

Fig. 5. Representative histology of an advanced stage mesotheliomas observed in rats treated with MWCNT. (a) Mesothelioma cells invaded and destroyed the smooth muscle layer of adjacent organs/tissues (in this case, the diaphragm). Under high magnification, the tumor consisted of (b) mesothelioid cells in the surface and (c) spindle-shaped sarcomatous cells in depth.

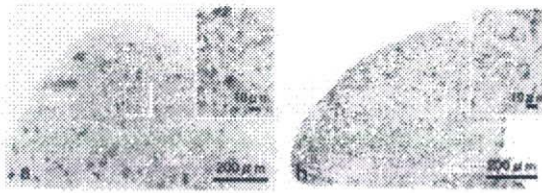


Fig. 6. Representative histology of granulomas observed in rats treated with (a) MWCNT and (b) crocidolite. Granulomas of MWCNT-treated rats were with a high cellularity and contained agglomerates and non-agglomerated particles of MWCNT (a, inset). On the other hand, granulomas of crocidolite-treated rats were with the lesser cellularity and contained fine fiber-shaped particles of asbestos within a rich collagenous matrix (b, inset).

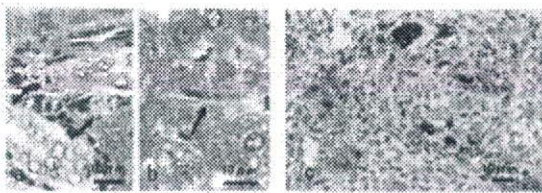


Fig. 7. Intraorgan distribution of the particles in rats treated with MWCNT. In the liver, fibrous MWCNT particles were observed in the cytoplasm of (a) macrophages present in the portal area (arrows) and of (b) a Kupffer cell present in the sinusoid (arrow). (c) MWCNT particle were detected also in the cytoplasm of multinuclear giant cells and macrophages in the mesenteric lymph node (asterisk).

similar range among 3 groups. These were thus considered as spontaneous lesions occurring without any relationship to the administration of test chemicals (data not shown).

DISCUSSION

The above data clearly indicates that MWCNT possesses carcinogenicity to cause mesothelioma at a considerably high rate in intact male rats under the present experimental conditions. Data regarding potential *in vivo* toxicity of carbon nanotubes have mostly been obtained by short-term studies featuring intracelomic, intratracheal or inhaled administration of test chemicals, simply indicating that carbon nanotubes are capable of producing inflammatory changes (Lam *et al.*, 2006; Poland *et al.*, 2008). The only long-term toxicity report available in the literature at this moment is the *p53* gene deficient mice study for MWCNT of Takagi *et al.* (2008a), and long-term toxicity of carbon nanotubes in intact animals is absolutely obscure. In this context, the present results are important and useful for the future risk assessment on MWCNT or related substances, even though this was a small-sized 1-dose study, the obtained data thus being still somewhat immature. More detailed and larger-sized studies are apparently demanded to elucidate long-term toxicity and carcinogenicity of MWCNT in intact animals, by aiming to elucidate dose-dependency and underlying mechanisms. In addition, it is especially necessary to assess whether and how MWCNT causes toxicity/carcinogenicity in intact animals when administered via human-relevant routes, and how differently MWCNT behaves according to its property and state upon exposure. These studies are now underway in our laboratories.

Histological characteristics of mesotheliomas induced by MWCNT in the present study were in good accordance with those previously published in the literature for animals and humans exposed to asbestos and other man-made mineral fibers (Adachi *et al.*, 2001; Blobel *et al.*, 1985; Davis *et al.*, 1976; Mackay *et al.*, 1987). Although the sarcomatoid portion of the advanced-stage tumors might need to be differentiated from fibrosarcoma, the differential diagnosis is not difficult, because the histological transition from the mesothelioid portion was apparently observed, and mitoses were much less than ordinary fibrosarcoma. Pleural mesothelioma lesions are conceived to be distant metastatic lesions, because peritoneal lesions/changes (inflammatory changes, fibrous thickening, granuloma, effusion, mesothelial hypertrophy and mesothelial hyperplasia) were absent in the pleura, except at the diaphragm, and pleural tumors were sufficiently distant from

the diaphragm lacking macroscopic or histological continuity from the peritoneal cavity and the diaphragm.

The present study, as well as the *p53* gene-deficient mice study (Takagi *et al.*, 2008a), was conducted to identify a potential hazard of MWCNT, and mechanistic assessments were not performed. Mechanisms underlying carcinogenicity of MWCNT are thus still obscure. Significant relation has been indicated between the size of substances and their tumorigenicity in the case of asbestos and other man-made mineral fibers, and number of such fibers must reach a sufficient level to cause chronic activation of inflammatory cell, genotoxicity, fibrosis and cancer in the target tissue (Davis, 1986, 1988 and 1989; Kamp, 1992; Miller *et al.*, 1999; Mossman and Churg, 1998; Kane, 1996; Pott *et al.*, 1987; Stanton *et al.*, 1981). In the case of MWCNT, the thinner and longer fibers are, the stronger the magnitude of asbestos-like inflammatory response is, when intraperitoneally administered (Poland *et al.*, 2008). In the present study, MWCNT had an average width of about 100 nm, and its length ranged between 100-20,000 nm, among which considerably long fibers with the 5,000 - 20,000 nm length occupied 27.5%. As aforementioned and also described by Takagi *et al.* (2008c), it is likely that dispersed and free fibrous particles are present and can also continuously come off agglomerates. Furthermore, large masses of MWCNT agglomerate present in the administering suspension are supposed to be trapped within the scrotal cavity before entering the peritoneal cavity at least partly and anyway in both cavities segregated from mesothelia by the granuloma formation. The supportive data obtained in the present study includes the presence of inflammatory changes throughout the peritoneal cavity, the lack of direct relationship between the granuloma formation and the mesothelioma development, and the detection of fibrous particles and small agglomerates of MWCNT in peripheral and resident macrophages or macrophage-oriented multi-nuclear giant cells in the liver and lymph nodes. The last data also suggest the circulatory spread of MWCNT, another important issue to be carefully assessed. Peritoneal mesothelia may thus be exposed to a sufficient amount of thin and long fibrous MWCNT particles that affect the peritoneum as a whole to make diffuse mesothelial hypertrophy and may introduce the environment sensitive for further carcinogenic stimuli in the region. It is conceivable that mesothelial hyperplasias are induced from some of such generally affected mesothelial cells receiving promoting stimuli by chance, and mesotheliomas are then developed from some of such preneoplastic lesions receiving progressive stimuli by chance.

It has been proposed that the exposure to MWCNT

causes chronic inflammation in which frustrated macrophages, mediators derived from such macrophages or other sources and oxidative stress are involved, and that these play major roles in the toxicity/carcinogenicity of MWCNT, similar to the case of asbestos and other man-made mineral fibers (Poland *et al.*, 2008; Shukla *et al.*, 2003; Takagi *et al.*, 2008a, 2008b and 2008c). Active granulomas possibly containing frustrated macrophages observed in MWCNT-treated rats in the present study may be participated in such chronic inflammation and then secondary involved in the carcinogenicity as a source of inflammatory mediators including reactive oxygen or nitrogen oxide species and cytokines. Peripheral and resident macrophages as well as multi-nuclear giant cells in organs/tissues containing MWCNT may also serve as frustrated macrophages. Iron has been believed to play a crucial role in the pathogenesis of asbestos-induced diseases, by acting as a major catalyst in oxidative stress reactions (Shukla *et al.*, 2003). In the present study, however, the iron content of MWCNT was only 1.2-1.3 hundredths of that of crocidolite (Matsuoka *et al.*, 2003; Poser *et al.*, 2003; Roller *et al.*, 1996). While Lam *et al.* (2006) described that single-wall carbon nanotube containing 2,300 ppm, a little less than the iron content of the presently utilized MWCNT, induces oxidative stress and inflammatory reactions in the lung when intratracheally administered, roles of iron in carcinogenicity of MWCNT should be clarified in the future.

Comparing the present study with the *p53* gene deficient mice study of Takagi *et al.* (2008a), there are 3 clear differences regarding the route to administer test chemicals, the detection of crocidolite's carcinogenicity and the dose of test chemicals. We administered test chemicals by an intrascrotal injection, not by an ordinary intraperitoneal injection used by Takagi *et al.* (2008a), in order to increase sensitivity. The background is based on the fact that in male Fischer 344 rats mesotheliomas are spontaneously developed from the tunica vaginalis adherent to the epididymis or the tunica albuginea of the testis, and chemically induced also specifically in the scrotum (Johnson *et al.*, 1986). Furthermore, we expect that the bursal and small space of the scrotal cavity disturbs the diffusion of test chemicals, then retains them at a relatively high level for a considerable period, and thereby causes efficient exposure in the region. The scrotal cavity of rats is, however, freely connected with the peritoneal cavity, and mesotheliomas were developed throughout the peritoneal cavity. It is thus possible that there are no essential differences in reality between intrascrotal and ordinary intraperitoneal administrations.

