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석면과 대체물질의 독성연구 '94

- 한국산 석재용구의 섬유성 물질분석
- 석면에 의한 거식세포의 분화
- 석면과 대체물질의 독성학적 연구

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한국산업안전공단
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목 차

Analysis of Mineral Fibers from Stone-K

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한국산 석재용구의 섬유성 물질분석

Abstract

To analyze mineral fibers found in serpentine, which is used for stone kettle manufacturing and to protect workers in this factory, samples obtained from the factory were analyzed for their metal contents using an electron microscope equipped with energy dispersive X-ray analyzer. In addition, the metal contents were compared with other known asbestos. Although many kinds of fibers similar to asbestos were observed, any known asbestos fibers were not found. These fibers may have similar toxicities to known asbestos even if their toxicities are not known at this time. It is suggested that pulmonary function test and other chest X-ray are needed for the protection of stone-kettle manufacturing workers. Further epidemiological study is also required.

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To analyze mineral fibers found in serpentine which is used for stone kettle manufacturing and to protect workers in this factory, samples obtained from the factory were analyzed for their metal contents using an electron microscope equipped with energy dispersive X-ray analyzer. In addition, the metal contents were compared with other known asbestos. Although many kinds of fibers similar to asbestos were observed, amy known asbestos fibers were not found. These fibers may have similar toxicities to known asbestos even if their toxicities are not known at this time. It is suggested that pulmonary function test and other chest X-ray are needed for the protection of stone-kettle manufacturing workers. Further epidemiological study is also required.

한국산 석재용구의 섬유성 물질분석

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I. 서 론

우리나라에서 석면광상은 사문암에서 섬유상으로 산출되는 사문암질 석면 (온석면), 석회암 또는 규질석회암중 섬유집합체로 맥을 이룬 석유피, 편마암 결정편암, 석회암중의 각섬석군의 변질에 의해 생성된 각섬석 석면의 세가지 형태로 분류된다 (김 옥준, 1987). 온석면은 충남홍성, 경기도 가평에서 산출되고 chrysotile계 석면이고, 석유피는 경북 문경, 충북 제원군등에서 산출되며 sepiolite라고 사료되고, 각섬석계 석면은 전북 진안 장수군에서 산출된다고 한다.

금번의 연구에서는 전북장수 지방에 산재하고 있는 석재용구 가공 공장에서 사용하는 사용되는 암석에서 발견되는 섬유성 물질을 분석하여 이런 물질이 석면관련 물질인지를 파악하고자 하였고, 이 결과는 향후 석재용구 가공 근로자의 건강보호나 역학조사의 자료가 되고자 하였다.

II. 재료 및 방법

석재용구 가공 공장에 산재해있는 석재분말을 채취하여 시험관에 보관하였다. 석재용구 암석조각은 비닐백에 넣어 보관하였다. 200 ml의 마개가 달린 삼각플라스크에 극소량의 시료 분말과 증류수 100 ml을 가해서 1시간 동안 초음파로 분산한후 흡입여과 (nuclear pore filter, 0.2 um diameter) 하였다. Filter에 탄소증착후 그 일부 (약 3㎟ 정사각형)를 탄소막을 장착한 전자현미경 grid (200 mesh, Ni)에 끼우고 chloroform vapor를 이용하여 필터를 용해하여 제거시켰다. KEVEX-7000Q형 에너지분산형 X-선 분석장치 (Energy Dispersive X-ray analysis equipment; EDX)가 부착된 Hitachi-800형 전자현미경을 사용하였고 석면의 정성분석은 섬유의 형상과 조성에 따라 실시하였다.

Table 1. Major constituents of Asbetos*

		Si	Fe	Mg	Na	Ca	Al
Serpentine	Chrysotile	●		●			
Amphibole	Crocidolite	●	●	□	□		
	Amosite	●	●	□			
	Anthophyllite	●	▲	□			
	Tremolite	●		●		▲	
	Actinolite	●	●			▲	

● : more than 20%

▲ : 10-20%

□ : 4-10%

* : Summarized from Environmental Health Criteria 53, 1986, WHO.

III. 결 과

4종의 시료를 채취하였다. 시료 A는 석재용구공장에 산재 해있는 석재용구 분진을 채취하였고, Figure 1A는 석재용구 파편을 보여준다. 시료 B는 Figure 1B에서 보는 바와 같아 검은 색깔을 띤 암석이고 시료 C는 또 다른 검은 색깔을 띤 암석이었고 (Figure 1C), 시료 D는 하얀색을 띤 암석조각이었다 (Figure 1D).

이런 시료들의 분진을 광학현미경으로 관찰하였을때 섬유상 물질을 발견할 수 있었다. EDX가 부착된 전자현미경으로 분석하였을때, 시료 A에서는 섬유상 물질이 관찰되었고 (Figure 2 A, B, and C), 2가지 형태의 섬유가 발견되었다. Si, Al, Ca이 주성분인 섬유 (type 1, Figure 3A)와 Si, Mg, Fe가 주성분인 섬유 (type 3, Figure 3B)가 발견되었다.

시료 B에서도 역시 섬유상 물질은 관찰되었고 (Figure 4 A, B, C, and D), 5가지 형태의 섬유가 발견되었다. Si, Al, Ca 이 주성분인 형태 (type 1, Figure 5A), Si, Al, Fe가 주성분인 형태 (type 2, Figure 5B), Si, Mg, Fe가 주성분인 형태 (type 3, Figure 5C), Si, Al, Mg, Fe가 주성분인 형태 (type 4, Figure 5D), 그리고 Si, Al, Mg, Fe, K가 주성분인 형태 (type 4, Figure 5E) 가 발견되었다.

시료 C에서도 섬유상물질이 발견되었고 (Figure 6), 4가지 형태의 섬유가 발견되었다. Si, Al, Fe가 주성분인 type 2 (Figure 7A), Si, Mg, Fe가 주성분인 type 3 (Figure 7B), Si, Al, Mg, Fe, K가 주성분인 type 5 (Figure 7C), 그리고 Si, Ca, Al, Fe, Mg가 주성분인 type 6 (Figure 7D)가 발견되었다.

시료 D에서도 섬유성 물질이 발견되었으며 (Figure 8 A & B), 3가지 형태의 섬유가 발견되었다. Si, Al, Fe 주성분인 type 2 (Figure 9A), Si, Al, Mg, Fe, K가 주성분인 type 5 (Figure 9B), Si, Al, Mg, Ca, Fe가 주성분인 type 6 (Figure 9C)가 발견되었다.

위에서 보는바와 같이 4종의 시료에서는 석면은 검출되지 않았다. 섬유성 물질의 대부분은 규산 (Si)과 알루미늄 (Al)이 주성분이었다. 에너지 분산형 X-선 분석에 의하면 섬유성 물질은 6 가지 형태로 나눌수 있었다. type 1은 Si, Al, Ca이 주성분이었고, type 2는 Si, Al, Fe가 주성분, type 3는 Si, Mg, Fe가 주성분, type 4는 Si, Al, Mg와 Fe, type 5는 Si, Al, Mg, K, Fe, type 6는 Si, Al, Mg. Ca, Fe 였다.

이런 6가지 형태의 섬유를 chrysotile과 amosite와 비교하였다. Figure 10A 에서는 chrysotile 섬유의 형태를 보여주고 있고 주성분은 Si와 Mg 인 것을 볼 수 있다 (Figure 10B). Figure 11A에서는 amosite 섬유의 형태를 볼수 있고, 주성분이 Si, Fe, Mg, Mn인 것을 볼수 있다 (Figure 11 B). Type 3가 Si, Mg, Fe를 주성분으로 하여 chrysotile과 유사한 점은 있었으나 chrysotile에서 발견되는 관상구조를 가지고 있지 않았고, Mg/Si의 비가 작고 (chrysotile의 경우는 Mg/Si= 약 1), Fe를 약 10% 이상 함유하고 있어, talc도 아니라고 추정된다. 그리고 이런 섬유들을 다른 알려진 석면과 비교하였을때 (Table 1), 기존의 알려진 석면과는 다른 형태를 보여주고 있다.

Figure 1. Samples used for EDX analysis

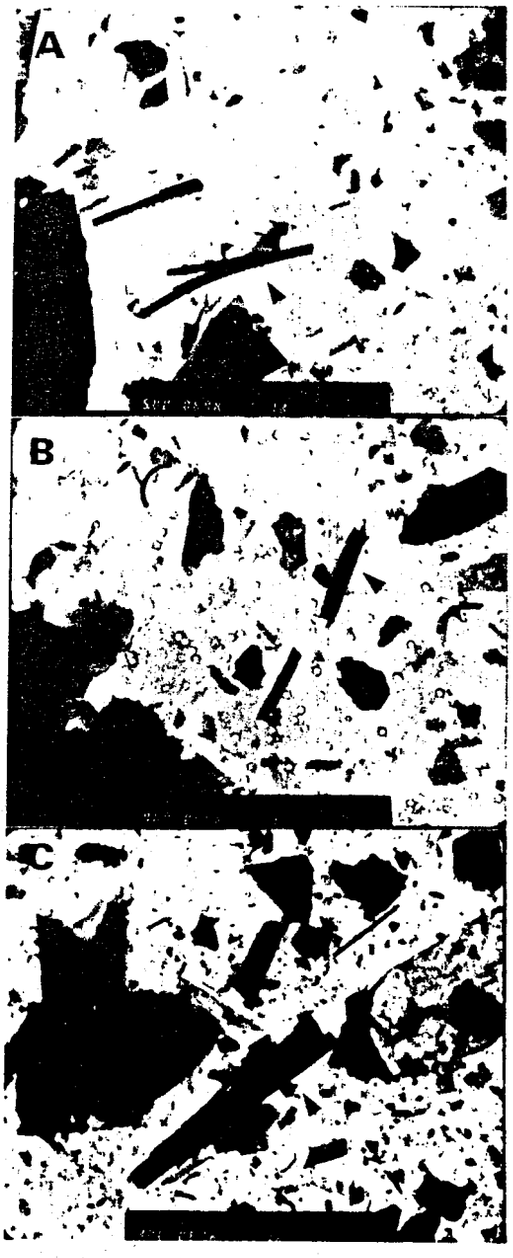

*Figure 2. Fibers found in sample A. A, magnification x5000; B, 7000x; C, x3000. Arrows indicate fibers.

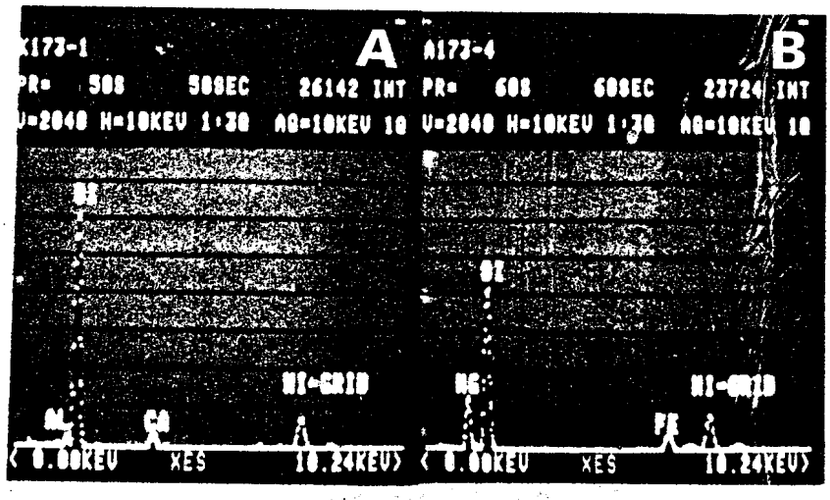

Figure 3. EDX patterns of sample A.

