8)

Recently, many kinds of man-made-mineral fibers have been used instead of asbestos. However, there is no reliable short-term test to assess their carcinogenicity. We studied whether we could evaluate the carcinogenicity of the fibers in vivo by monitoring 8-OH-Gua in DNA and its repair activity in the lung after a single intratracheal instillation of either crocidolite or glass fibers to hamsters and rats.

MATERIALS AND METHODS

Materials The crocidolite was an International Union Against Cancer (UICC) reference sample. The glass fibers were obtained from a binderless glass fiber filter (GB100R Advantec Tokyo Co., Tokyo). It was previously reported that no tumors, pneumoconiotic nodules, or interstitial fibrosis were found in any of the rats exposed to these glass fibers by chronic inhalation for 1 year.9)

Fluorescently labeled DNA containing 8-OH-Gua was prepared using Fluoro Prime Fluorescein Amidite (Phar-
macia) by Japan Bio Services Co., Ltd. (Saitama). Deprotection was carried out using concentrated ammonia containing 0.1 M mercaptoethanol.

**Animal treatment**

**Time course study (hamster and rat):** Nine-week-old male Syrian hamsters or 9-week-old male Wistar rats (n=6 for each data point) were killed under ether anesthesia at 1, 3, 5, 7, and 9 days after a single intratracheal instillation of saline or fibers (2 mg of crocidolite or glass fibers suspended in 0.2 ml of saline). The lungs were immediately removed and then used for the repair experiments. A portion of the lung was frozen and kept at −80°C for the 8-OH-Gua analysis.

**Dose dependency study (rat):** One day after a single intratracheal instillation of saline or fibers (0.5, 2, or 10 mg of crocidolite asbestos, or 2 mg of glass fibers, suspended in 0.2 ml saline), the rats were killed and the lungs were isolated for the repair assay and the analysis of 8-OH-Gua.

**Determination of 8-OH-Gua in DNA** The method for the determination of the 8-OH-Gua level in the DNA was described in detail by Yamaguchi et al. Briefly, the tissues were homogenized in lysis buffer and the DNA was extracted with a commercial kit (WB kit, Wako Biochemicals, Osaka). The extracted DNA was enzymatically digested and injected into an HPLC column on an HPLC apparatus equipped with an ECD (ESA Coulochem II, Chelmsford, MA). Twenty microgram aliquots of dG (0.5 mg/ml) and 8-OH-dG (5 ng/ml) solutions were injected as standard samples. The value of 8-OH-Gua was calculated as the number of residues per 10⁵ Gua. Control levels (mean±SD) of 8-OH-Gua in hamster and rat lung DNA were 0.143±0.020 and 0.163±0.010, respectively. In Figs. 1 and 2, all the data were expressed as the ratio (%) to these control levels.

**Measurement of 8-OH-Gua repair activity** The method of measuring the 8-OH-Gua repair activity was described in detail by Yamaguchi et al. Briefly, the tissues were homogenized with Tris-HCl buffer containing protease inhibitors. The homogenates were centrifuged to obtain the crude extracts, which were kept at −80°C until use.

**Fig. 1. Changes in the 8-OH-Gua levels in hamster lung DNA after the instillation of fibers (2 mg).** These are expressed as the ratio (%) to the average of those after the instillation of saline (control). Values are mean±SD; n=6. *P<0.01 vs saline as evaluated by applying the Wilcoxon rank sum test (using the exact table). □ saline, ■ glass fibers, ■ crocidolite.

**Fig. 2. Dose-dependent increase of the 8-OH-Gua levels in rat lung DNA 1 day after the instillation of fibers.** Values are mean±SD; n=6. *P<0.01 vs saline as evaluated by applying the Wilcoxon rank sum test (using the exact table). For comparison, data from hamster experiments (2 mg fibers, after 1 day) are also shown.
The crude extracts were incubated with the $^{32}$P-end-labeled (hamster experiments) ds-DNA substrate. After two ethanol precipitations, the pellets were dissolved in loading buffer and denatured. The excised fragment, generated as the consequence of base excision repair activity, was analyzed by 20% denaturing polyacrylamide gel electrophoresis. For comparison, a hot piperidine-treated oligonucleotide was also analyzed as a fragment marker. After electrophoresis, the autoradiograms were processed and the radioactivity was analyzed using a Bioimage analyzer system. The repair activity was calculated as the ratio (%) of the excised fragment activity to the total activity (substrate activity plus fragment activity). In Figs. 3 and 4, all the data were expressed as the ratio (%) to control values.