Crocidolite did not cause carcinogenicity in the present

study. This may be simply a matter of the dose of crocidolite. Takagi *et al.* (2008a) used 3 mg/mouse corresponding to 80.79×10^8 particles/mouse (Moalli *et al.*, 1987), 120 mg/kg body weight (estimating an average body weight to be 25 g) and 3516×10^8 particles/kg body weight, whereas we used 0.47 mg/rat corresponding to 13.77×10^8 particles/rat, 2 mg/kg body weight and 58.60×10^8 particles/kg body weight. Previous studies to show the induction of mesotheliomas by asbestos administered intraperitoneally (Adachi *et al.*, 2001; Cullen *et al.*, 2002; Davis, 1976; Mackay *et al.*, 1987) or intrathoracically (Vasilieva *et al.*, 1998; Wagner *et al.*, 1984; Whitaker *et al.*, 1984) were generally performed with higher doses and/or longer periods than those in the present study. The dose of crocidolite in the present study may thus be too low to induce mesothelioma, which is also supported by the observation of inactive granuloma in crocidolite-treated rats. The reason why we set the dose of crocidolite as was used, was in order to make it in an equivalent range with that of MWCNT at least as a weight basis. It should be noted, however, that the present results cannot be used for the comparison of the strength of carcinogenicity between crocidolite and MWCNT, because even though their weight-based doses were in a similar range, their particle number-based doses were quite different.

The presently utilized dose of MWCNT was also far lower than that of Takagi *et al.* (2008a); ours being 0.24 mg/rat, 0.85×10^8 particles/rat, 1 mg/kg body weight and 3.62×10^8 particles/kg body weight, whereas theirs being 3 mg/mouse, 10.65×10^8 particles/mouse, 120 mg/kg body weight and 426×10^8 particles/kg body weight. Takagi *et al.* (2008a) achieved the 87.5% incidence of mesotheliomas within 25 weeks, which is not so different from the incidence of 85.7% and the earliest onset at the end of week 37 of the present study. Assuming the higher sensitivity of animals (because of the genetical modification; not considering possible species difference) and the 120-times higher dose as a weight per unit body weight basis in the *p53* gene deficient mice study (Takagi *et al.*, 2008a) than in the present study, it is suggested that MWCNT is capable of exerting its carcinogenicity by its substantially low dose level. In fact, Takagi *et al.* (2008b, 2008c) preliminarily described that MWCNT seemed to induce mesotheliomas in their model even at a dose of 3 μ g/mouse, 1,000 times lower than their previous dose as a weight basis. In any case, the exact dose-dependency of the carcinogenicity of MWCNT must be critically evaluated, when the data of ongoing detailed studies becomes available.

The *p53* gene deficient mice study (Takagi *et al.*,

2008a) has been faced with criticism in terms of its methodology; low relevance to supposed human situation and highly artificial conditions in a certain sense (Donaldson *et al.*, 2008; Ichihara *et al.*, 2008). It is easily imagined the present study will have to deal with a similar criticism. One must understand, however, that this is the first step of the hazard identification stage in which the presence or absence of a hazard must be assessed under the most severe exposure conditions in the most sensitive animal species/models (Takagi *et al.*, 2008a, 2008b and 2008c). Human relevant conditions must of course keep in mind but are well capable of being assessed in the later steps/stages of the risk assessment processes. Needless to say, a chemical with a serious hazard can be without a high risk, if such a hazard occurs only under the human irrelevant conditions, or a risk can be properly managed. On the other hand, the nature of MWCNT used in the *p53* gene-deficient mice study (Takagi *et al.*, 2008a), in which agglomerates were present in association with fibrous/rod-shaped particles, was another target of criticism (Donaldson *et al.*, 2008; Ichihara *et al.*, 2008). It is well known that nanomaterials can exist as either truly nanometer-scale materials, over-nanometer scale materials or their mixture, and physical, chemical and biological characteristics may differ among such different states of a particular substance, which is one of the important issues for the risk assessment of nanomaterials. Potential hazard of a newly introduced substance, however, should principally be assessed at first using its sample as is, because its exposure will occur in such a state. This is why the *p53* gene deficient mice study (Takagi *et al.*, 2008b) and the present study were performed using the MWCNT sample as is. Influence of different states, including scale, on biological effects of MWCNT should then be assessed in the later steps/stages of the risk assessment processes.

In conclusion, the present data identifies a carcinogenic hazard of MWCNT. While such a hazard was detected under the particular condition, the obtained fact will serve as one of the indispensable evidences to be used for the risk assessment crucial for not only protection and improvement of human health and welfare, but also safe and acceptable development and prevalence of MWCNT and similar upcoming materials.

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ナノ粒子・ナノ材料の健康問題 —その3— 「ナノ粒子の毒性・健康問題」

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Health Effects of Nanoparticles and Nanomaterials (III) Toxicity and Health Effects of Nanoparticles

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Abstract As described before in the first Frontier Report of this series, there are two types of nanoparticles to be considered in hygiene science; One is the environmental nanoparticle emitted from automobiles and the other is the manufactured nanoparticle. In general nanoparticles (less than 100 nm) are reported to be permeable through cell membrane and tissues and their large surface area is responsible for the greater toxicity compared to larger particles. However, there are contradictory reports on the health effects of nanoparticles. Recent reports suggest that carbon nanotubes, fiber-shaped biopersistent nanoparticles, resemble asbestos in the pathogenesis of granuloma and mesothelioma. As such we summarize health effects of environmental and manufactured nanoparticles in the literature so far including our studies, in this report.

Key words: environmental nanoparticle (環境ナノ粒子), manufactured nanoparticle (工業用ナノ粒子), health effect (健康影響), dose metric (用量計測量)

はじめに

粒子状物質の生体影響は、溶解した物質としてではなく固体物質との生体反応を起点としており、界面での反応を考慮に入れる必要があるため、他の有害化学物質とは毒性学的な考え方がやや異なる。もっとも、生体内で速やかに溶解してしまう粒子状物質やその成分の影響を考える際には、主として体内への粒子の沈着パターンを考慮すれば、一般の有害物質と同様に扱うことが可能である。生体内に取り込まれた粒子状物質は、主として網内系で処理されるため、マクロファージなどの貪食細胞が粒子のクリアランスや影響に関して重要な役割を担っているが、超微小粒子であるナノ粒子（ナノ材料の分野では100nm以下、大気環境では概ね50nm以下の粒子を

指す。）は、非常に小さくマクロファージに認識されにくいと考えられている。そのため、ドラッグデリバリーシステム（DDS）分野においては、ナノサイズの粒子状物質を使用することにより、網内系でトラップされることなく体内の標的組織に効率よく薬剤を到達させる手法が研究されている。しかし、一般にはナノ粒子は分散状態ではなく凝集体として存在することの方が多いため、一次粒子としてはナノサイズであっても、見かけの挙動はミクロンサイズに近い粒子体になっていることも往々にしてある。このため、毒性試験を行う際にはナノ粒子の実効粒径について十分留意しておく必要がある。

ナノ粒子の取込み

一般に大きい粒子は、貪食（Phagocytosis）により細胞内に取り込まれるが、これは細胞膜が細胞骨格を形成するアクチンの働きにより異物を囲むようにして細胞内に取込む過程のことを指す。同様に、アクチンの作用により細胞膜が隆起して液滴粒子を細胞内に取込む過程は貪

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飲 (Pinocytosis) と呼ばれている。一方、非常に小さい粒子は、細胞表面の粒子が細胞膜と一緒に細胞内に引き込まれる過程で細胞内に取込まれるが、この過程はエンドサイトーシス (Endocytosis) と呼ばれており、食食や貪飲とは区別されている (1)。しかし、粒径の異なる蛍光ポリスチレン粒子 (1, 0.2, 0.078 μm) をラットに吸入させた研究では、超微小粒子の細胞への取込みはエンドサイトーシスより、むしろ拡散作用により起こっているのではないかと推測されている (2)。一方、マクロファージにおいては細胞表面に発現している macrophage receptor with collagenous structure (MARCO) が、オプソニイズ化されていない粒子の貪食に関与していることが報告されている (3)、MARCO が細胞へのナノ粒子の取込みに関与しているかどうかを、20, 200nm 及び 1 μm の蛍光ポリスチレンビーズを用いて、蛍光顕微鏡、蛍光分光光度計および原子間力顕微鏡を用いて調べた研究では、MARCO がナノ粒子の細胞への結合および取込みにも関与していることが報告されている (4)。

マンガン電池作業員においてパーキンソン様の症状が多いことは労働衛生分野でよく知られているが、その発症機序として吸入したマンガンが嗅神経を介して脳に達するのではないかと考えられてきた。ラットを用いた実験では、MnO₂ 曝露群の嗅球における Mn 濃度は対象曝露群と比較して有意に上昇しており、また、線条体、前頭葉皮質、小脳における同濃度も有意に上昇していたことが報告されている (5)。

¹³C ラベルした超微小カーボン粒子を、ラットに吸入曝露させ、安定同位体比質量分析計を用いて組織の ¹³C を測定した実験では、曝露 1 日後に大脳、小脳、嗅球で ¹³C の濃度の増加が測定されたことから、カーボンナノ粒子が少量ではあるが脳に移行することが示唆されている (6)。鼻腔に沈着したナノ粒子が嗅神経を通過して嗅球に移行し、さらに大脳、小脳に移行すると考えられているが、一度血流に入った粒子が脳組織で検出されている可能性も否定できず、嗅神経を経由したナノ粒子の体内移行についてはさらなる研究が必要である。また、¹³C ラベルした ultrafine カーボン粒子の吸入曝露実験において、0.5 時間後に肝臓への移行が観察されたことから、ナノ粒子が肺胞から肺以外の組織に移行することが示唆されている (7)。しかし、この報告においても、粒子が直接肺胞壁を通過して血管に入っているのか、あるいは消化管を経由しているのかの検討が必要である。