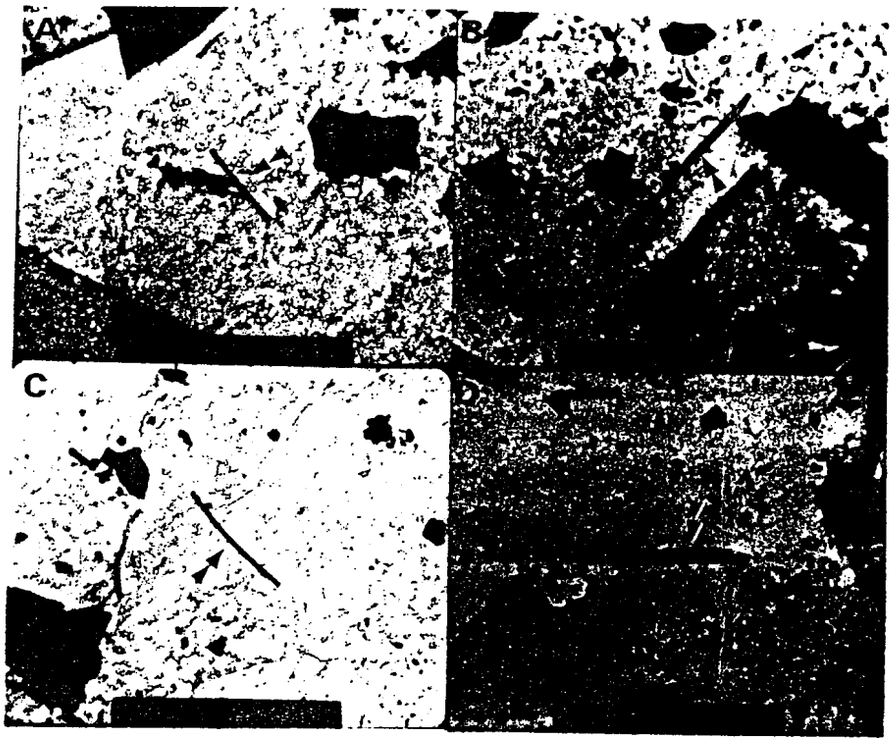

Figure 4. Fibers found in sample B. A, magnification x5000; B, x4000; C, x4000; D, 5000. Arrows indicate fibers.

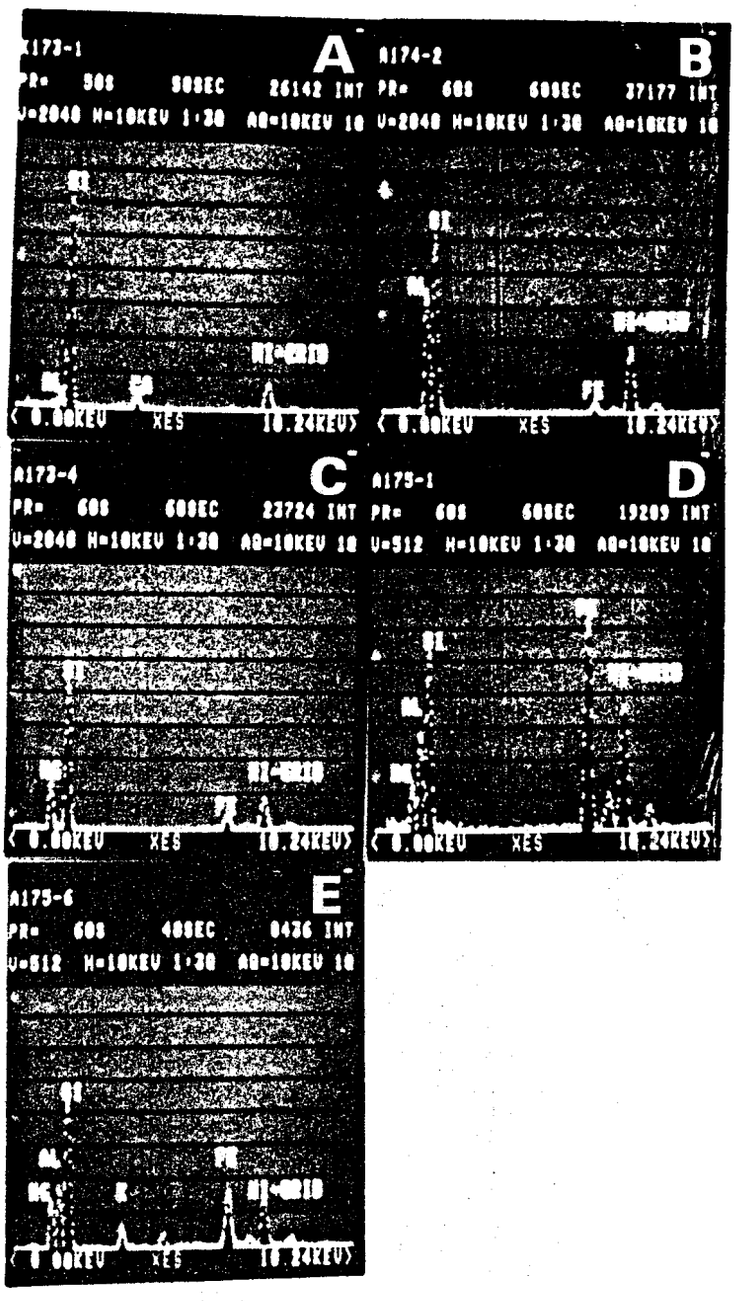

Figure 5. EDX patterns of sample B.

Figure 6. Fibers found in sample C. A, magnification x5000: B, x3500: C, x4000. Arrows indicate fibers.

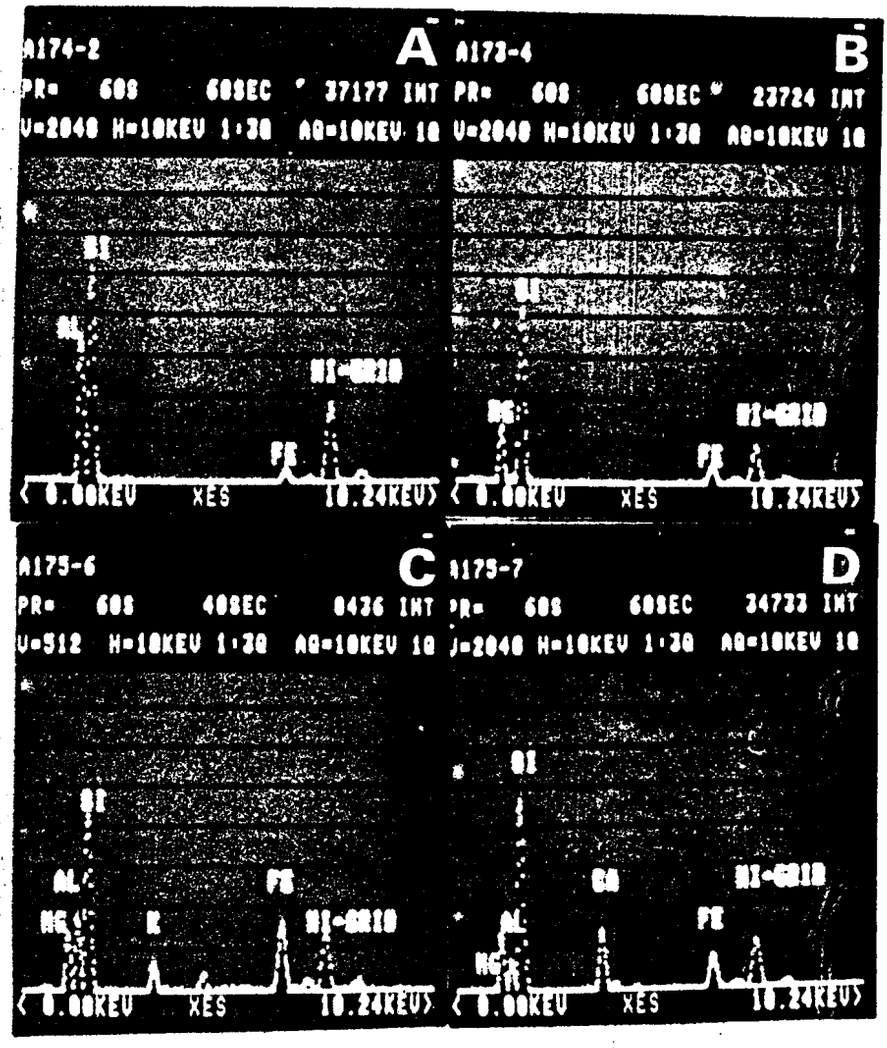

Figure 7. EDX patterns of sample C.

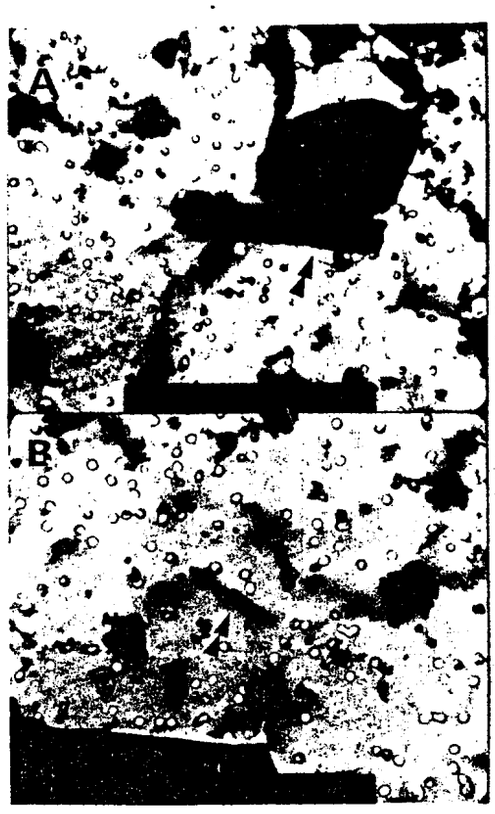

Figure 8. Fibers found in sample D. A, magnification x8000; B, x10000. Arrows indicate fibers.

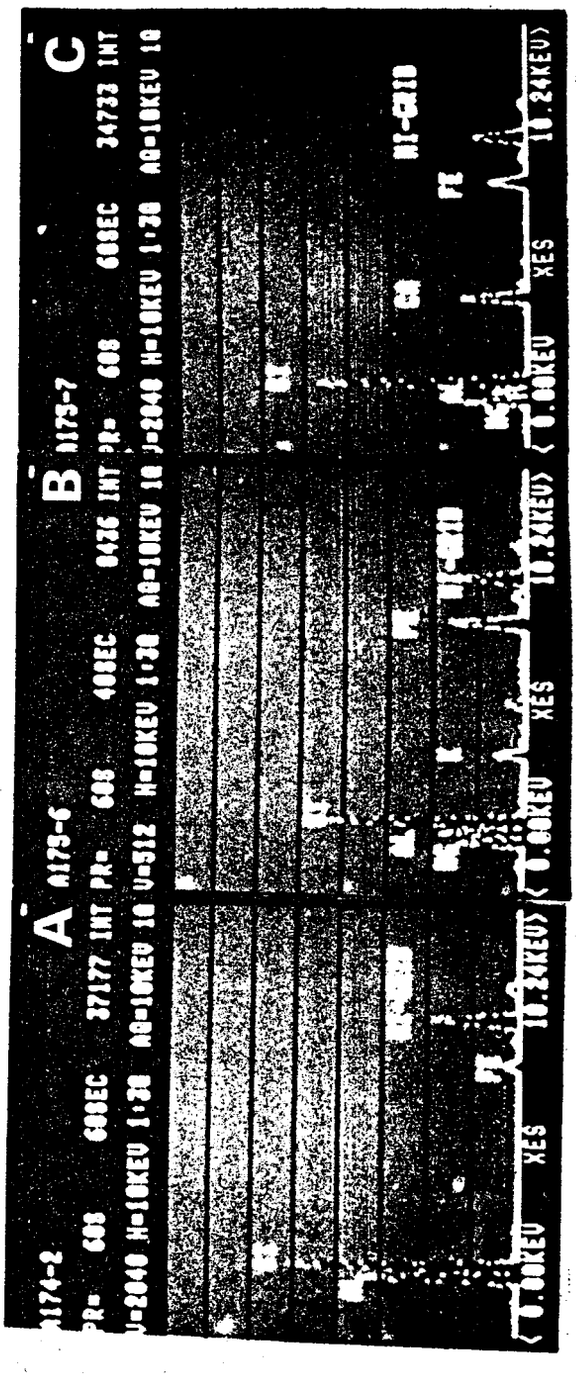

Figure 9. EDX patterns of sample D.

