Letter to the Editor

Dear Editor,

My colleagues and I have several concerns with regard to a recent article published in the journal: "Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube" by A. Takagi *et al.* 33(1):105-116, 2008. While we strongly support the efforts to identify and characterize potential health effects of nanoparticles on workers and consumers as well as evaluation of possible environmental impact, we believe that inherent flaws in the study prevent meaningful hazard identification and risk assessment.

First, little information to support risk assessment is gained by using highly unrealistic exposure doses. The authors of this study intraperitoneally instilled 3mg of MWCNT into the abdomen of a mouse. MWCNT are low density materials. Therefore, 3mg represents an inordinately large volume of material. Such exposure is beyond the realm of reality. Therefore, artifactual responses are likely.

Secondly, the authors argue that fiber counts for asbestos exposure were an order of magnitude greater than that for the MWCNT exposure. The authors are correct in stating that fiber number is the most appropriate dose metric to use in evaluating the toxicity of fibers. However, the number of fibers per mass for the MWCNT was vastly underestimated. By the authors' own admission, the MWCNT sample was highly agglomerated with structures as large as 50-200 micrometers in diameter (Figs. 2c and 2d). They counted particles not individual nanotubes. A 50 micrometer structure could contain thousands of individual nanotubes. In contrast, asbestos was well dispersed into individual fibers (Fig. 3a). Therefore, exposure dose expressed as fiber number could be orders of magnitude higher for MWCNT than asbestos in this study.

Thirdly, histological evidence shows "typical epithelial mesotheliomas" with "large tumors" after exposure to asbestos (Fig. 7). In contrast, MWCNT cause "peritoneal adhesion and fibrous thickening" "due to the formation of fibrous scars" with an associated "spectrum of peritoneal mesothelial lesions" (Fig. 6). According to the text, it seems that this spectrum of lesions included typical mesotheliomas, but the authors did not present any photomicrographs demonstrating the presence of such mesotheliomas following exposure to MWCNT. As a consequence, the paper did not show any histological evidence that typical mesotheliomas were associated with MWCNT.

Fourthly, gross inspection of abdominal structures in the MWCNT-exposed mice showed "moderate to severe fibrous adhesions." The authors report that a "major cause of death was construction ileus due to severe peritoneal

adhesion." Therefore, the higher death rate in the MWCNT vs. asbestos group may have been simply strangulation of the intestine; not mesothelioma.

Finally, poor material characterization in the study as presented severely compromises interpretation of the results, or comparison with other similar studies. SEM photographs used to determine the size distribution in Fig. 1 should be shown, in addition to further information on how the material was prepared for the size assessment presented. Further details are also needed on the degree to which this preparation technique might have altered the dispersion and characteristics of the fibers; especially whether heating samples to such a high temperature of 480°C might have altered their characteristics. The optical micrographs presented in Fig. 2 are of insufficient resolution to determine the physical nature of the MWCNT. At a minimum, we suggest toxicity studies using nanomaterials should include electron micrographs (TEM and SEM) of these materials that allow their shape, form and aggregation state to be assessed. These should ideally be augmented with additional physicochemical information on the material before and after administration. This is essential where small differences in nanotube construction, diameter, length, aggregation state and levels of impurities such as amorphous carbon and graphitic carbon, might lead to profound differences in toxicity.

In summary, evaluation of the potential biological impact of nanomaterials such as MWCNT is essential, if they are to be developed and used safely. However, exposure doses must be realistic for the results to be relevant to issues of occupational or environmental health, and sample characterization needs to reflect the complexity and nanoscale structure of the material being studied. We believe the current study is flawed on both accounts.

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Response to Letter to the Editor

Dear Editor,

With regards to the letter from Dr. Gaku Ichihara *et al.* about our recent publication in Journal of Toxicological Sciences (33, 105-116, 2008), our responses to the letter are as follows:

1.

This study is designed to identify hazard characteristics of the MWCNT, of which mechanism is shared with asbestos in terms of mesothelioma induction. Whether MWCNT reaches the pleural mesothelium when inhaled is beyond the scope of our article and, therefore should be tested elsewhere.

Our study covers the highest end of dosage used in the past including those performed before guidelines are made. Again, the aim of our study is to identify the hazard whether MWCNT could induce mesothelioma or not and provide information for further dose-response study if tumor was induced. In such a first and limited animal numbers study, false-negative results should be avoided. Therefore, we think that the selection of high dose in our study is rational.

Among CNT researchers, there seems a consensus that the specific gravity of nanotubes is close to graphite (around 2.2). The assumption is that the specific gravity of MWCNT is around 2.2. The specific gravity of crocidolite is reported to be around 3.2. Therefore, the amount of MWCNT is not far fetched from the asbestos given in this study.

The argument of maximum tolerated dose does not apply to the hazard identification study. From the clinical observation of mesothelioma patients in Japan, maximum tolerated dose would not be the proper parameter for this type of exposure that the clearance of the responsible particle is very slow, but cumulative dose is the important factor for further risk assessment. And yet, there are two issues to be mentioned in relation to this claim. Firstly, in Japan, there are, unfortunately, a group of workers exposed to a very high cumulative dose of asbestos for considerably long time so that severe pleural thickening and respiratory disturbance are the first clinical symp-

toms. Secondly, mesotheliomagenesis by asbestos does not seem to have threshold. Indeed, 75% of new mesothelioma patients have no pleural anomaly by CT/MRI or X-ray, and recent study by the Ministry of Environment of Japan showed that 40% of mesothelioma patients cannot identify where and when they were exposed to asbestos. We consider that it is very important to identify hazard of MWCNT of which mechanism is very likely identical to asbestos before widespread exposure takes place in the work place and the market place.