放電により発生させた金微小粒子 (MMD=16nm) を Wistar-Kyoto ラットに曝露した実験においては、肺組織の 0.03 から 0.06% の金が生検組織から検出されていることから、呼吸器に沈着したナノ粒子は、血流を介して多臓器へ移行する可能性が示されている (8)。また、酸化カドミウム (9)、あるいは銀ナノ粒子 (10) を Fischer ラットに吸入させたところ (粒径はそれぞれ 40 と 15-20nm)、これらの粒子が肝臓や腎臓、嗅球に移行していることが報告されている。しかし、これらの金属粒子は呼吸器内で一部

溶解することも考えられるので、粒子として体内移行しているのか、金属イオンとなってからタンパク質などのキャリアーに結合して体内を移行したのか確認する必要がある。摘出したラットの肺に 18nm の放射性イリジウムナノ粒子を曝露してナノ粒子の血管への通過を調べた実験 (11) や、摘出ウツギ灌流肺を用いて、24, 110, 190nm の蛍光ポリスチレンナノ粒子の肺胞壁における透過性を調べた実験 (12) では、ともに粒子の肺胞腔から血管内への移行は認められていない。しかし、ヒスタミンや過酸化水素の処理により血管透過性を亢進させておくと、ナノ粒子の血管への移行が認められていることから、肺-血管バリアー機能が低下すると、ナノ粒子が肺胞腔から血管内へ透過し易くなることが示されている。

一方、17-20nm の放射ラベルした Ir をラットに吸入させた研究において、ナノ粒子はミクロオーダーの粒子に比べ、肺胞マクロファージに取り込まれる割合は少なく、肺の間質に取り込まれた後、暫くして上皮に再出現し喉頭側に移行している可能性があることが報告されている。これは、ナノ粒子がどのように肺からクリアランスされるかに関して前例のない画期的な仮説ではあるが、実験を繰り返して確認する必要があると考えられる (13)。以上まとめると、ナノ粒子の肺胞から血流内への移行に関しては、必ずしもはっきり証明されているわけではないが、肺の炎症時などの血管透過性が亢進している場合は、明らかに粒子の肺胞壁通過が起こっていることから、僅かではあるがナノ粒子は肺胞から直接血流に入ることが推測される。

また、小児の吸入曝露のリスクアセスメントのために ICRP と RfC のモデルを使って成人と小児とを比較した研究においては、粒子の肺への沈着量は粒径にも依存するが、小児の表面積あたりの沈着が成人に比べて 2 から 4 倍多くなっていること、特に 10nm のナノ粒子では 4 倍沈着が多いことなどが報告されており、今後ナノ粒子のリスク評価において考慮すべきことと考えられる (14)。

工業用ナノ粒子の生体影響

ナノサイズの物質・材料の工業的製造と多方面にわたる利用は、いわゆるナノテクノロジーと呼ばれ、電磁光学、構造材料を中心として一般家庭用品から食品にいたるまでの新しい応用の展望が期待され、薬物輸送を含む医療への展開も期待されている。この様に特定の機能を意図して合成された純粋な単体としてのナノ物質が生体に及ぼす影響については多くの点で未知である。ナノテクノロジーに対する期待が大きいだけに、生体影響の大枠を明らかにし、ナノ粒子でもあるナノマテリアルの利用が安心して進められるように対応することが求められている。様々な分野に応用が見込まれるため、ナノマテリアルも様々な種類のもが開発されてきている。その分類法も様々ではあるが、基となる化学物質の種類から以下のように分類できると考えられる。

- ・酸化金属・金属：二酸化珪素 (SiO_2)、二酸化チタン (TiO_2)、アルミナ (Al_2O_3)、酸化鉄 (Fe_3O_4 , Fe_2O_3)、酸化亜鉛 (ZnO)、酸化インジウムスズ (ITO) など
- ・炭素系：フラーレン、カーボンナノチューブ (CNT)、カーボンファイバーなど
- ・ナノクレイ：特殊な層構造を持たせたケイ酸塩
- ・有機ナノ粒子：ナノ粒子化された薬品・化合物 (医薬品、ビタミン、色素など) ポリマー、高分子、ミセル、リポソームなど

その他、ナノコンポジットとして、ナノ粒子を特殊な役割のために構成成分としてポリマーやセラミック、金属マトリックス製品中に再配合することもある。

このように多岐にわたる工業用ナノ粒子を一括して生体影響を検討することは不可能であり、そのため国際的には、安全性評価を念頭においた各ナノマテリアルの標準化作業も進んでいる。将来的には、これらの標準化されたナノマテリアルに関する評価が進んでいくものと考えられるが、現時点では、個別の物質についての断片的な生体影響に関する情報しか得られていない。しかも、生産量や使用量を反映して、二酸化チタンやフラーレン、カーボンナノチューブといった物質に関する情報に限られているという状況である。

二酸化チタン

酸化チタン自体は、古くから白色顔料として使われてきており、着色の目的で食品添加物としても使用されてきている。顔料としては、一次粒径は2~300nm ぐらいであるが、通常大きな凝集・集合体を形成している。近年は、紫外線防護や光触媒活性を目的としたより一次粒径の小さいナノ粒子 (1-50nm) が使用されるようになってきた。工業的製品の多くは粒子の形状としてルチル型とアナターゼ型に分類され、アナターゼ型の光触媒作用がより強いと考えられている。顔料としての使用が主流であった1989年のIARCの発がん性評価では、ラットへの吸入実験で高用量群においてのみ肺線維腫の増加が認められる(15)ものの、経口、皮下、気管内および腹腔内投与のいずれにおいても動物実験において催腫瘍性が認められず、不十分な疫学データのためグループ3に分類された(16)。しかし、その後、顔料タイプおよびナノ粒子の両方において、吸入および気管内投与によるラットでの肺がん発生率の増加を示す報告を考慮し、2006年2月のIARCの評価では、Group2Bに変更された(17)。ナノサイズの粒子 (ultrafine particle) とサブミクロンサイズの粒子 (fine particle) の吸入曝露による炎症性を比較した研究からは、ナノサイズ粒子による炎症反応の方が強いとする研究が報告されている(18, 19)が、二酸化チタンを気管内滴下した研究では、顔料系のサイズとナノサイズの粒子で炎症反応に違いのないことも報告されている(20)。しかし、ルチル型とアナターゼ型を比較した研究では、粒子サイズが同等でもアナターゼ型の方が、炎症反応が強く、表面活性の違いが重要な因子であるこ

とも示唆されている(21)。一方酸化チタンは、日焼け止め剤の中に紫外線防護の目的で使用され、近年は使用時の透明性を高める等の目的でナノサイズ化されたものが使用されており、ナノサイズ粒子の皮膚曝露による影響は、検討すべき曝露経路の一つである。しかし、局所刺激や感作性、全身影響に関してサイズの違いによる影響を検討した報告はなく、吸収性に関して行われた少数の研究がある。日焼け止め剤中の二酸化チタンが、角質層や毛嚢の中に浸透していることを示した報告があるが、この毛嚢への浸透部分は角質層に覆われている部分のみであった(22)。また別の報告では、二酸化チタンナノ粒子による皮膚への透過性はほとんど示されていない(23)。しかし、5-20nm というような超微細二酸化チタンが皮膚を透過し、皮内の免疫系と相互作用する可能性は指摘されており(24)、今後、慢性曝露による影響を考慮した研究が必要であると考えられる。

フラーレン

フラーレンは、空気から分子酸素を容易に吸着させることができ、光照射により得た余分な励起エネルギーを近くの酸素分子に渡し、反応性の高い一重項酸素を生成することが知られている。遺伝子突然変異誘発性に関しては、可視光線照射時と代謝活性化系存在時にいくつかのサルモネラ菌種で変異原性が示された(25)。発生した一重項酸素から間接的にラット肝臓ミクロゾームの作用により生成した過酸化脂質が酸化的DNAを引き起こしたことが示唆されている。しかし、マクロファージを用いた初期の実験では、フラーレンによる活性酸素生成に対する影響はほとんど認められていない(26)。フラーレンの毒性に関する報告は、ほとんどが修飾されたフラーレンに関するもので、未修飾のフラーレンに関する情報は少ない。未修飾のフラーレンと水酸化フラーレンの *in vitro* での細胞障害性に関する研究では、未修飾のフラーレンの方が細胞毒性の強いことが示されている(27, 28)が、両フラーレンに関して3mg/kg までのラットへ気管内滴下による *in vivo* 単回投与実験では、どちらも一過性の炎症反応を示し、その反応性には違いが認められなかった(29)。経口投与した未修飾のフラーレンの毒性影響を調べた報告はないが、フラーレンはマウスの皮膚塗布に対しての局所炎症作用や発がん促進作用を示さないことが報告されている(30)。また、UVA 照射下でC60 フラーレンのトルエン溶液をマウスの皮膚に反復投与した実験では、紅斑はみられたが、皮膚がんはみられなかった(30)。未修飾フラーレンについては、溶媒や培地への分散化が極めて困難であり、*in vitro* 系では使用した溶媒や分散剤の影響を受ける他、*in vivo* 系における情報も少なく、系統だった毒性研究の進展が望まれる。