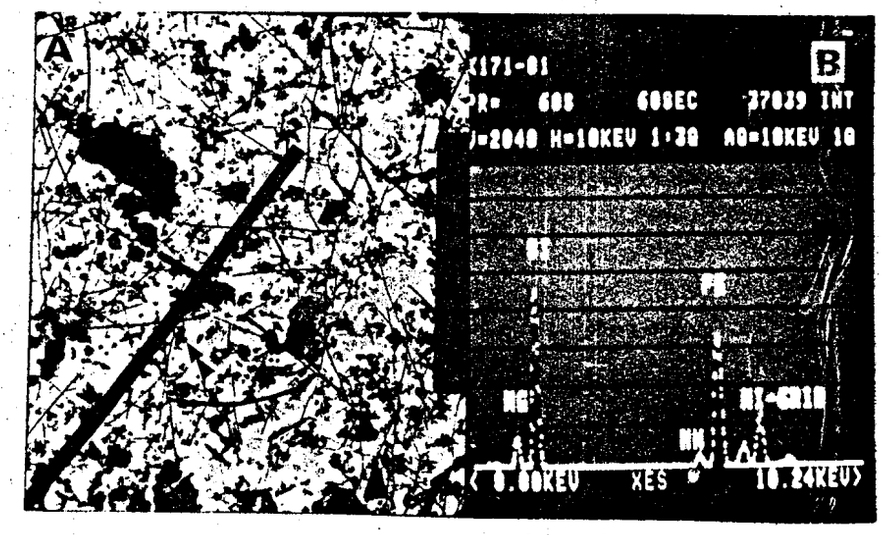

Figure 10. Amosite fiber and EDX pattern. A, magnification x4000: B, EDX pattern. Arrows indicate fibers.

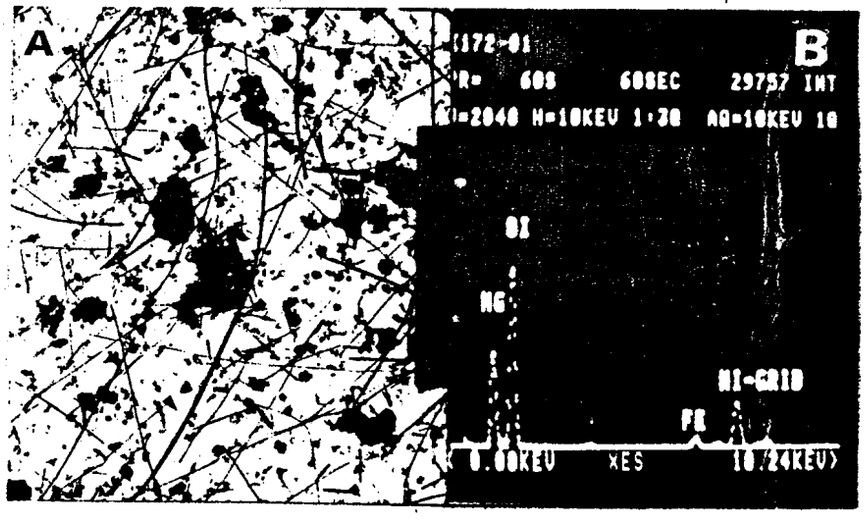

Figure 11. Chrysotile fiber and EDX pattern. A, magnification x5000: B, EDX pattern of chrysotile.

IV. 고 찰

4종의 시료분석결과에서 보이는 바와 같이 석재용구에서는 공정과정에서 여러 종류의 섬유성 물질의 발견되었다. 이 섬유성 물질은 일반적으로 알려진 석면물질 즉 chrysotile이나 crocidolite, amosite등과 전혀 다른 광물의 조성을 보여주고 있다. 이런 물질의 유해성이나 독성학적인 연구는 전혀 되어있지 않은 상태이다. 그러나 이런 섬유들의 유해성은 석면섬유와 유해성이 거의 동일하다고 한다 (Nozaki, personal communication). 일반적으로 분진중에서도 섬유성 분진의 유해성이 가장 높다고 알려져 있으므로 석재용구 가공에 일하고 있는 근로자의 폐기능검사와 흉부X-선 촬영등의 정밀건강진단과 역학조사가 시급하다고 생각된다.

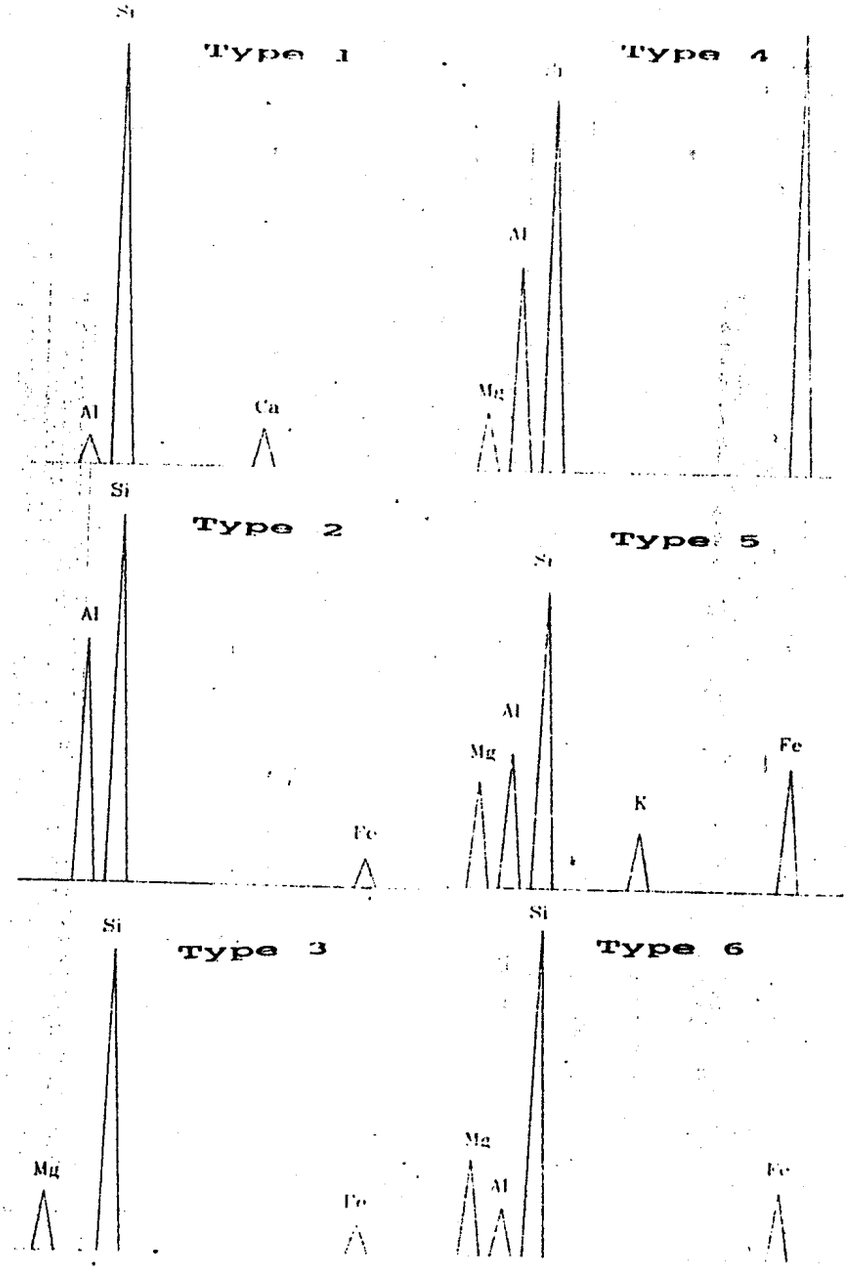

Figure 12. EDX patterns of fibers from stone-kettle.

V. 결론 및 요약

우리나라 석재용구 가공 공장에서 사용되는 암석 (사문암)에서 발견되는 섬유성 물질을 분석하여 석면 관련 물질인지를 조사하고자 석재가공 근로자의 건강보호나 역학조사의 기초자료가 되기위해 석재용구 가공공장에서 시료를 취하여 전자현미경에 장착된 X-ray 분석장치를 이용하요 성분을 분석하고 기존 석면과 비교하였다. 많은 종류의 광물 섬유성 물질이 발견되었고 이들의 성분을 분석한 결과 기존 석면은 발견되지 않았으나 석면과 유사한 물질들이 발견되었다. 이들의 유해성은 아직 알려지지 않았으나 석면과 거의 동일한 유해성을 보여준리라 추정된다. 석재용구 가공 공장에서 근로자의 폐기능 검사나 흉부 X-선 촬영 등의 전밀건강 진단과 역학조사가 시급하다고 생각된다.

VI. 참고문헌

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Growth and Differentiation of Macrophages by Asbestos

Il Je Yu

석면에 의한 거식세포의 분화

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— Abstract —

Asbestos has been implicated in lung fibrosis and mesothelioma. The disruption of cell division cycle as been suggested for on of mechanism for the diseases processes. To understand the possible role of asbestos fibers on the cell division cycle, RAW macrophage and Chinese hamster lung cell (CHL) were cultured for 6 days with 200 ug/ml of chrysotile fibers (average size 4 um). In each day, the duplicate cultures were processed for histopathology and immunohistochemistry. The histopathology of RAW macrophage treated with asbestos fiber showed continual cellular differentiation from the third day, the macrophages were developed into multinuclear cells and the asbestos fibers were completely covered with cellular materials. In contrast, macrophages without asbestos fibers showed continual cell division. The histopathology of CHL cell treated with asbestos showed massive cell death from the beginning, while CHL cell without asbestos fiber showed continual cell division. Antibody against p34cdc2 kinase, an essential enzyme for cell division cycle in eukaryotic cells, was employed to monitor changes of p34cdc2 kinase. In addition, p34cdc2 specific peptide was also used for measuring the kinase activity. The macrophages differentiated by asbestos fiber showed significant decreases on p34cdc2 kinase level. Immunoblottings of p34cdc2 also coincided with the results of the kinase activity level. Taken together, our results indicated that asbestos fiber induces cellular differentiation for macrophages by suppressing cell division cycle.

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I. 서 론

석면에 의한 asbestosis나 중피종 (mesothelioma)은 이미 널리 알려진 사실이다. 그러나 이런 asbestosis나 중피종의 기전은 면역학과 분자생물학의 발달로 조금씩 서서히 밝혀지고 있다. Asbestosis는 폐 구종의 파괴와 결합조직 (connective tissue)의 과다성장에 의한 diffuse interstitial fibrosis와 small airway fibrosis로 특징지어질 수 있다. 석면폭로에 위한 섬유세포 (fibroblast)의 과다성장의 자극은 백혈구에서 cytokine, tumor necrosis factor (TNF), Interleukin (IL)의 분비가 증가함에 따라 일어난다. Brown 등은 (1991) 흰쥐의 기관지에 amosite를 주입 (instillation)했을때 폐거식세포 (BAL macrophages)에서의 IL-1과 TNF의 증가를 관찰했다. 그리고 TNF 분비는 길이가 긴 석면섬유가 짧은 섬유일때보다 더 많은 분비를 유도됨을 보고하였다. 기관지내로 주입한 chrysotile에 의해 발생되는 물질들에 의해 fibroblast들이 분열하고 궁극적으로 fibrosis를 일으켰다고 보고했다 (Lemaire et al., 1986). Fibronectin 같은 물질이 BAL macrophage에 의해 생성되어 세포의 성장을 촉진시킬수도 있다고 한다. 석면에 폭로된 동물에서 증가된 fibronectin이 관찰되었다고 Begin 등 (1986)은 보고하였다.

성장 호르몬 (growth factor)에 의한 cell division cycle 조절은 최근 많이 연구되고 있는 분야이다. 이에 관여하는 growth factor는 platelet derived growth factor (PDGF), fibronectin, IL-1, insulin-like growth factor (IGF), transforming growth factor (TGF) 들이 cell cycle에 중요한 역할을 한다고 한다. Alveloar macrophages는 이런 물질 (IL-1, PDGF, fibronectin, TNF-α, FGF, EGF, TGF-β 들을 생성하여 분비한다 (Kovacs, 1991). Asbestos에 의해 이런 물질이 PDGF의 생성자극을 보고했고(Kumar, 1988), 증가된 IGF-1의 mRNA를 asbestosis 환자의 alveoloar macrophage에서 관찰할 수 있었다고 한다 (Rom et al., 1988). 석면폭로후 1주에서 24주까지 관칠한 결과 조직병리학적인 과정과 동일하게 흰쥐의 alveolar macrophage에서 부터 FGF나 MDGF (macrophage-derived growth factor) 등의 분비를 볼 수 있었다고 한다 (Lemaire et al., 1986). Fiber의 길이에 상관없이 fibroblast 분열 호

르몬을 분비한다는 보고도 있지만 (Adamson & Bowden, 1990), long fiber에서는 fibroblast의 분열을 촉진하는 호르몬을 관찰할 수는 없었지만 병리학적인 영향은 관찰할 수 있었고, short fiber에서는 병리학적인 영향이 없이 fibroblast의 분열을 촉진하는 호르몬을 관찰할 수 있었다고 한다 (Davis et al., 1986).