We are aware that the dosage was at high end range in this study. Therefore, we immediately started a follow up dose-response study with MWCNT dosage 10 times (high-dose group, 300µg/animal), 100 times (medium-dose group, 30µg/animal), and 1000 times (low-dose group, 3µg/animal) lower than this study. More than 350 days have already passed, and macroscopic findings of the moribund animals preliminarily indicate that mesotheliomas were induced in all dosage groups, including the low-dose group where no severe peritoneal adhesion was observed.

2.

Again, the specific gravity of MWCNT is close enough to asbestos. Therefore, it is unlikely that the numbers of fibers are greatly different between MWCNT and asbestos. Moreover, as shown in the Materials and Methods, MWCNT was fully dispersed onto the mesh before the width/length measurement under electron microscope. And the weight of the MWCNT on the mesh was measured. As a result, the fiber number per mg MWCNT is not biased as the letter indicates. The total number of fiber applied to a mouse includes both dispersed and aggregated MWCNT. In this respect, considerable portion of MWCNT was embedded in the granulation and scars as a form of aggregate, so that the effective freely-dispersed MWCNT fibers might have been smaller in number than asbestos.

It is important to use biologically low-toxic medium to apply test chemicals to animals in any toxicology study. The medium we used for dispersing MWCNT has limited ability in terms of dispersing all MWCNT into indi-

vidual fibers. Even though, as shown in Fig. 1a, there are considerable portion of dispersed fibers in the suspension. More over, from the surface of the clump, fibers are falling off as shown in Fig. 1d. And it is also highly possible that the particulate matters in the abdominal cavity would unevenly distribute because of the folds and dips of the complicated shape of the serosal surface, and aggregates of considerable size would form. And above all, as shown macroscopically in Fig. 5 and microscopically in Fig. 6, the major aggregates or clumps of both asbestos and MWCNT in the abdominal cavity are embedded deep in fibrous scars (Pigmented portions in Figs. 5a and b) and segregated from the mesotheliomatous lesions (Clumps of fibers are not seen in close proximity to the mesothelial lesions in Fig. 6). Histopathology supports our assumption that major portion of clumps and aggregates are segregated from mesothelium by fibrous scar and granulation. And the asbestos and MWCNTs that were effective for induction of mesotheliomas were the free, well dispersed portion of the applied suspension. In other words, there still were wide surface of peritoneum left to encounter with dispersed fibers and frustrated phagocytosis to happen in our study. In other words, fibers that are dispersed onto the surface of mesothelium are a portion of administered asbestos or MWCNT so that the effective fiber numbers are smaller than the total.

3.

We were more than happy to post more photographs of the lesions from both groups if the space allowed. However, it is a common sense among toxicologist and pathologist that presentation of representative lesion is sufficient for conveying the result. We believe that the photographs in this paper are necessary and sufficient for the average readers. Fig. 6 shows a typical early mesothelial lesion induced by MWCNT, and we consider that this figure conveys sufficient evidence that MWCNT induced a spectrum of mesothelioma lesions including typical ones.

Nonetheless, we are happy to present other representative photographs from MWCNT treated mice as below.

4.

The “context of the tumor” is the important information for the analysis of the effect of treatment over control. In essence, “lethal” tumors and “incidentally found” tumors should be separately monitored, statistically analyzed and, then combined in the end to reach a conclusion whether the treatment had enhanced the yield of tumor. In this study, the context of the tumor was carefully assessed. Every case accompanies peritoneal fibrosis. And yet, it is possible to classify, for example small mesotheliomas with severe fibrosis with a sign of ileus-derived emaciation was classified as incidental tumor, and a large mesothelioma with hemorrhagic ascites and constriction of the intestine by the tumor as lethal even there is fibrosis. The judgment was not difficult as a whole. But if such case existed, we put it into incidental context. Therefore, lethal tumor yield is not exaggerated by peritoneal adhesion. And for those who are suspicious about these “context” criteria, we also showed total yield of tumor over effective animal number. In both ways of expression, the result of the study is the same, i.e. MWCNT was as potent

as crocidolite.

5.

The 480 degree heating process of the MWCNT was applied only for electron microscope (EM) samples, in order to measure length, width and fiber number. This process was essential for removing TX-100, which was used only for EM analysis. The heating process causes no significant influences as for measuring length, width and fiber number. As mentioned above, we gave preference to the usage of biologically low-toxic medium for injection to animals over the stronger detergents used for EM analysis.

An important aspect of hazard identification of an identifiable product under mass production is to promptly inform the toxicity of the product “as is” to the producer and users, simply because it may be readily used “as is”. Whether the precise effect of the MWCNT on mesotheliomagenesis is augmented or attenuated by the small differences in nanotube construction and other factors are the second issues to be assessed. What is understood from the beginning of this study is that, as the light microscopy shows and the EM based measurement shows that this MWCNT consists of particles of various length and width, i.e. it is a mixture. And there have been a series of carcinogenesis studies on fibrous materials in the past saying that particles of a certain shape and length are mesotheliomagenic. We consider that this study contains sufficient information on the biological effects of the sample MWCNT “as is”, and that it includes at least asbestos-type toxicity. The sample MWCNT is retained so that further physical nature can be explored. However, pursuing such information is beyond the scope of this study.

In summary, this study is not assessing the biological effects of nano level particle, but is assessing the micrometer-sized rod or fiber shaped particles that are found in the sample MWCNT “as is”. The detection system used for this study is sensitive to mesotheliomagenesis. The specificity can be checked by histopathological observation of the induced tumor types. Hazard of the nano-sized particles in this MWCNT sample is not assessed in this study at all and, hence remains totally unknown. Further studies are essential for identification of such toxicity. The type of study for that purpose should be different from the method used in this study.

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