カーボンナノチューブ (CNT)

CNTは単層または多層の形状を持ち、それぞれSWCNTおよびMWCNTとして分類されるが、その製法により層

の数や構造、繊維の長さ、使用する触媒金属などが異なる様々な種類が存在する。また、ある種の MWCNT の形状がアスベストに類似していることから、その潜在的な懸念について関心が持たれている。まず、SWCNT について、ラット及びマウスに気管内投与し、肺への影響を検討した実験において、肉芽腫形成と間質性炎症を引き起こすことが報告された (31, 32)。Warhaeit らのラットの実験では多発性肉芽腫が観察され、高倍率の検査によりナノチューブの固まりの周りを覆う単核性の肉芽腫が認められた。この変化は用量非依存的で生体の単なる異物反応と捉えることができ、生理学的関連性を持たないかもしれないと考えられている。また、Lam らのマウスを用いた気管内投与実験では、慢性曝露で傷害性のあるクォーツ粒子より炎症反応の強いことが示された。一方、SWCNT を咽頭吸引によりマウスに曝露させた実験では、BAL の炎症細胞、炎症サイトカイン、蛋白質の迅速な増加により、SWCNT が急性炎症反応を起こすことを示したが、SWCNT のマクロファージとの反応性は一過性で、炎症性細胞浸潤を伴わない間質の繊維化が認められている (33)。さらに、咽頭に滴下した実験では、大動脈ミトコンドリアのグルタチオン量、蛋白カルボニル化活性の変化を伴うミトコンドリア DNA 障害が示され、ApoE-ノックアウトマウスで、アテローム性動脈硬化症の進行を増強することが示され、体循環に吸収されて、全身影響を示す可能性が示唆されている (34)。ラットに気管内投与した MWCNT は、投与後 60 日後にも肺に残存し、濃度依存性の炎症反応を起こしコラーゲンリッチな肉芽腫を形成した。この実験では、平均 5.9 μm と 0.9 μm に粉碎した MWCNT の投与による比較研究が行われ、粉碎前の MWCNT の多くが気道に蓄積し炎症を示していたが、粉碎した方は肺胞域に達し肺実質における肉芽腫を形成していた (35)。マウスに MWCNT (200-400 μg) を気管内滴下した実験では、肺の炎症反応は一過性で弱かったが、投与に依存した血小板の活性化と凝固作用の活性化を促進している可能性が示唆された (36)。一方、アスベストとの形状の類似性から想定される慢性影響としての発がん性や中皮腫の誘発性の懸念について長期の試験が必要であると考えられるが、CNT に関する吸入曝露法が確立していない状況において、最近腹腔内投与による 2 つの研究が報告された。一つは、p53 のヘテロノックアウトマウスに 3mg/mice を腹腔内単回投与した場合に、投与後半年までの間に、ほとんどのマウスに中皮腫の発生が認められたものである (37)。この実験では同時に投与したクロシドライトにおいても、ほとんどのマウスに中皮腫を誘発しているが、未修飾フラーレンを同用量 (3mg/mice) を投与した場合は、対象群と同様に中皮腫の発生は認められていない。もう一つの研究では、長さの違う 4 種類の MWCNT を 50 μg/kg の用量で腹腔内単回投与して、7 日後の中皮細胞の増殖性を検証したところ、長いタイプの 2 種類について腹腔側に CNT を取り込んだ群においてのみ細胞が集合した肉腫の形成が認め

られた (27)。これらの結果は、様々な形状の MWCNT において、体内残留性や適切な長期の吸入曝露研究の必要性を示唆している。

その他の工業用ナノマテリアルに関する研究は、ほとんどがさらに断片的な研究しか報告しかされていない。しかし、上記 3 種のナノマテリアルに関する報告を見る限りにおいても、粒子の大きさや表面積だけでなく、表面活性や形状に依存した毒性研究が必要であることが示されている。また、検出するエンドポイントが使用する溶剤/分散剤により影響を受ける可能性もあるため、毒性手法の標準化/最適化が必要であるとされている。この観点に立って、OECD や ISO などの国際的機関を中心に毒性影響を検証するためのナノマテリアル標準化や、毒性試験法の調和化に関する活動がおこなわれている。しかし、各ナノマテリアルの中でも毒性影響を把握するためには、少なくとも数種の標準物質が必要であると考えられ、本格的な検証作業に入る前に、さらに物性に基いて基礎的な研究が必要であると思われる。

大気中の環境ナノ粒子の生体影響

大気汚染物質の中でも、粒子状物質による健康影響の対象は、総粉塵、浮遊粒子状物質、PM2.5 とより粒径の小さい粒子状物質に関する健康影響研究に移行し、5 年ほど前よりさらに小さい 50nm 以下の環境ナノ粒子に関する研究が始められているところではあるが、これらの経緯については既に報告している (38)。ここでは、疫学研究において報告されている微小粒子と循環器系疾患による過剰死亡を (39)、実験的に証明しようとの試みから、超微小粒子 (Ultrafine or Nanoparticle) が肺組織を透過して循環系に入り凝固線溶系や循環器に直接作用する影響機序も推定されている。

一般大気環境中に観測される環境ナノ粒子の主たる発生源はディーゼル車由来であり、元素状炭素よりも高級アルカンなどの有機性炭素を主成分とするが、その他硫黄分、重金属なども含まれている。これに対して、これまで扱われてきたディーゼル排気粒子とは、元素状炭素 (Elemental Carbon, すす) を主としており、そこに多環芳香族化合物などが吸着している黒い粒子状物質をさす。工業用ナノ粒子の生体影響に関する研究に比べて、環境ナノ粒子の吸入曝露実験ができる施設は限られていることから、環境ナノ粒子の生体影響に関する報告例は極めて少ない。国内の吸入曝露実験に於いて (図 1) これまでに得られている実験結果からは、環境ナノ粒子の呼吸器への影響はこれまでの粒子状物質と比較して必ずしも高いとはいえない (40)。また、環境ナノ粒子の主成分である高級アルカンは、肺表面活性に相転移を伴う変化を起こすことが知られている (41)。ナノ粒子は、拡散運動が大きく、基本的にはガス状物質と分離することができないので、ディーゼル排気粒子の影響にガス状物質の影響が相加・相乗的に働いていることも考えられる。

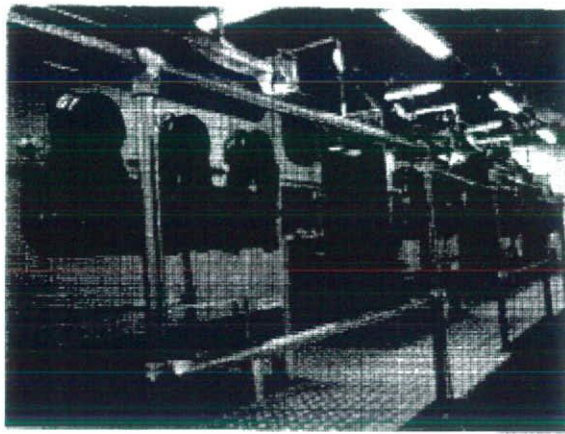


図 1 独立行政法人国立環境研究所内に設置されている、ディーゼル排ガス由来環境ナノ粒子の吸入曝露装置。慢性吸入実験も可能なように設計されている。

諸外国においては、大気中の粒子状物質を吸入したヒトボランティアの実験や、捕集した超微小粒子状物質を細胞などに曝露した実験などが行われている。デンマークのコペンハーゲンのヒトボランティアを対象として、12, 23, 57 及び 212nm の4サイズの都市大気中粒子を個数濃度の影響として比較した研究では、末梢血単核細胞 (PBMCs) のDNAに対する酸化的損傷と関連する修復能力を調べたところ、57nm モードの個数濃度とDNA損傷との間には有意の用量反応関係がみられたが、オゾン、酸化窒素および一酸化炭素などの付随する曝露は、これらの結果に影響を及ぼさなかったと報告されている。このことは、超微小粒子状物質が血球に影響を及ぼしていることを示唆している (42)。

ロサンゼルスで捕集された、粗大 (2.5-10 μm)、微小 (<2.5 μm)、超微小粒子状物質 (<0.1 μm) の、マクロファージや上皮細胞への取り込みとそれらの酸化ストレス活性を調べた報告では、UFPs は最も強い heme oxygenase-1 (HO-1) の発現誘導を示したこと、超微小粒子状物質と微小粒子は、ミトコンドリア内に局在しており、構造的な損傷をおこして酸化ストレスの誘導に関連する可能性があることが示唆されている (43)。都市大気中からサイズごとに捕集した粒子状物質をヒト気管支上皮細胞に曝露し、炎症や免疫修飾の過程に重要なサイトカイン産生に与える影響について検討した報告では、0.18 μm 以下の最も小さいサイズの粒子 (ultrafine/fine) の活性が高く、特にその化学的成分が影響していることが示されている (44)。cDNA マイクロアレイを用いて米国 North Carolina の Chapel Hill で捕集した ultrafine particles をヒト肺動脈内皮細胞に曝露して遺伝子発現を検討したところ、ultrafine particles 曝露により 320 遺伝子が増加、106 遺伝子が減少しており、その中には血管内皮細胞に凝固に関与する遺伝子発現の誘導も含まれていた (45)。このことは、疫学で指摘されている微小粒子状物質と循