석면에 의한 cell cycle에 영향을 주는 호르몬에 대한 연구는 다소 진행이 되었으나, 궁극적인 과정의 하나로 석면에 의한 cell cycle의 영향에서 cell cycle에 관계하는 중요한 조절 단백질의 역할은 전혀 연구가 되어있지 않다. Cell cycle의 가장 중요한 효소의 하나인 p34cdc2 kinase는 eukaryotic cell cycle을 진행하는데 있어서 가장 중요한 작용을 하고 있다. 이것의 cyclin이라는 regulatory subunit과 p34cdc2의 catalytic subunit으로 되어있는데 세포가 분리하는 M phase에서 이 효소의 활성도가 최대가 된다. 그리고 분열하지 않는 세포에서는 이 효소의 활성도는 매우 낮은 것을 볼 수 있다.

본 연구에서는 석면이 fibroblast나 macrophage의 cell cycle에 어떻게 영향을 주는가를 연구하고자 한다. 모델 시스템으로서는 RAW macrophage cell line과 CHL fibroblast (Chinese Hamster Lung cell)을 이용하였다. 물론 성숙한 macrophge는 분열하지 않지만 손쉽게 배양하여 언제든지 사용할 수 있다는 점에서 이 세포들을 이용하였다. 본연구에서는 석면을 탐식한 세포들이 어떤 조직병리학적인 형태를 보여주는가를 조사하였고, 이런 과정들이 분자생화학적인 면에서 cell cycle에 어떤 영향을 끼치는가를 연구하였다.

II. 재료 및 방법

1. 석 면

UICC chrysotile (평균 4 um)을 사용하였다.

2. 세포배양

RAW cell은 mouse macrophage cell line으로서 heat inactivated 10% fetal bovine serum과 penicillin과 streptomycin (100 unit/ml) 함유된 RPMI 1640 (RPMI-FCSHI)에서 37°C 배양기에서 배양하였다. CHL (chinese hamster lung cell)은 lung fibroblast로서 heat inactivated 10% fetal bovine serum과 penicillin과 streptomycin (100 unit/ml) 함유된 DMEM (DMEM-FCSHI)에서 37°C 배양기에서 배양하였다.

3. 조직병리학

100,000 개의 세포는 24 Well 배양용기에 넣어둔 유리 coverslip (12 mm diameter) 위에서 밤새 37°C에서 배양하였다. 그리고 나서 석면섬유를 적절한 농도로 더하였다. 석면의 농도는 200 ug 부터 0.1 ug 까지 였다. 석면섬유를 더한후 일정기간이 지난 배양세포는 Wright법을 이용한 Leukostat (Fisher)에 의해 염색하였다. 조직병리학적 검사는 Olympus 현미경을 사용하였다.

4. Protein kinase assay

Peptide를 이용하여 protein kinase activity를 측정하였다. Peptide는 ADAQHK TPPKKKRKVEDPKDF (CSH 103)의 아미노산 서열을 가지고 있고 SV40 LTAg의 아미노산서열을 지니고 있었다. 이것을 이용하여 p34cdc2 kinase의 activity를 측정하였다. 측정방법은 유 일재 등에 의한 방법을 사용하였다. [$^{32}PO_4$]가 p34cdc2 특이기질 peptide의 threonine에 들어가는 것을 방사성 동위원소를 이용하여 측정하였다. 먼저 시료 5 μl를 p34cdc2 buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothritol(DTT))에 넣어 1 mM 기질과 100 uM [γ-^{32}P]-ATP (1000-3000 cpm/pmol) 함께 총 30 μl가 되게 한다. 효소반응은 방사성동위원소 ATP를 더함으로서 시작되게 한다. 반응은 trichloroacetic acid를 최종 10% 가 되게 더하여 정지시킨다. 효소반응의 정도는 인산이 phosphocellululose paper에 흡착된 것을 동위원소측정방법으로 측정한다 (Kuenzel & Krebs, 1985; Marshak & Carroll, 1991).

5. 전기영동과 Immunoblotting

sodium dodecyl sulfate (SDS)존재하에서 12.5% (w/v)의 polyacrylamide gel을 이용하여 전기영동을 하였다 (Laemmli, 1970). 전기영동후 분리된 단백질은 전기적으로 nitrocellulose paper (NC)에 옮겨 졌다 (Towbin et al., 1979 & 1984). NC paper는 3% (w/v) bovine serum albumin (BSA)로 비특이적인 흡착을 방지하기 위해 blocking 하였다. 그리고 적절하게 희석한 anti-p34cdc2 kinase 항체 (G6)로 반응시킨 후, goat anti-rabbit IgG alkaline phosphatase가 부착된 2차 항체에 의해 현상하였다.

III. 결 과

석면 (200 ug)을 chrysotile 석면을 더하여 배양한 세포에서 세포의 형태에따라 전혀 다른 세포적 반응을 볼 수 있었다. Figure 1 A (1d), C (2d), E (3d), G (4d), I (5d), K (6d)는 석면을 더하지 않고 1 day 부터 6 day 까지 배양한 CHL 세포로 세포가 점점 분열해 나가면서 세포수가 증식되고 배양용기를 가득채운 것을 볼수 있다. 이와 반대로 석면을 더하여 배양한 CHL 세포는 (Figure 1 B (1d), D (2d), F (3d), H (4d), J (5d), L (6d)) 1 day 부터 cell death를 볼수가 있었고 2 day 부터는 거의 살아있는 세포를 관찰할 수 없었다. 이 실험의 결과로 CHL lung fibroblast는 chrysotile fiber에 아주 민감한 반응을 보여 cell death 일으키고 배양용기에 부착하여 성장 (growth with attachment)하는 능력을 상실케 하는 것을 알 수 있었다.

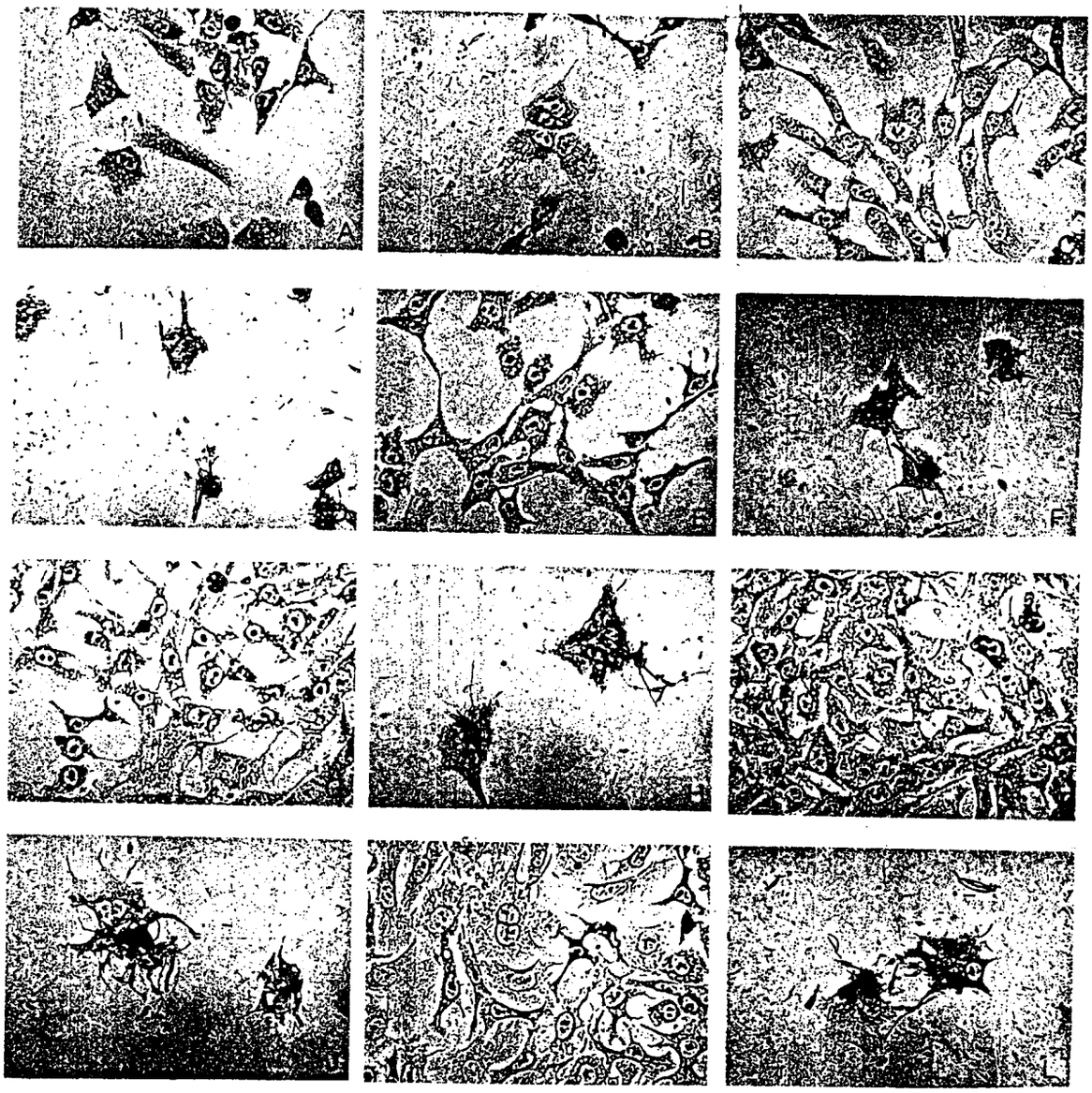

Figure 1. CHL cells cultured with absence of chrysotile fibers. A (1 d), C (2 d), E (3 d), G (4 d), I (5 d), and K (6 d), and CHL Cells cultured with presence of chrysotile fibers B (1 d), D (2 d), F (3 d), H (4 d), J (5 d), and L (6 d).

RAW cell (mouse macrophage cell line)은 CHL cell 과는 달리 석면에 의한 세포의 분화를 관찰할 수 있었다. Figure 2 A (1d), C (2d), E (3d), G (4d), I (5d), K (6d) 에서 보이는 것과 같이 RAW cell은 석면이 없을때는 둥근형태를 가지면서 신속히 분열하여 배양용기를 6일째에는 완전히 채우는 것을 볼 수 있다. 이와 반대로 석면을 더한 RAW cell은 Figure 2 B (1d), D (2d), F (3d), H (4d), J (5d), L (6d)에서 보이는 것과 같이 chrysotile 석면에 의해 세포분열을 멈추고 2 day 부터 multinuclear cell을 형성하여 4, 5, 6 일 째에는 giant multinuclear cell로 변하는 것을 볼수 있다. 많은 세포들이 석면을 탐식한 것도 볼수 있다.

이렇게 석면에 의해 세포분열을 멈추고 분화하는 현상을 규명하게 하기 위해, 세포분열에 (cell cycle) 가장 핵심적인 작용을 하는 p34cdc2 kinase 의 activity와 양(amount)이 어떻게 조절되는 지를 연구하였다. Figure 3에서 보는 바와 같이 p34cdc2 kinase의 activity는 석면을 더하지 않은 세포에서는 큰 변화가 없으나 chrysotile을 더한 세포에서는 배양일수가 증가하면서 점점 activity가 떨어지는 것을 볼 수 있다.

또한 p34cdc2의 양을 immunoblotting을 이용하여 측정하였을 때에도, 석면을 더하지 않은 세포에서는 p34cdc2 kinase의 양에서 큰 변화를 보여주지 않았으나. 석면을 더하여 배양한 RAW cell에서는 p34cdc2의 양이 배양일수에 따라 감소하는 것을 볼 수 있었다 (Figure 4).