In the rat experiments, fluorescently labeled DNA with the same sequence, instead of $^{32}$P-labeled DNA, was used. After incubation, the cleaved fragment was detected by a Pharmacia ALF DNA sequencer (Fragment Manager, Ver. 1.1; Pharmacia).

**RESULTS**

**Hamster experiments** The 8-OH-Gua level in the DNA and its repair activity after the fiber instillation are expressed as the ratio (%) to those after the saline instillation (control). A significant increase in the 8-OH-Gua level in the lung DNA was observed at 1 day after the exposure to crocidolite, as compared to saline ($P<0.01$) (Fig. 1). Thereafter, the 8-OH-Gua level in the lung DNA decreased gradually and reached that of the saline treatment at 9 days. The repair activity increased significantly at 7 days ($P<0.05$) and decreased to that of the saline treatment at 9 days (Fig. 3). On the other hand, after exposure to the glass fibers, the increase in the level of 8-
OH-Gua in the lung DNA was not its repair activity also did not significantly change.

**Rat experiments** One day after the intratracheal instillation of various amounts of crocidolite asbestos to rats, a dose-dependent increase of 8-OH-Gua was observed in the lung DNA, whereas the increase was not significant in the lungs of the glass fiber (2 mg)-treated rats (Fig. 2). Seven days after the single instillation of 2 mg of crocidolite asbestos, the repair activity for 8-OH-Gua was increased to 2 times that of the control, while that of the glass fiber treated rats was increased to 1.3 times (Fig. 4).

**DISCUSSION**

Asbestos is a well-known causative agent for lung cancer and mesothelioma. However, its mechanisms of action are unknown. Two hypotheses have been proposed: First, the specific size and shape of the fibers increase the carcinogenicity, but it is difficult to explain the carcinogenicity of asbestos fibers only in terms of this hypothesis. Second, reactive oxygen species play an important role in asbestos-related DNA damage and carcinogenicity. Hydroxyl radicals, generated by the reaction of iron in the fibers and H$_2$O$_2$, form in the injured cells, especially in the lungs of the glass fiber (2 mg)-treated rats (Fig. 2). The mechanism of the time lag is still unclear. However, we observed the similar phenomenon that the repair activity arose on the 5th day, while 8-OH-Gua formation was detected on the 1st day in the crocidolite-treated hamsters. The mechanism of the time lag is due to the recognition for 8-OH-Gua by repair mechanisms. These mechanisms should be investigated.

In this study, we examined whether we could evaluate the carcinogenicity of the fibers in vivo, by measuring 8-OH-Gua in DNA and its repair activity. Two types of fibers were used. Crocidolite, which is the most potent carcinogenic type of asbestos fibers, was used as a positive control of carcinogenicity. The other was glass fibers, which were previously reported to produce no tumors, pneumoniaconlue nodules, or interstitial fibrosis in any rats exposed to chronic inhalation of these fibers for 1 year, as a negative control of carcinogenicity.

In conclusion, crocidolite asbestos, as a positive control of carcinogenicity, significantly increased both the 8-OH-Gua level in DNA and its repair activity in hamster and rat lung. Glass fibers, as a negative control of carcinogenicity, induced little or no increase in these parameter. These assays of 8-OH-Gua and its repair activity in short-term (9 days) animal experiments will be useful for evaluating the carcinogenicity of fibers. In addition, we observed that the repair activity appeared on the 7th day while 8-OH-Gua formation was detected on the 1st day in the crocidolite-treated hamsters. The mechanism of the time lag is still unclear. However, we observed the similar phenomenon that the repair activity arose on the 5th day, while 8-OH-Gua formation appeared on the 1st day in the DNA of ferric nitritoliatecetate-treated rat kidney. Based on these observations, we speculate that the time lag is due to the recognition for 8-OH-Gua by repair mechanisms. These mechanisms should be investigated.

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