環器系影響との関係を指示するものである。

一方、粒径の小さな大気粒子の影響はそれほど大きくない、あるいは粒径の影響はほとんど見られないという報告もされている。ヨーロッパ6都市 (Duisburg, Prague, Amsterdam, Helsinki, Barcelona, Athens) の Coarse (PM10-2.5), fine (PM2.5-0.2) と ultrafine (PM0.2) 粒子をマウスに気管内投与し、肺胞洗浄液中の総細胞数、タバコ濃度、乳酸脱水素酵素活性、炎症性サイトカインを測定して時間経過、投与量、各都市の粒子の違いによる毒性を調べたところ、一般的には粗大粒子が微小粒子より炎症誘導能が高く、超微小粒子はほとんど炎症をおこさなかったと報告されている (46)。

また、オランダの都市で捕集した超微小 (<PM0.1)、微小 (PM0.1-2.5)、粗大 (PM2.5-10) の粒子状物質 (PM) がヒト肺胞マクロファージの炎症性サイトカイン、食食活性などに及ぼす影響を比較検討した研究では、影響の強さは PM2.5-10 > PM0.1-2.5 > 超微小粒子であり、その理由として、粗大粒子の分画に検出される微生物産物が炎症反応に関与している可能性が示唆されている (47)。

このように、環境ナノ粒子、超微小粒子状物質が粗大粒子状物質より大きな影響を示すことが示唆されている一方で、必ずしも粒径の小さい粒子の毒性が高いとは限らないという報告も出されており、はっきりした結論が出せない状況である。また、大気汚染の疫学的研究として環境ナノ粒子の健康影響を調べることは重要であることには間違いはないが、環境ナノ粒子の濃度が測定場所により極端に異なること、粒子の化学組成の変化が大きく揮発性成分を含むことから、実際のフィールド研究として環境ナノ粒子の影響を捉えることは難しいと考えられている (48)。

終わりに

ナノ粒子の生体影響に関するこれまでの知見をまとめると、概ね粒径が小さい粒子ほど生体に与える酸化的ストレスが大きく、単位重量当たりの毒性が高くなることはかなり確からしいと考えられる。これは、粒子の表面が反応の場となっており、粒径が小さいほど粒子と生体との反応の面積が大きくなっているためであると考えると理解しやすい。また、呼吸器以外の臓器へのナノ粒子の生体影響機序としては、粒子として直接移行したための影響であると断定することはできないが、心臓などの機能をはじめとしてなんらかの影響を及ぼしていることを否定できない。粒子状物質の生体影響を把握する上において、個数、表面積、重量のうちどのような用量計測量 (dose metric) が最も適切であるかに関して多くの議論がある。気管内投与した粒子状物質による肺の炎症を調べた実験において、粒子の表面積が最も良い dose metric であるという報告が多い中で、それに反対する意見もある。ナノ粒子の毒性を表現する上でのメトリックとしては、粒径の総和相当量 (joint length) が有効

であるとの報告もあり注目に値する (49)。いずれにしても、粒子状物質にはアスベストのような繊維状粒子も多く存在することから、一義的にメトリックスを決めるのは難しいと思われる。

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Original Article

Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube

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ABSTRACT — Nanomaterials of carbon origin tend to form various shapes of particles in micrometer dimensions. Among them, multi-wall carbon nanotubes (MWCNT) form fibrous or rod-shaped particles of length around 10 to 20 micrometers with an aspect ratio of more than three. Fibrous particles of this dimension including asbestos and some man-made fibers are reported to be carcinogenic, typically inducing mesothelioma. Here we report that MWCNT induces mesothelioma along with a positive control, crocidolite (blue asbestos), when administered intraperitoneally to p53 heterozygous mice that have been reported to be sensitive to asbestos. Our results point out the possibility that carbon-made fibrous or rod-shaped micrometer particles may share the carcinogenic mechanisms postulated for asbestos. To maintain sound activity of industrialization of nanomaterials, it would be prudent to implement strategies to keep good control of exposure to fibrous or rod-shaped carbon materials both in the workplace and in the future market until the biological/ carcinogenic properties, especially of their long-term biodegradability, are fully assessed.

Key words: Multi-wall carbon nanotube (MWCNT); Asbestos; Fullerene; Mesothelioma; P53 heterozygous mouse; Micrometer particles

INTRODUCTION

A rapid increase in the usage of nanomaterials in consumer products and medical applications in the near future underlines the importance of understanding its potential toxicity to people and the environment (Lam *et al.*, 2006; Donaldson *et al.*, 2006). Among them, carbon nanotubes and fullerenes have been one of the most extensively researched and developed nanoparticles. Carbon nanoparticles tend to aggregate into micrometer particles due to their cohesive characteristics (Lam *et al.*, 2006; Luo *et al.*

2004). And they are considered to be very stable in the organism. These two elements lead us toxicologists to consider a concern of the chronic toxicity of micrometer-sized particles before any consideration is made for their pure nanometer-sized properties in our body. Once inside the body, the long-lasting scavenging and inflammatory activities towards the non-degradable micrometer-sized particles would lead to the continuous oxidative stress at their deposit sites, which eventually lead to tissue destruction and, on some occasion, carcinogenesis (Coussens and Werb, 2002). Additional concern is given to the fibrous or

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rod-shaped particles of micrometer length that share the dimension of asbestos reported to be carcinogenic to humans and experimental animals (Hei *et al.*, 2006; WHO, 1986, 1998). Another factor reported to relate with carcinogenic potency of asbestos is the iron (Fe) content. The most potent asbestos (crocidolite or blue asbestos) contains the highest amount of Fe (WHO, 1986). It is explained that Fenton reaction would accelerate the generation of oxygen radical species that lead to carcinogenesis (Jiang *et al.*, 2006; Gulumian and Wyk, 1987).

MWCNTs form micrometer-sized particles of fiber or rod-shape. The diameter ranges from 0.01 to 0.2 micrometer (Hou *et al.*, 2003) and lengths may reach tens of micrometers that correspond to the size and shape of asbestos. Additionally, some CNTs are reported to contain a considerable amount of Fe due to its manufacturing process (Lam *et al.*, 2006). Deduced from those factors, we hypothesized that MWCNT might have carcinogenic potency similar to asbestos when administered to organisms via the same route of exposure. Here, we adopted a short-term bioassay, i.e., the p53 heterozygous mouse intraperitoneal exposure model reported to be sensitive to asbestos and develop mesotheliomas fast (Marsella *et al.*, 1997; Vaslet *et al.*, 2002). This mouse model has been reported to be sensitive not only to genotoxic carcinogens (Pritchard *et al.*, 2003) but also to reactive oxygen species (ROS)-related carcinogenesis (Tazawa *et al.*, 2007) and therefore fits with the postulated carcinogenesis mecha-

nisms of asbestos and asbestos-like particles (Marsella *et al.*, 1997; Vaslet *et al.*, 2002).

MATERIALS AND METHODS

Experimental animals

The p53-heterozygous (p53(+/-)) mice were generously given by Dr. S. Aizawa (Tsukada *et al.*, 1993). This p53 (+/-) mice were bred with normal wild-type C57BL/6 females (SLC, Shizuoka, Japan). After more than 20 generations of backcrossing, seventy-six male p53(+/-) mice of an age of 9 to 11 weeks were used in this experiment (nineteen per group). All mice were housed individually under specific pathogen-free conditions, with a 12 hr light-dark cycle at the animal facility of NIHS. They were given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., Ltd.) *ad libitum*. Experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences (NIHS), Tokyo, Japan.

Histology

For evaluation of carcinogenicity, visceral organs including liver, kidney, spleen, lung, digestive tract and macroscopic tumors (*en bloc* in case of severe peritoneal adhesion) were fixed in 10% neutral buffered formalin. After conventional processing, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and

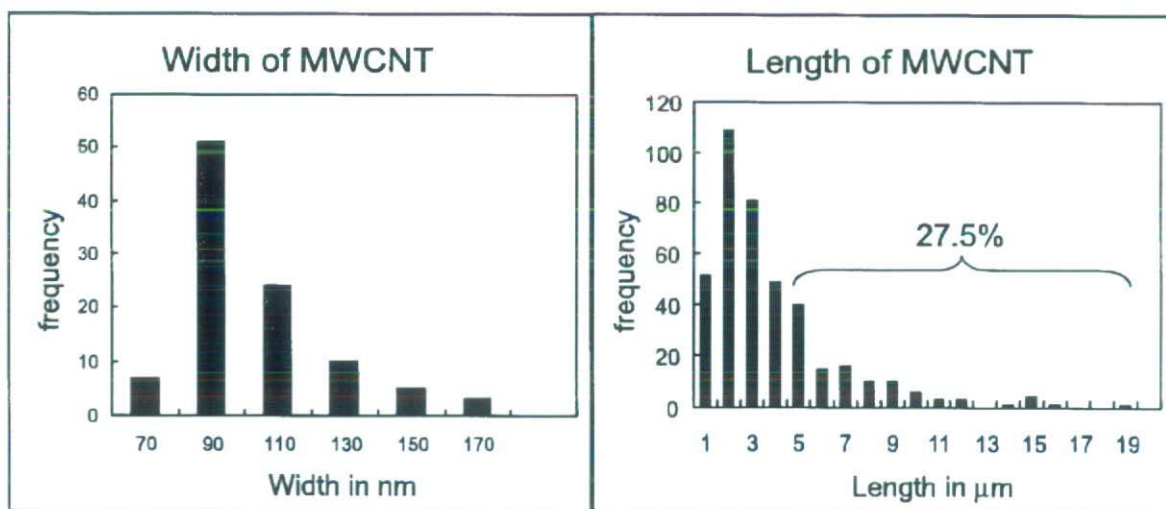


Fig. 1. Width and length distribution of MWCNT:

Width and length distribution of MWCNT (MITSUI MWCNT-7, Lot NO. 060125-01k) was measured at Tokyo Metropolitan Institute of Public Health. The average width was about 100 nm, and 27.5% of the particles were longer than 5 micrometer.

examined histopathologically under a light microscope.