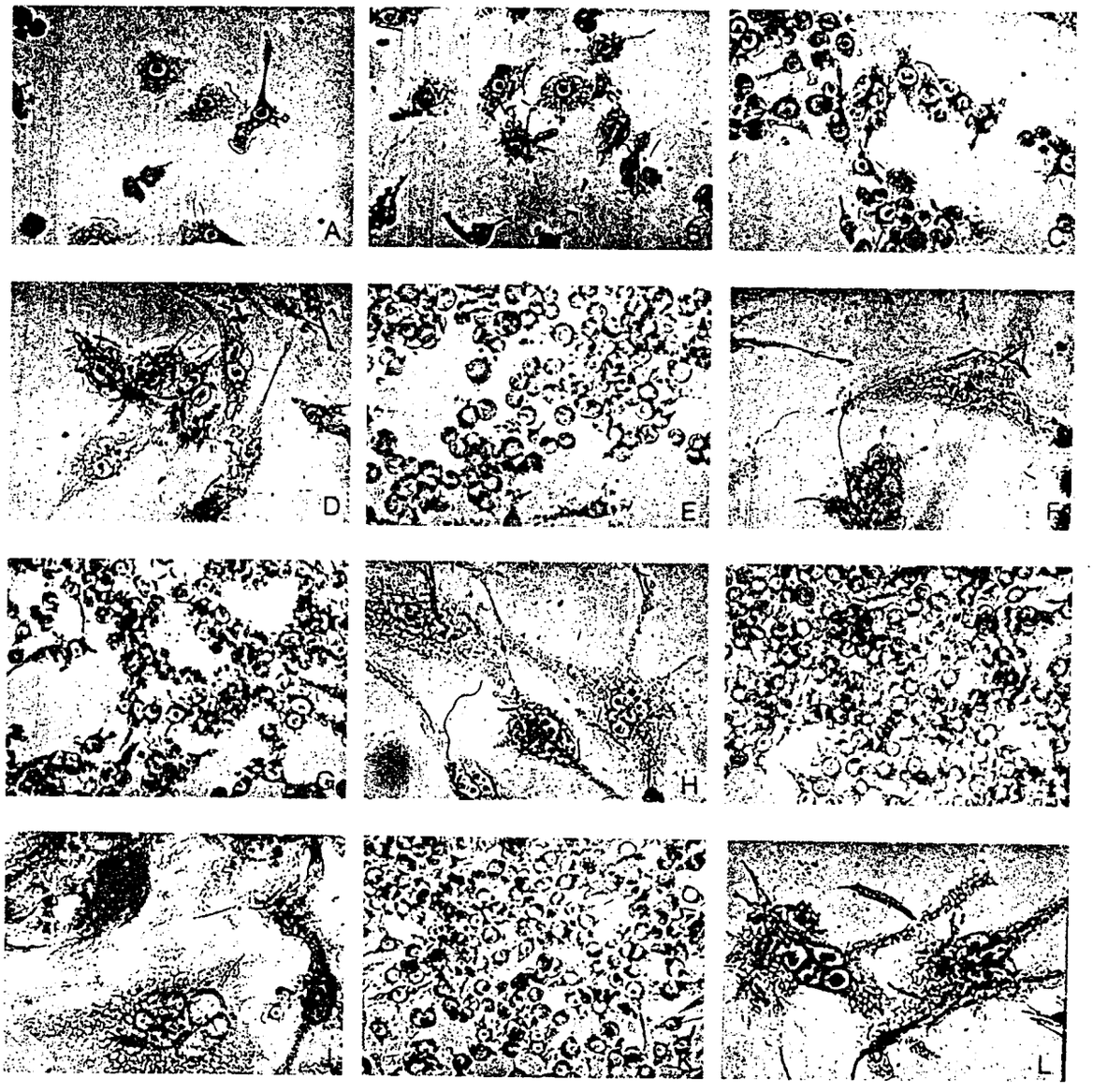

Figure 2. RAW cells cultured with absence of chrysotile fibers. A (1 d), C (2 d), E (3 d), G (4 d), I (5 d), and K (6 d), and RAW Cells cultured with presence of chrysotile fibers B (1 d), D (2 d), F (3 d), H (4 d), J (5 d), and L (6 d).

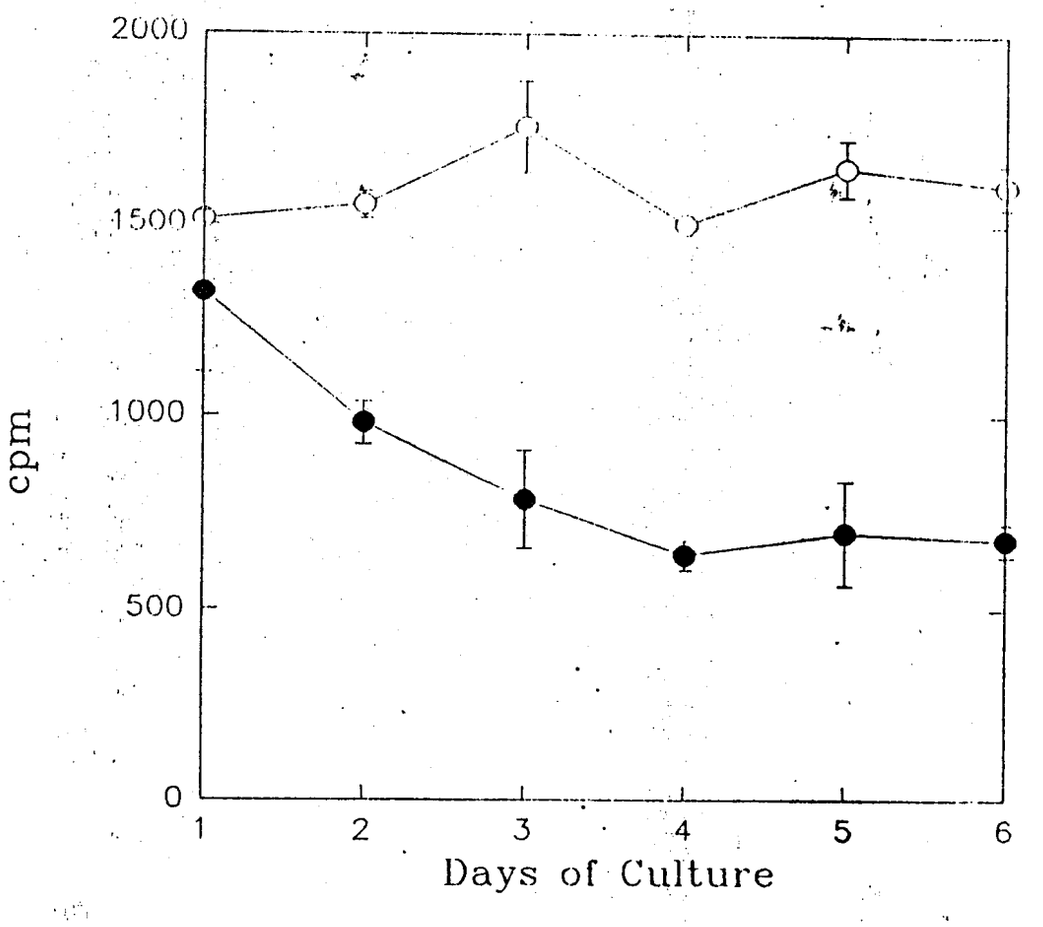

Figure 3. p34cdc2 kinase activity of RAW cells cultured with chrysotile (closed circles) and without chrysotile fibers (open circles).

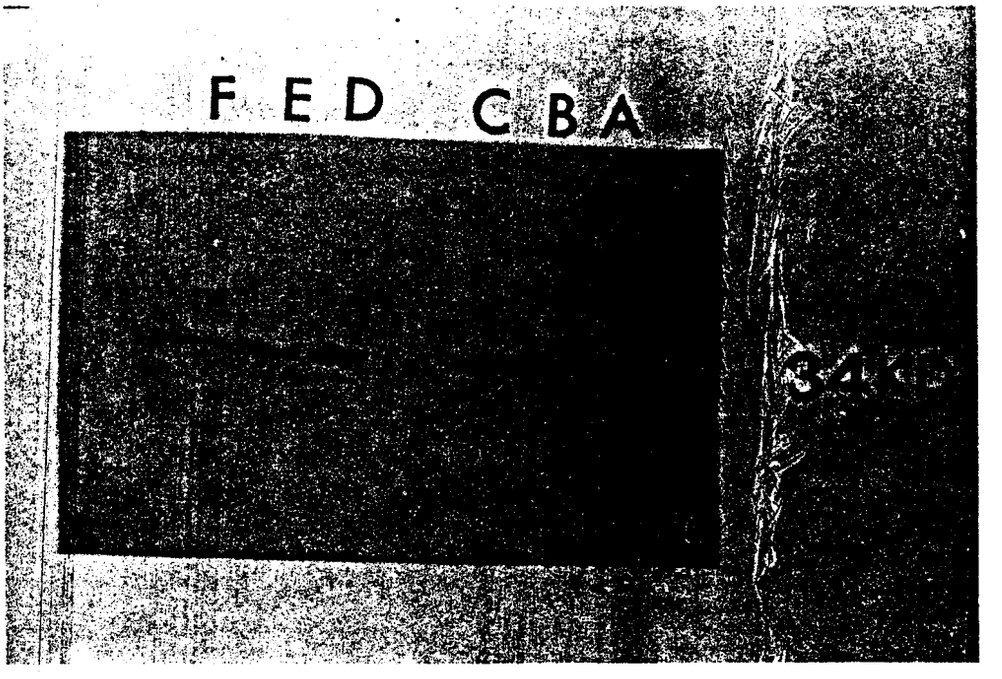

Figure 4. Immunoblotting of p34cdc2 kinase of RAW cells cultured with chrysotile (lane 1, 2, and 3) and without chrysotile fibers (lane 4, 5, and 6). lane 1 & 4 (1 d), lane 2 & 5 (3 d), lane 3 & 6 (5 d).

IV. 고 찰

이번 연구 결과에서는 chrysotile 석면이 세포반응에 어떤 영향을 끼치는 가를 조사 하였다. Fibroblast와 macrophage는 전혀 다른 세포반응을 보여주었는데 석면에 의하여 fibroblast는 급격한 cell death를 RAW cell은 분열을 멈추고 분화하는 것을 볼 수 있었다. 석면이 어떻게 세포분열을 멈추게 하는지는 아직 잘 알수 없으나 석면을 탐식한 세포의 시호전달계 (Signal transduction pathway)가 fibroblast와 macrophge는 전혀 다른 양상임을 제시하고 있다. CHL fibroblast는 급격한 apoptosis로 이끄는 신호전달계를 가지고 있고, 금번 연구에서 보듯이 RAW macrophage는 cell division cycle을 멈추고 분화 (differentiation)하게하는 신호전달계로 가는 것을 볼수 있다. 금번 연구에서는 이런 apoptosis에 관계한다고 알려진 항암유전자 p53을 조사하지 않았지만 p53의 역할을 석면을 탐식한 세포에서 알게 된다면 p34cdc2와 p53의 관계로 부터 cell death와 cellular differentiation을 좀더 명확하게 알 수가 있을 것이다.

이 결과를 in vivo 상황에서 고찰해보면 흡입된 석면은 대부분 alveolar macrophage나 interstitial macrophage에 의해 탐식되어 interstitial fibroblast에게는 미량이 영향을 미치리라 생각된다. 만약 석면 fiber가 interstitial fibroblast에 의해 탐식되면 interstitial fibroblast는 곧 죽게 될 것이고 다시 macrophge에 의해 탐식되리라 생각된다. 그리고 석면 fiber를 탐식한 macrophage는 더욱 분화하여 cellular factor를 분비하여 fibrosis를 촉진하리라고 생각된다.

금번 실험에서는 석면을 탐식한 macrophage가 분비하는 물질들을 조사하지는 못하였다. 석면을 탐식한 macrophage의 culture supernatant를 fibrobalst culture에 더하여 ^{3}H thymididine을 이용한 labelling을 하여 세포분열이 촉진되는가를 살펴보는 연구가 산행되어야 할 것이다. 그리고 supernatant에서의 PDGF, fibronectin, TNF-α, INF-γ, EGF, FGF 등의 fibroblast 성장 조절물질을 규명하는 연구가 필요하다고 하겠다.

V. 결론 및 요약

석면은 폐의 fibrosis와 종피종을 유발한다고 알려져 있다. 이런 과정에 세포분열사이클 (cell division cycle)의 붕괴가 하나의 기전으로 제시되고 있다. 석면이 cell cycle에 미치는 영향을 이해하기 위해 RAW 거식세포 (macrophage)와 CHL 세포 (chinese hamster lung cell)을 6 일간 배양하여 조직병리학적으로 또 생화학적으로 조사하였다. 조직병리학적으로 3일째 부터 거식세포가 분화하여 다핵의 거대세포로 변해 많은 석면을 탐식한것을 볼수 있었다. 이와 달리 석면을 넣지 않은 거식세포는 계속 분열하는 것을 볼 수 있었다. CHL cell을 조직병리 학적으로 보았을 때, 석면을 더하지 않은 CHL 세포는 계속 분열하였지만 석면을 더한 CHL 세포는 처음 부터 석면이 심한 세포의 cell death를 유발하는 것을 볼 수 있었다. 세포분열에 필수적인 효소인 p34cdc2에 대한 항체와, 또 이 효소의 활성도를 측정할 수있는 특이 기질 펩타이드를 이용하여 p34cdc2 kinase의 level을 조사하였다. 석면에 의해 분화된 세포에서는 효소의 활성도가 감소함을 발견할 수 있었고, immunoblotting (면역항체흡착법)으로 조사하였을 때도 효소수준의 감소를 볼 수 있었다. 이런 결과로 석면에 의해 거식세포의 분열이 억제되고 분화를 촉진하는 것을 볼 수 있었다.

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In vitro toxicity testing for asbestos and particles

석면과 대체물질의 독성학적연구

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Abstract

To substitute inhalation toxicology which requires experimental animals, high cost, intensive labor, and time, in vitro toxicology has been attempted to test asbestos and man-made mineral fibers. To evaluate cytotoxicity of asbestos, asbestos (amosite, anthophyllite, chrysotile, crocidolite, and sepiolite) and particles (SiO_2, and TiO_2) were evaluated using MTT assay and trypan blue exclusion assay. Degree of toxicity to macrophage showed amosite> anthophyllite> chrysotile> crocidolite> SiO_2> sepiolite> TiO_2. Degree of toxicity to fibroblast showed chrysotile> amosite> anthophyllite> sepiolite> crocidolite> SiO_2 > TiO_2. It is suggested that in vitro fiber toxicity test was influenced by three factors; cell type, duration of coculture, and fiber type. Generally, macrophage showed more strength to fibers or particles than that of fibroblast.

In vitro toxicity testing for asbestos and particles

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- Abstract -

To substitute inhalation toxicology which requires experimental animal, high cost, intensive labor, and time, in vitro toxicology has been attemted to test asbestos and man-made mineral fibers. To measure cytotoxicity of asbestos, asbestos (amosite, anthophyllite, chrysotile, crocidolite, adn sepiolite) and particles (SiO_2, and TiO_2) were evaluated using MTT assay and trypan blue exclusion assay. Degree of toxicity to macrophage showed amosite> anthophyllite> chrysotile> crocidolite> SiO_2> sepiolite> TiO_2. Degree of toxicity to fibroblast showed chrysotile~ amosite~ thophyllite> sepiolite> crocidolite> SiO_2 > TiO_2. It is suggested that in vitro fiber toxicity test was influenced by three factors: cell type, duration of coculture, and fiber type. Generally, macrophage showed more strength to fibers or particles than that of fibroblast.

석면과 대체물질의 독성학적연구

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I. 서 론

일반적으로 석면과 석면대체물질등 fiber의 독성학적인 연구는 고도의 기술과 경험을 요하는 연구이다. Fiber Inhalation Toxicology (섬유성물질의 흡입독성)는 gas 흡입독성이나 mist 흡입독성보다는 한단계 높은 흡입독성이다. 이것을 하기위해서는 일정한 호흡성 fiber (Inhalable fiber)를 만들어야하는데 이것도 쉬운 일은 아니고, 일정한 크기로 만들었는지를 검정하기 위해서는 전자현미경적인 분석이 뒤따르게 된다. 또 이것을 만들기위해서는 연구자나 일반인들이 피해를 입지 않게 아주 보호가 잘되고 차단된 시설이 필요하다. 그리고 이것을 일정한 농도로 유지시켜 실험동물에게 폭로시키는 것도 많은 경험과 고도의 기술을 요하는 것이다. 특히 fiber inhalation toxicology는 가장 비용이 많이 드는 연구로서 발암성시험의 경우 상당한 비용이 소요되는 것으로 알려져 있다.

이렇게 비용과 시간 많은 인력이 소모되는 실험동물을 통한 흡입독성의 문제점개선의 방법으로 단기간의 in vitro fiber toxicology 방법이 대두되고 있다. In vitro fiber toxicology는 두가지의 목적으로 행하고 있는데, fiber에 인한 장애 기전을 세포생물학적 단계에서 연구하여 in vivo와 관련지을 수 있고 이것을 이용하여 단기간에 fiber의 toxicity를 스크린 할 수 있는 점이다. 그리고 독성학적인 시험방법의 예측된 정확도를 향상시켜 fiber 생산자들에게 안전한 fiber생산을 위한 방향제시를 할 수 있게 한다 (Hesterberg et al., 1993). In vitro 연구의 문제점은 fiber toxicity의 in vivo에서만 측정이 가능한 lung deposition이나 clearance, durability등의 중요한 지표를 측정하기가 어렵다는 점이다. 이의 개선방법으로 단기적인 동물흡입시험을 병행한 in vitro fiber toxicity 시험이 Hesterberg et al. (1990)에 의해 제시 되고 있다.

In vitro fiber toxicity 시험은 주로 cell culture 시스템을 이용하는데 Mossman and Sesko (1990)은 세가지의 시험방법을 이용한 접근방법을 제시하고 있는데, 대상세포형태로서는 alveolar macrophage, tracheal epithelial cell과 lung fibroblast를 사용하고, 이런 결과로 부터 fiber toxicity의 세포특이적인 측면을 알 수 있고, 그래서 한가지 세포형태로 시험했을때 보다 in vivo와 쉽게 연계시킬수 있다. 그리고 다른 접근 방법으로서는 단기적인 in vivo inhlation 폭로후 in vitro에서 평가하는 방법으로 Warheit 등(1991)은 동물에세 fiber를 흡입이나 주입 (instillation) 시킨 후 lung lavage 한 세포들을 평가하는 방법을 사용하였다. 또 다른 방법으로는 몇가지 다른 세포들을 섞어서 배양하여 시험하는 방법이 있다. Fiber 폭로된 macrophage가 fibroblast에 끼치는 영향 연구 (Bauman et al., 1990), macrophage로 부터 neutrophil chemotactic factor 생성에 대한 연구 (Hayes et al., 1990) macrophage의 암세포에 대한 세포독성연구 (Bissonnette et al., 1990), 그리고 기관지 배양 연구 (Mossman and Craighead, 1980) 등은 모두 이런 방법을 이용하여 fiber toxicity를 시험하였다.

In vitro fiber toxicology는 많은 제한점이 있지만 단계적인 연구에 많은 도움을 주고 있고 새로운 in vitro 방법이 점점 개발됨에 따라 단기적 시험방법으로 점점 많은 연구자들이 이 방법을 사용하고 있다.

본 연구에서는 미래의 산업보건연구원에서 fiber 흡입독성 (fiber inhalation toxicology)를 하기위해서 기술축적의 방법으로 지금은 우리 연구원의 장비나 연구수준으로 가능한 "석면과 대체물질의 독성연구"를 in vitro 방법으로 시도해보고자 한다. 대체물질 즉 MMMF (Man-Made Mineral Fibers)와 man-made organic fiber등이 최근에 많이 사용되고 있는데, 특히 MMMF는 여러가지 조성이 다른 물질들이 최근에 사용되고 있고 그리고 많은 MMMF가 개발단계에 있다 (참고로 일본의 경우 사용되고 있는 대체물질의 list를 표 1에서 볼수 있다). 그러나 이런 물질의 독성이 아직도 많이 규명되지 않은 것도 많아서 in vitro 방법으로 스크린할 수 있는 방법의 연구가 절실한 실정이다.

Table 1. Substitute of asbestos in Japan

석면함유제품	대체재료
Construction	
건축내장용석면규산 Calcium판	Pulp fiber
	Alkaline-resistant glass 섬유
건축외장용판	Pulp fiber
	Alkaline-resistant glass 섬유
	Vinylon
건착물내이화수관	Vinylon
Asbestos spray	Pulp fiber
	Rock wool
건축재료(unspecified)	Polyvinyl chloride fiber
	Mica
	Vermiculite
선박용규산 Calcium판	Wollastonite
Friction material	
Disk pad	Steel fiber
소형차용 Disk pad	Aramid fiber(Aromatic polyamide)
	Glass fiber
Brake lining	Aramid fiber(Aromatic polyamide)
	Glass fiber
중기계용 Brake lining	Phenol fiber
Clutch facing	Aramid fiber(Aromatic polyamide)
	Glass fiber
중기계용 Clutch facing	Phenol fiber
차동차 Radial-tire belt	Para-type aramid fiber
Joint sheet (Gasket, Packing, etc).	Aramid fiber(Aromatic polyamide)
	Glass fiber
	Phenol fiber
	pulp fiber
	Carbon fiber
	Polybenzoimidazol fiber
방직품	Glass fiber
	Carbon fiber
	Aramid fiber
	Polybenzoimidazol fiber

Paint	Polyorephine (Polyethylene synthetic pulp)
Asbestos paper	Aramid fiber
Fire-proof sheet for welding	Acryl염화섬유 Phenol fiber
Heat-resistant cloth	Acryl염화섬유 Para-type aramid fiber
Heat-insulaton	Ceramic fiber
Plastic 보강재	Acrylic fiber
Rubber 보강재	Acrylic fiber
Filter aid	Potassium titanate fiber

II. 재료 및 방법

1. 세포배양

HeLa, CHL, RAW 세포는 100 mm 배양접시에 DMEM-FBSHI 이나 RPMI-FBSHI의 배양액으로 37^{d} C의 배양기에서 배양하였다. Fiber toxicity 시험은 96 well 배양접시에서 한 well에 세포가 10000 개 정도가 들어가게 하여 배양하였고, 시험대상 세포는 일정한 양의 fiber를 첫 배양 다음날에 더하여서 여러가지 독성시험을 행하였다.

2. Fibers and particles

Chrysotile과, crocidolite, amosite, anthophyllite는 UICC standard reference sample을 사용하였고, 이런 석면류와 sepiolite (Kuzuo, Tochigi, Japan)는 Hisanaga 박사 (일본 산업의학총합연구소) 에게서 공여받았다. 그리고 SiO_2, TiO_2는 Sigma에서 구입하였다.

3. Trypan Blue Exclusion

2.5%의 trypan blue stock solution을 증류수에 만든다. 20 μl의 trypan blue 용액을 200 μl의 세포배양액이 넣어 잘섞이도록 pipetting 한후 20 μl의 용액을 채취하여 hemocytometer 아래에서 세포를 헤아려 생존도를 측정한다. 살아있는 세포는 핵에 청색의 염색이 되지않고 죽은 세포는 핵이 파랗게 염색된다.

4. Cytotoxicity Assay

Fiber의 세포독성을 측정하기 위하여 MTT방법을 사용하였다. MTT의 원리는 3H-thymidine incorporation과 유사하다. MTT 측정은 MTT가 미토콘드리아의 효소인 succinate dehydrogenase에 의해 푸른색을 띤 formazan으로 바뀌는 것을 측정하는 것이다. 이런 변형은 항상 살아있는 세포안에서만 일어나고, formazan의 양은 살아있는 세포의 수에 비례한다 (Denizot and Lang, 1986).

MTT (1-[4,5-dimethrylthiazol-2yl]-3,5-diphenyl formazan)은 Sigma로 부터 구입하였다. MTT를 PBS에 녹여 5 mg/ml 용액을 만들어 여과 하여 무균상태와 찌꺼기가 없도록 한다. MTT assay는 10 μl의 MTT 용액을 96 well 배양용기의 각 well에 더하여 2시간 동안 37 C배양기에 놓아서 반응시키고, 두시간 후에는 100 μl의 10% SDS in 0.01N HCl을 더하여 세포를 죽여 formazan이 세포밖으로 나오게 한 다음 2시간 더 37°C에서 반응시킨다. 각 well로 부터 정량을 취하여 UV spectrophotometer에서 570 nm에서 측정한다. Cytotoxicity는 fiber를 더하지 않은 control세포의 값으로 나누어 % of viable cell을 나타낸다.

III. 결 과

1. 세포의 형태에 따른 Chrysotile의 독성

A. RAW cell과 chrysotile

RAW cell은 mouse macrophage cell line으로 정상적인 macrophage의 특성을 많이 지고 있다. Cytotoxicity를 측정하기 위해 세포를 각 well당 약 10000 넣어 하루 배양하고 난 뒤 200, 100, 50, 20, 10, 1 μg의 chrysotile을 각각의 well에 넣어 1, 3, 5 day 후에 MTT assay방법으로 측정을 하였다. Figure 1에서 보는 바와 같이 chrysotile의 농도에 따라 viability가 감소 (cytotoxicity가 증가) 하는 것을 볼 수 있다. 1 day, 3 day, 5 day 에서 모두 이런 현상을 보여준다. 그리고 fiber와의 배양기간도 중요한 영향을 미쳐 1 day 보다는 3 day, 5 day가 훨씬 viability 가 낮은 것을 알 수 있다. 그리고 25 μg 정도에서 50%의 viability에 이르는 것을 볼 수 있다 (3-5 day).