Materials

Multi-wall carbon nanotube (MITSUI MWCNT-7, Lot NO. 060125-01k), UICC-grade Crocidolite (NIHS material stock), and fullerene (C₆₀, Nanom purple, Frontier Carbon Corporation, Tokyo, Japan) were used in this study.

The number of particles per weight and size distribution of MWCNT was determined as follows: 1.03 mg of MWCNT was suspended in 5 ml of 5% Triton X-100 (Qbiogene, CA, USA) and sonicated for 30 min, immediately diluted x100 by 5% TX-100, and then an aliquot of 5 microliters was mounted on a glass plate. The plate was heated up to 480 °C for 20 min by an electric oven, metal-

ized by platinum and palladium, and subjected to scanning electron microscope observation. All visual fields were photographed. Number and length of the particles were measured on the enlarged photo prints. As a result, one gram of MWCNT corresponded to 3.55×10^{11} particles. The length and width distribution is shown in Fig. 1. The number of particles per weight of the UICC Crocidolite was reported as 2.93×10^{12} fibers/g (Moalli *et al.*, 1987). The contents of elements in the MWCNT were determined by collision type inductively coupled plasma mass spectrometer (ICP-MS 7500ce, Agilent Technologies, Inc. Santa Clara, CA, USA) and combustion ion chromatography (DX-120, Dionex Corporation, Sunnyvale, CA, USA). The average content of Fe was about 3,500 ppm (0.35%) by a microwave-assisted dissolution procedure with a mix-

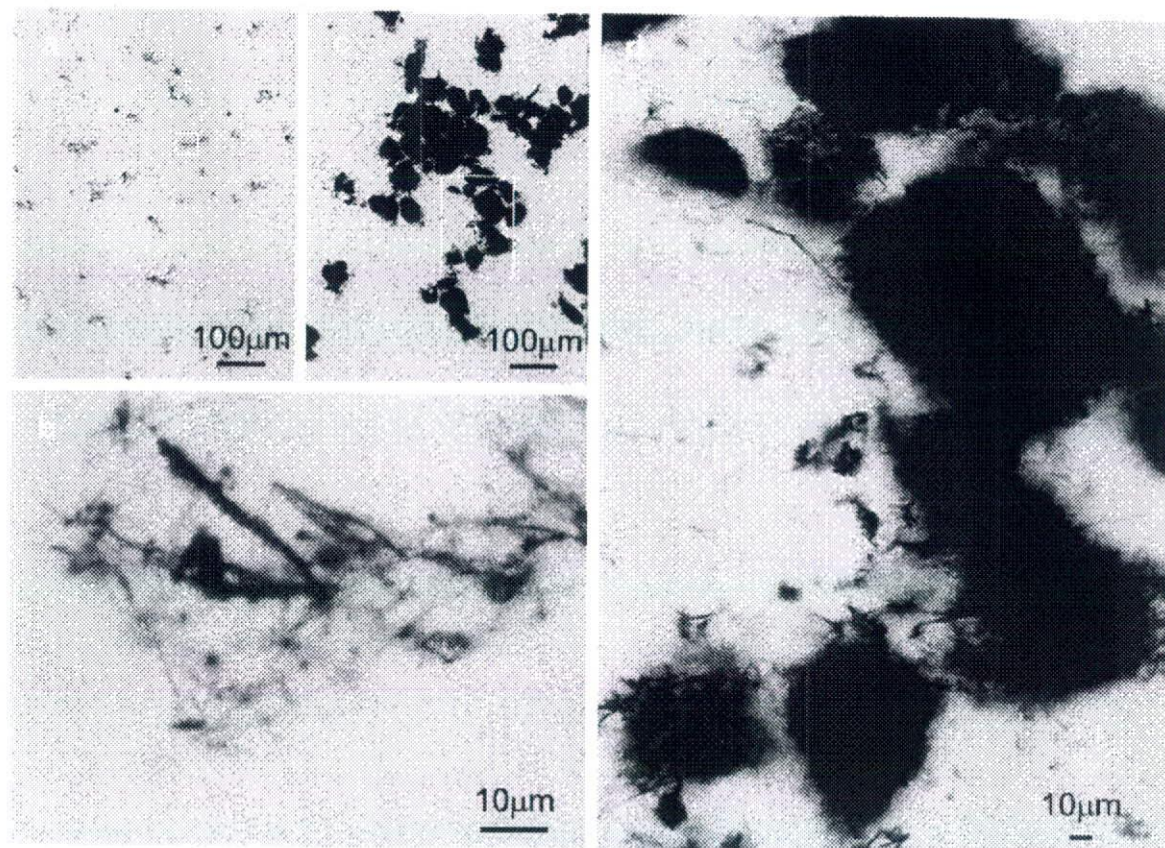


Fig. 2. Light microscopic view of administered MWCNT:

Light microscopic view of sonicated MWCNT sample suspension mounted on slide glasses. a) Well-dispersed area of the preparation. b) Close-up view of the boxed area in a). Fine fiber or rod-shaped particles longer than 10 micrometers are seen. c) Aggregated MWCNT. d) Close-up view of the boxed area in c) Aggregates are 50 to 200 micrometers in dimensions.

ture of nitric acid and perchloric acid. Sulfur content was about 470 ppm. Chlorine was 20 ppm and fluorine and bromine were below detection levels (5 and 40 ppm, respectively).

Preparation of particle suspension

MWCNT, crocidolite and fullerene were suspended at a concentration of 3 mg/ml to 0.5% methyl cellulose (Shin-Etsu Chemical Co., Ltd.) solution and autoclaved (121 °C, 15 min). After addition of Tween 80 (Tokyo Chemical Industry Co., Ltd.; final 1.0% conc.), the solutions were subjected to sonication by ultrasonic homogenizer (VP30s, TAITEC Co. Japan) (cf. Fig. 2).

Treatment of mice

Nineteen male p53 (+/-) mice at the age of 9 to 11 weeks were given single i.p. injection of 1×10^9 of MWCNT particles (corresponding to 3 mg/head) in 1 ml suspension. The number of the particles was set to a moderate value of the reported ranges (Roller *et al.*, 1997) which corresponds to the maximum value recommended by the draft guideline for man-made mineral fibers (Bernstein and Riego Sintes, 1999). Another 19 mice were given single i.p. injection of 3 mg/head suspension (1 ml) of fullerene, and as a positive control of this carcinogenesis study, another 19 mice were given 1×10^{10} of crocidolite in 1 ml of suspension (corresponds to 3 mg/head) at the first day of experiment. Vehicle solution (1 ml) was given to 19 mice as negative controls. Satellite groups consisting

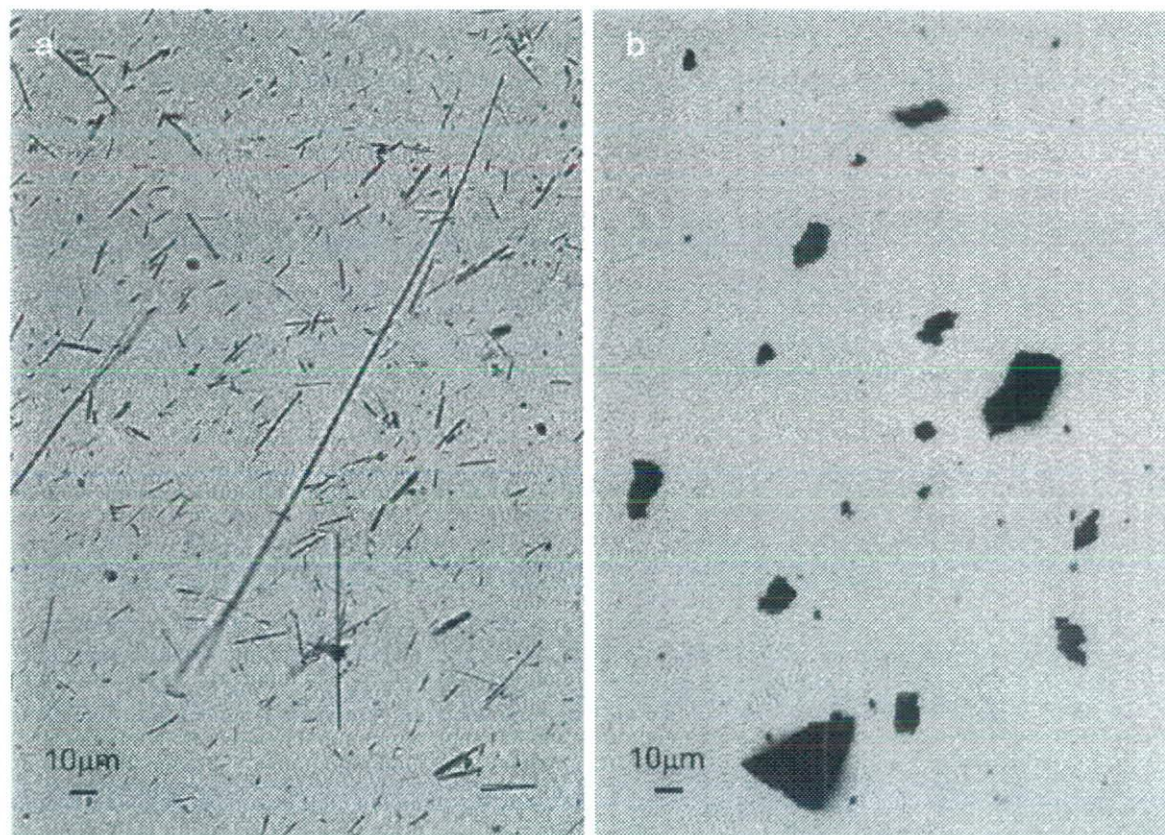


Fig. 3. Light microscopic views of administered crocidolite and fullerene: Light microscopic views of administered crocidolite and fullerene. a) Crocidolite sample consisting of various lengths of rod-shaped particles. b) Fullerene sample consisted of sand grain-like particles of sizes ranging up to 50 micrometers.