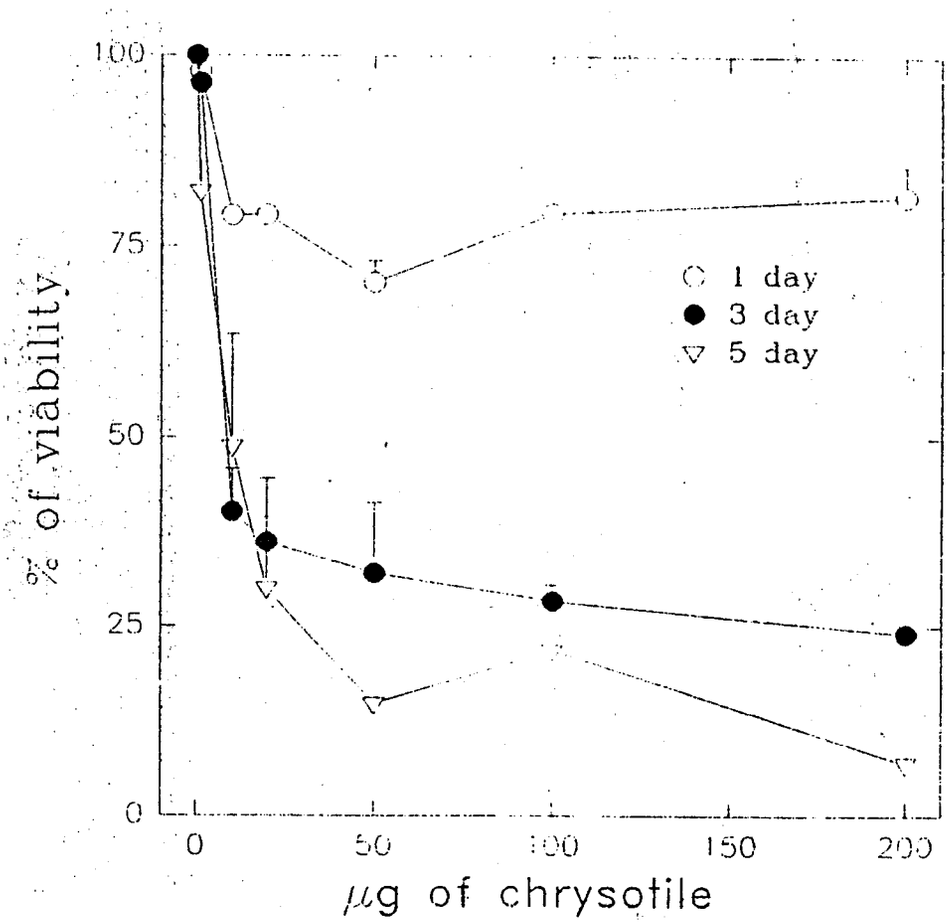


Figure 1. Cytoxicity of chrysotile fibers to RAW macrophages. MTT assay was used for cytotoxicity test. Results are presented as % of viability.

B. WI 38 lung fibroblast과 chrysotile

WI 38 lung fibroblast는 태아의 폐의 fibroblast에서 고정된 것이다. 이 세포도 위의 방법과 동일하게 20, 10, 1, 0.1 ug의 chrysotile을 더한 후 1, 3, 5 day 후에 MTT assay 방법으로 viability를 측정한 것이다. Figure 2에서 보이는 것과 같이 여기에서도 농도에 따른 독성을 보여주고 있고, 3-5 day 에서는 1 μg 이하에서 50% 정도의 viability를 보여준다. 그리고 여기에서도 역시 fiber와의 배양기간도 영향을 미치는 것을 보여주어서 1 day보다는 3 day, 5 day가 더욱 높은 toxicity를 보여주는 것을 알수 있다.

C. HeLa cell과 chrysotile

HeLa cell은 cervical carcinoma cell에서 고정한 세포로서 epithelial cell 계통의 세포이다. 이 세포도 위의 방법과 동일하게 20, 10, 1, 0.1 μg의 chrysotile을 더한 후 1, 3, 5 day 후에 MTT assay 방법으로 cytotoxicity를 측정한 것이다. Figure 3에서 보이는 것과 같이 여기에서도 농도에 따른 독성을 보여주고 있고, 1 day 에서 10 μg 정도에서 50% 정도의 viability를 보여준다.

D. HeLa cell과 TiO_2

TiO_2가 HeLa cell에 미치는 cytotoxicity를 MTT assay 방법으로 측정을 하였다. Figure 4에서 보이는 것 같이 TiO_2 는 농도에 따라 크게 viability를 주지 않는다 그래서 TiO_2 는 control particle로 사용될 수 있을 것이다.

2. 여러가지 Fiber와 particle의 cytotoxicity 비교

A. RAW cell

5, 10, 20 μg의 fiber와 particle을 RAW cell에 더하여 1, 3, 5 day 후 이들의 독성을 MTT assay로 상호비교하여 보았다. Figure 5 (5 μg), Figure 6 (10 μg), Figure 7 (20 μg) 에서 보이는 것과 같이 amosite와 anthophyllite는 1 day 경과시 낮은 농도 (5 μg) 에서도 50% 이하의 세포독성을 보여주지만 crocidolite와 sepilite는 높은 농도 (20 μg)에서 40% 이하의 viability을 보여주고 있다. amosite는 지속적인 독성을 보여주지만, 많은 fiber 와 particle들이 RAW cell의 경우에는 배양일이 경과할수록 cytotoxicity가 낮아지는 것을 볼수 있다. 그리고 TiO_2는 cytotoxicity에 큰변화가 없는 것을 볼 수 있다. Cytotoxicity의 등급은 amosite> anthophyllite> chrysotile> crocidolite> SiO_2> sepiolite> TiO_2 순이다. Figure 8에서는 amosite (A), anthophyllite (B), crocidolite (C), chrysotile (D), SiO_2 (E), sepiolite (F), TiO_2 등을 탐식한 세포를 보여주고 있다. 화살표는 석면을 탐식하여 세포가 비대하여진 것을 나타내고 있다.

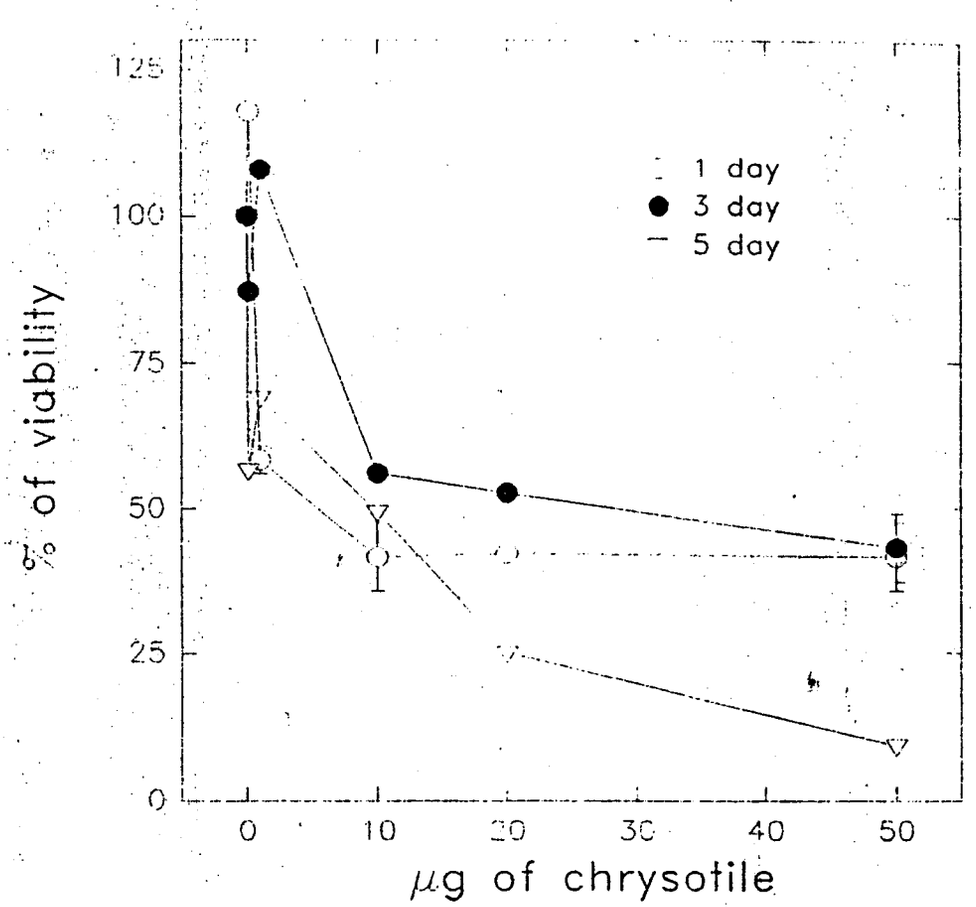


Figure 2. Cytoxicity of chrysotile fibers to WI 38 lung fibroblasts. MTT assay was used for cytotoxicity test. Results are presented as % of viability.

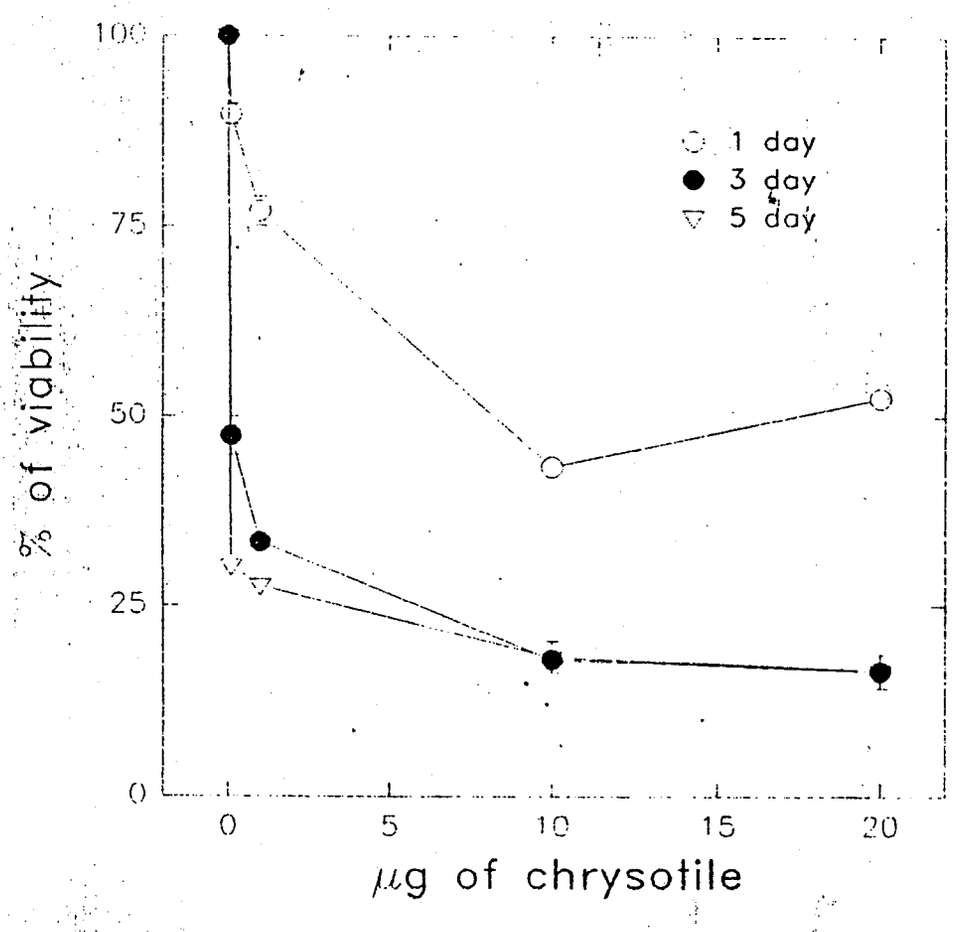


Figure 3. Cytoxicity of chrysotile fibers to HeLa cells. MTT assay was used for cytotoxicity test. Results are presented as % of viability.