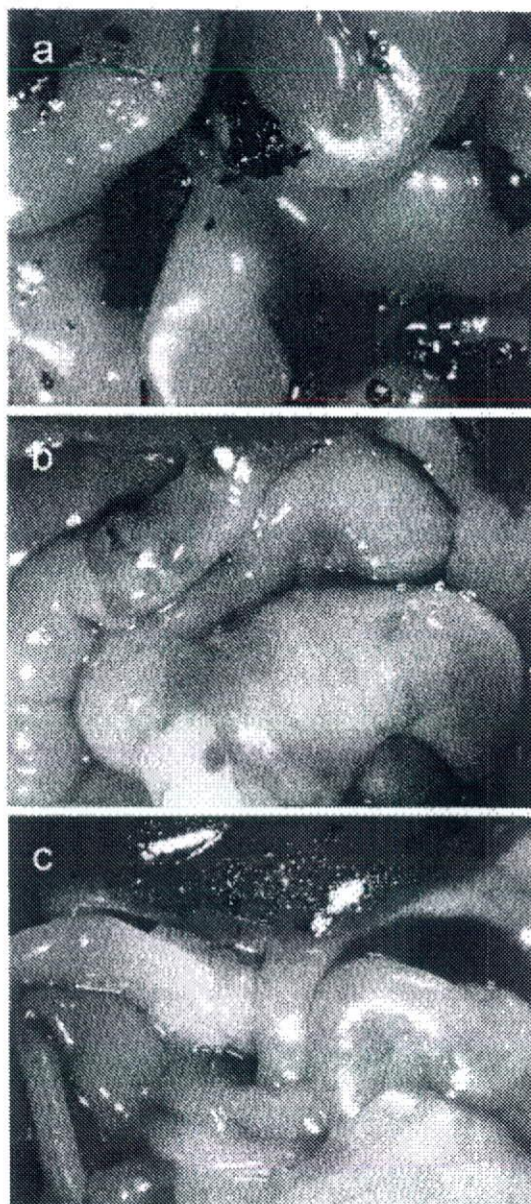
Mesothelioma by MWCNT in p53 $-/-$ mouse.

Fig. 4. Early peritoneal responses to MWCNT, crocidolite, and fullerene (10 days after i.p. injection): Early findings of peritoneal cavity 10 days after i.p. administration of a) MWCNT inducing slight fibrinous deposit, adhesion, ascites retention, and edematous and hypertonic intestinal loops, b) crocidolite inducing slightly edematous intestinal loops, and c) fullerene with no obvious change except for black patchy deposits on the serosal surface.

of 6 wild-type C57BL/6 male mice each were similarly treated and sacrificed at day 10 for the observation of early peritoneal responses.

RESULTS

Although rigorously agitated prior to i.p. injection, the MWCNT sample contained aggregates among dispersed rod-shaped or fibrous particles (Fig. 2). Crocidolite sample was made of evenly dispersed rod-shaped or fibrous particles (Fig. 3a). Fullerene was in polygonal particles of micrometer size (Fig. 3b).

At day 10, the satellite groups were monitored for early

responses (Fig. 4). MWCNT mice showed slight fibrinous adhesion with a trace amount of ascites with scattered black spots of MWCNT aggregates. The intestine loops were edematous and hypotonic. Crocidolite mice showed similar responses but to a lesser extent, and there were no overt peritoneal adhesions. Bluish green spots of crocidolite aggregates were seen on the peritoneal surface. The Fullerene group showed minimal changes except for the black spots of aggregates on the serosal surfaces.

The vehicle control mice showed no overt change in peritoneal cavity.

The mice of main groups were monitored until one of the groups reached 100% mortality. The highest lethality

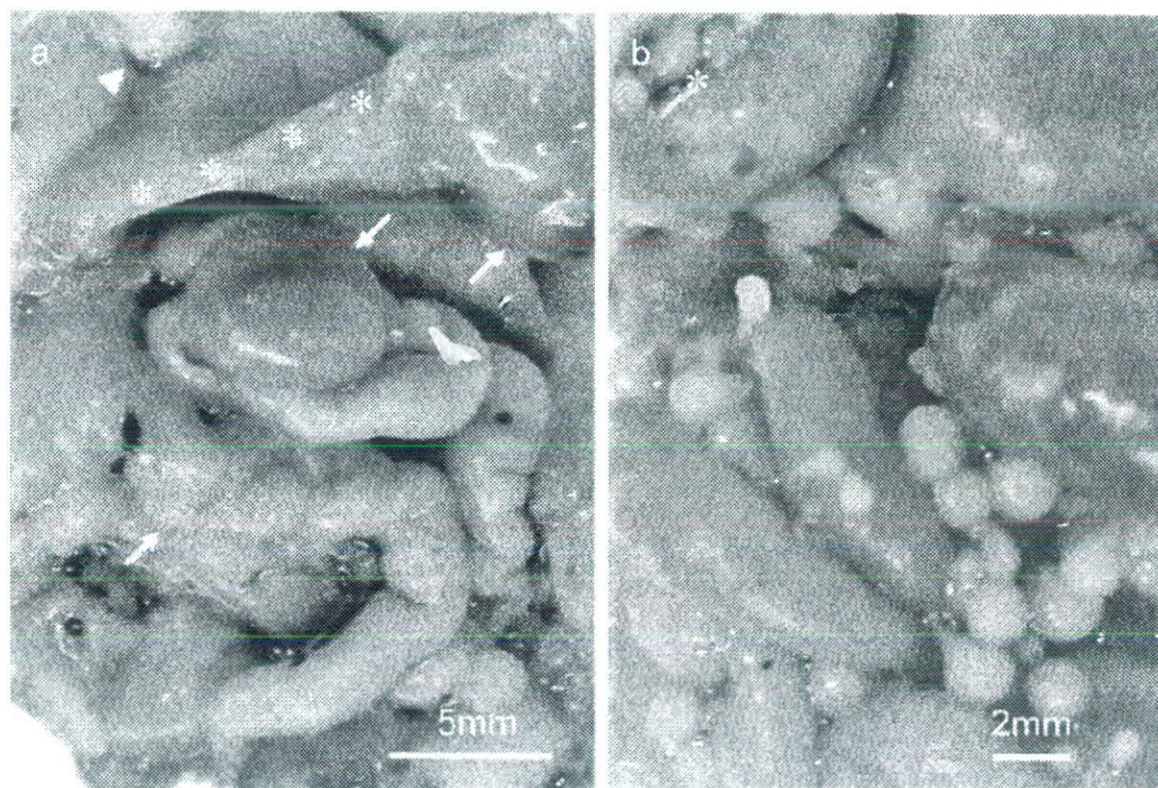


Fig. 5. Macroscopic view of abdominal viscera of MWCNT-treated and crocidolite-treated mouse:

Macroscopic view of the abdominal viscera excised *en bloc* of a) MWCNT-treated mouse that died at day 147, and b) crocidolite-treated mouse moribund on day 172 due to ileus. a) Fibrous adhesions of the visceral organs and multiple peritoneal tumor formation (arrows) are seen. Asterisks indicate the ventral cut end of diaphragma. One tumor penetrates the diaphragma and protrudes into pleural cavity (arrow head). Black spots are the aggregates of MWCNT. b) Multiple nodules up to 2 mm in diameter are induced on the serosal surface including liver (asterisk). Bluish green spots are the aggregates of crocidolite. Histology of the nodules is shown in Fig. 7a.

Mesothelioma by MWCNT in p53 +/- mouse.

was seen in the MWCNT group followed by the Crocidolite group, and the study was terminated at week 25 (day 180) and all mice of the Control and the Fullerene groups and 6 of the Crocidolite group were subject to autopsy. MWCNT-treated mice revealed moderate to severe fibrous peritoneal adhesion with slight ascites, fibrous peritoneal thickening with occasional black-colored depositions and a high incidence of macroscopic peritoneal tumors up to 2.7×1.5 cm in size (Fig. 5a). Similar findings but to a lesser extent with bluish green deposits were seen in asbestos-treated mice. In some cases, small polyp-like nodules were seen over the serosal surface (Fig. 5b). The Fullerene group showed no peritoneal adhesion, fibrous thickening nor tumor induction. Only small black plaques were scattered on the serosal surface.

Histologically, peritoneal adhesion and fibrous thicken-

ing of the MWCNT group mice was due to the formation of fibrous scars and foreign body granulomas against the MWCNT with phagocytic cells including multinucleated giant cells. Adjacent to those fibrogranulomatous lesions, a spectrum of peritoneal mesothelial lesions was seen, from nodular mesotheliomatous pile-ups of atypical mesothelial cells (Fig. 6), typical epithelial mesotheliomas with occasional hobnail appearance and mild to moderate fibrovascular stem formation (Fig. 7a), to large tumors measuring up to 2.7×1.5 cm in size composed of anaplastic cells with high mitotic rate and occasional central necrosis compatible with the diagnosis of high-grade malignant mesothelioma (Fig. 7b). Large tumors are invasive to the abdominal wall, diaphragm, liver parenchyma, and pancreas, and in some cases involving the thoracic cavity. No distant metastasis was observed so far as exam-

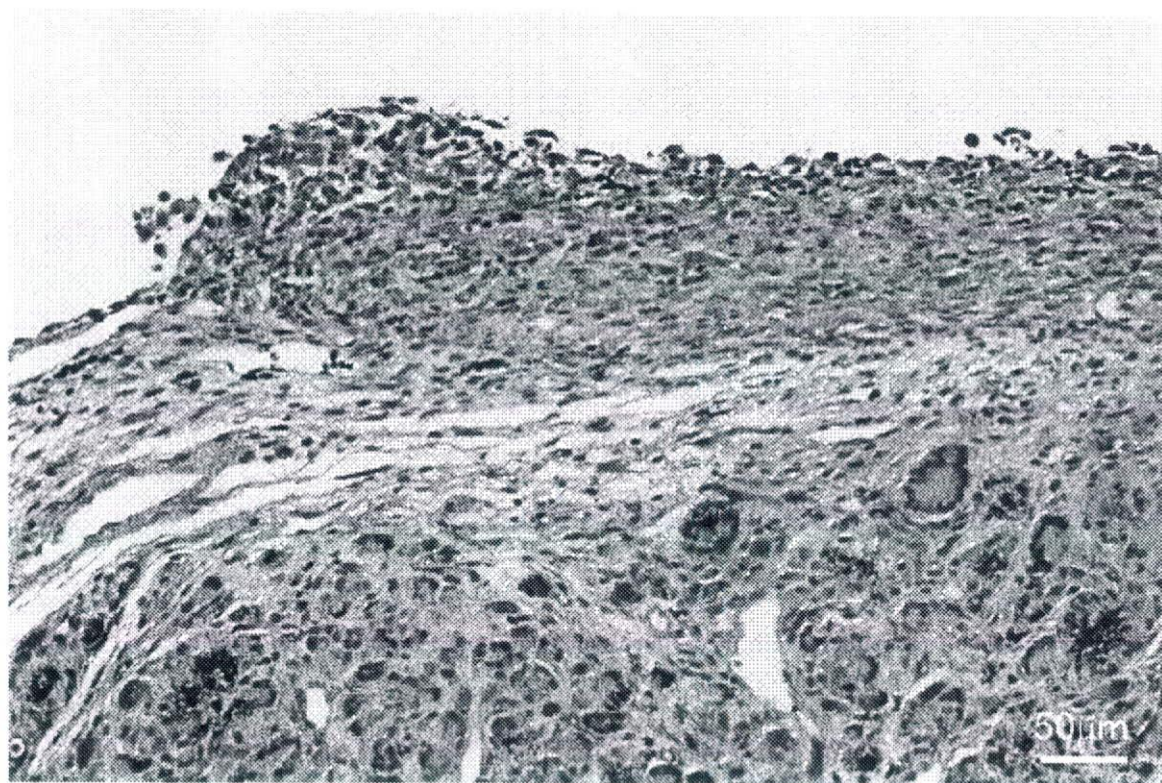


Fig. 6. Mesothelial response in MWCNT-treated mice:

Fibrous thickening of the peritoneum and foreign body granulomas against the MWCNT with phagocytic cells including multinucleated giant cells are formed in the MWCNT-treated mouse. Mesothelial lesions were found in the vicinity of fibrosis and granulomas. Microscopic mesotheliomatous plaques on the fibrotic peritoneum above a granuloma (MWCNT-treated mouse moribund on day 144 due to multiple mesotheliomas with severe peritoneal adhesion).

ined.

Cumulative mortality rate by mesothelioma is shown in Fig. 8. Mice with large/invasive mesotheliomas considered as cause of death are plotted by Kaplan-Meier method. Second major cause of death was constriction ileus due to severe peritoneal adhesion. Among those moribund/dead or terminated at week 25, there were 3 mice with incidental mesotheliomas in the MWCNT group (cause of death: all three by ileus) and 6 incidental mesotheliomas in the Crocidolite group (cause of death: three by ileus and three terminated at week 25). The overall incidence of mesothelioma after the first incidental case found in the MWCNT group at day 84 were 14/16 (87.5%, 11 found as cause of death, 3 as incidental) in MWCNT and 14/18 (77.8%, 8 found as cause of death, 6 as incidental including 3 terminated at week 25) in the Crocidolite group. Neither tumor induction nor interim death was observed in the Control and the Fullerene groups except for one moribund mouse by chronic pyelonephritis at day 152.

In large fibrous scars/granulation, aggregates similar to those shown in Fig. 2c and 2d were found embedded. Dis-

persed fibers of MWCNT and crocidolite were found extracellular in the fibrotic lesions or phagocytized by the phagocytic cells. Such fiber-laden cells were found not only in the peritoneal lesions but also in the liver within the hepatic sinusoids or along with the fibrous septum between the hepatic lobes, and in the mesenteric lymph nodes (Fig. 9).

In the Fullerene group, peritoneal lesion was minimal. Only small brownish black plaques were seen on the serosal surface. Histologically, the plaques contained polygonal clefts and lacunae surrounded by a thin layer of foamy cells and separated by thin fibrous septa (Fig. 10). The clefts/lacunae corresponded to the injected fullerene aggregates in size and shape. Since fullerene dissolves well in organic solvents, especially in xylene, the embedded particles were washed away during histology preparation, leaving clefts behind. It is noted that the edge of the clefts are tinted brown, indicating possible biodegradation of the surface of the fullerene particles by the phagocytic cells, blending proteins and/or other organic components so that the sub-micrometer fullerene grains become resis-

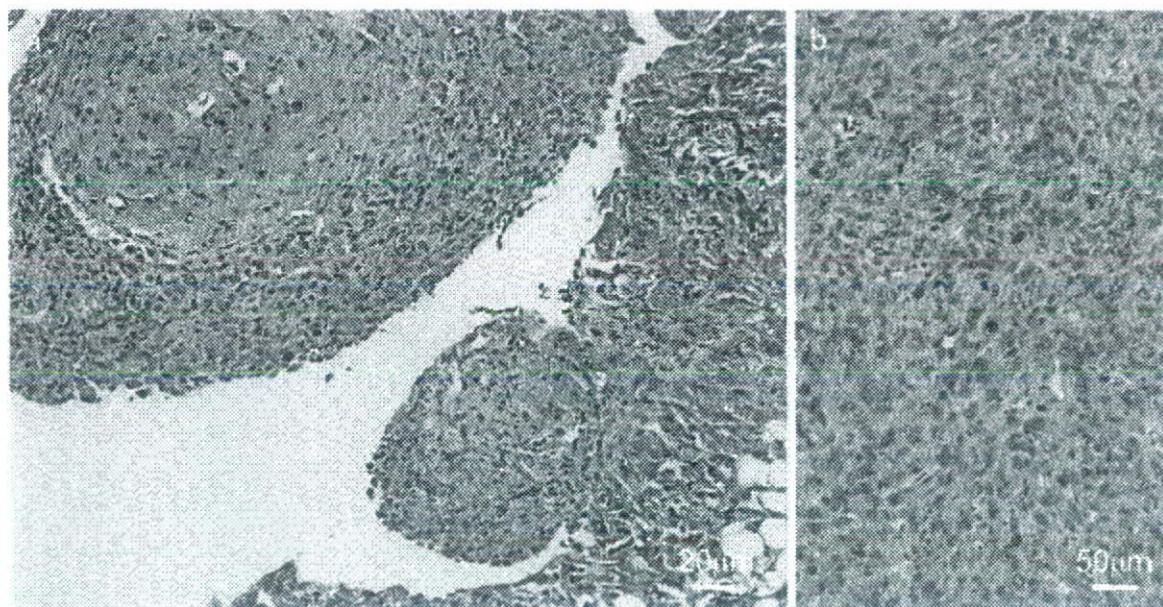


Fig. 7. Mesotheliomas in the Crocidolite group:

a) Typical mesothelioma nodules with fibrous stem induced in crocidolite-treated mouse (moribund on day 172 with multiple mesotheliomatous nodules with hemorrhagic ascites and peritoneal adhesion). b) Undifferentiated form of mesothelioma (so-called high-grade malignant mesothelioma) found as an invasive tumor of 1×1 cm in size (moribund case on day 170 with multiple invasive mesotheliomas up to 1×1.5 cm in size, severe peritoneal fibrosis and jaundice).