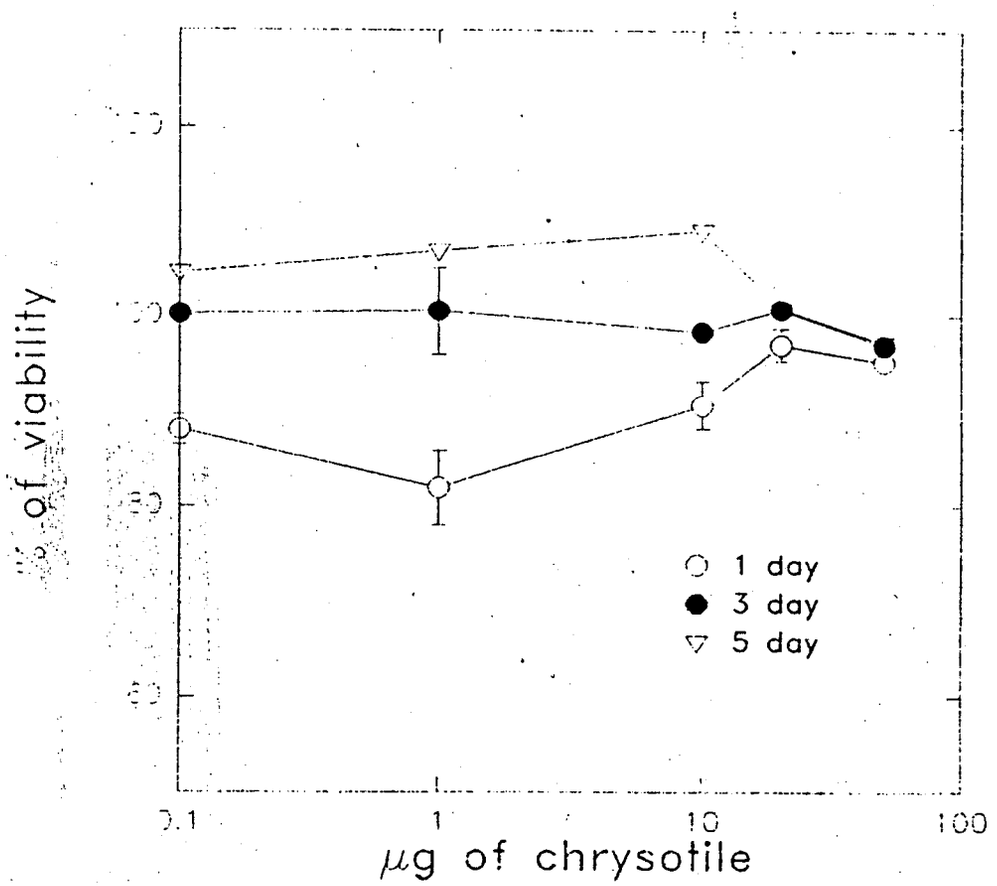
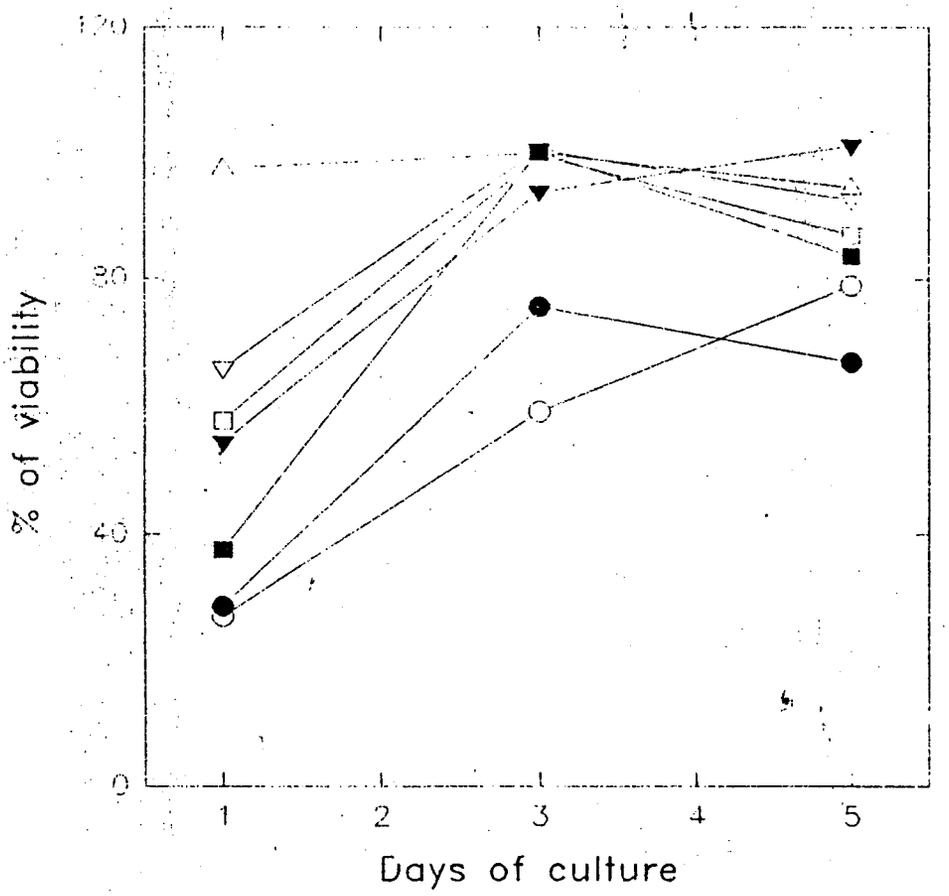


Figure 4. Cytoxicity of TiO_2 to HeLa cells.
MTT assay was used for cytotoxicity test.
Results are presented as % of viability.



○ amosite
● anthophyllite
▽ TiO_2
▼ crocidolite
□ SiO_2
■ Chrysotile
△ Sepiolite

Figure 5. Cytotoxicity of several fibers and particles to RAW cells.
MTT assay was used for cytotoxicity test.
Concentrations of fibers and particles were 5 ug.
Results are presented as % of viability.

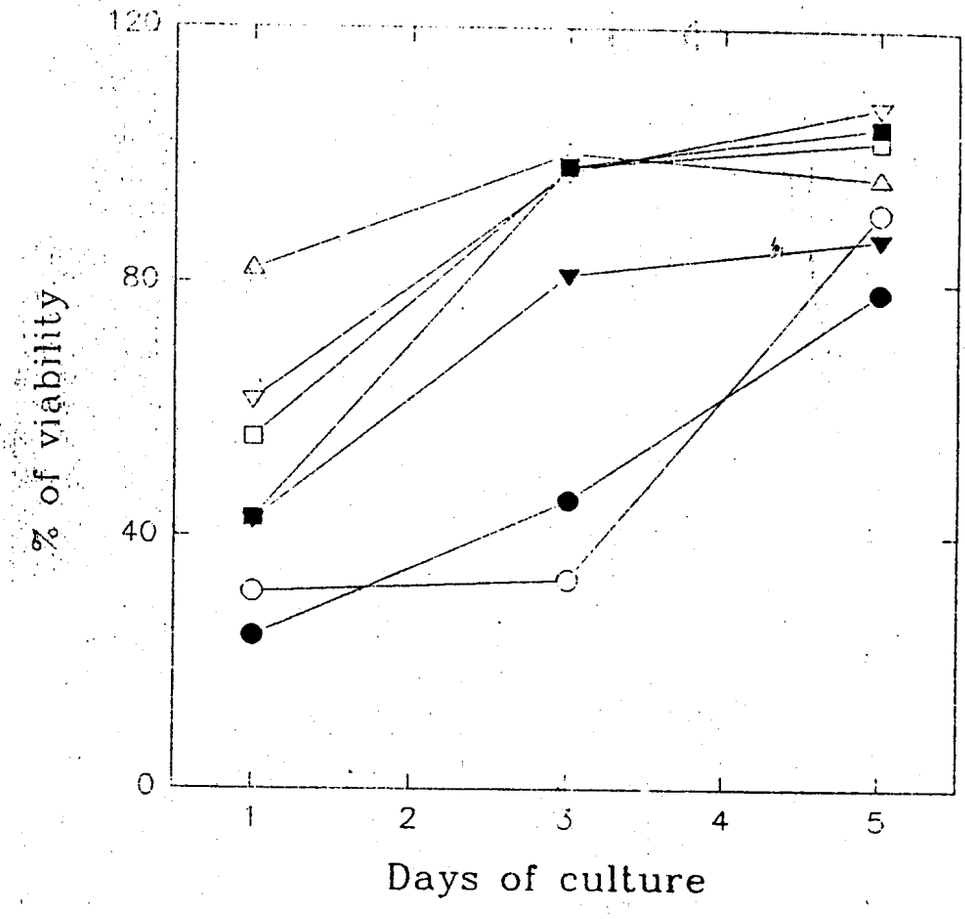


Figure 6. Cytotoxicity of several fibers and particles to RAW cells. MTT assay was used for cytotoxicity test. Concentrations of fibers and particles were 10 ug. Results are presented as % of viability.

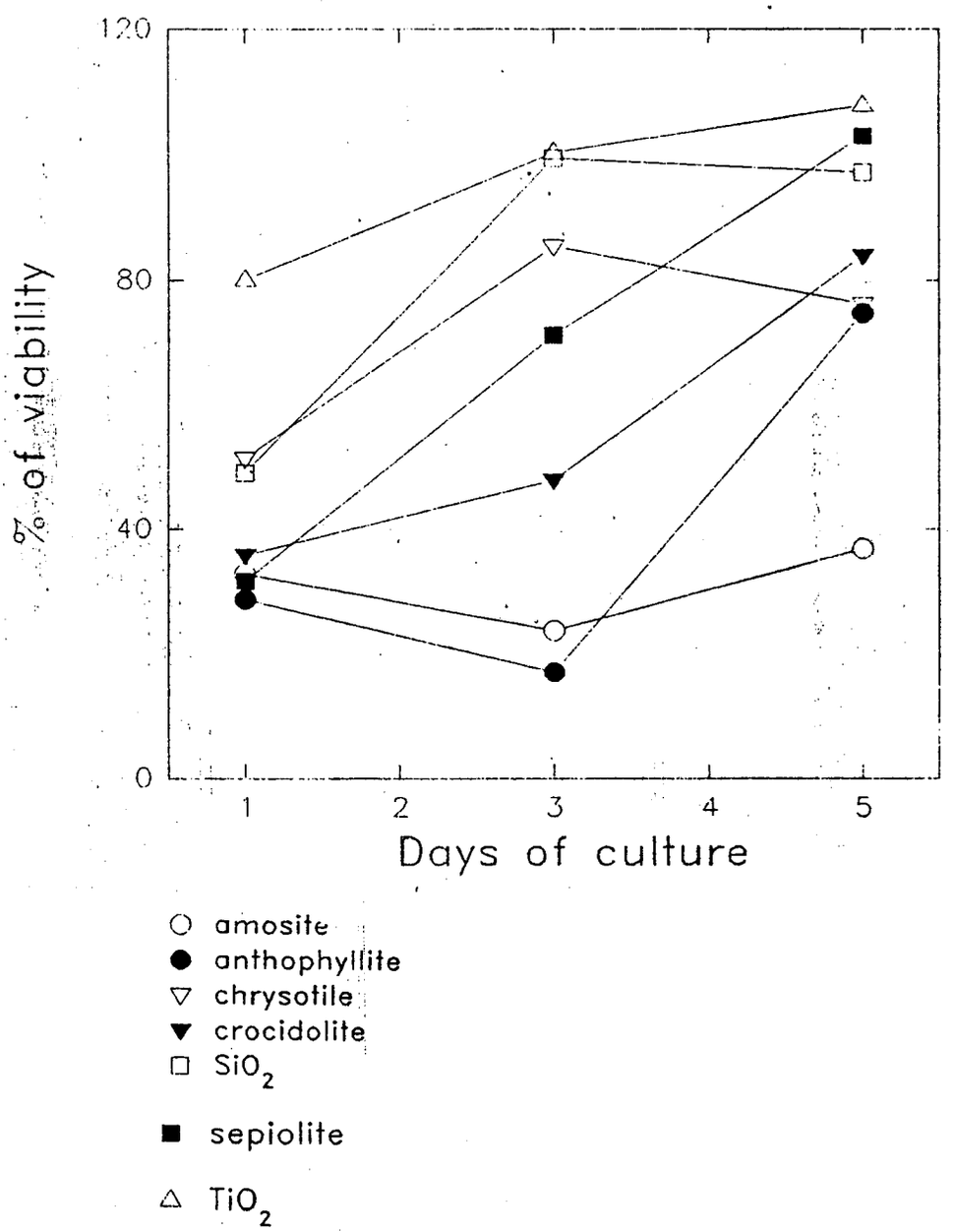


Figure 7. Cytotoxicity of several fibers and particles to RAW cells.
MTT assay was used for cytotoxicity test.
Concentrations of fibers and particles were 20 ug.
Results are presented as % of viability.

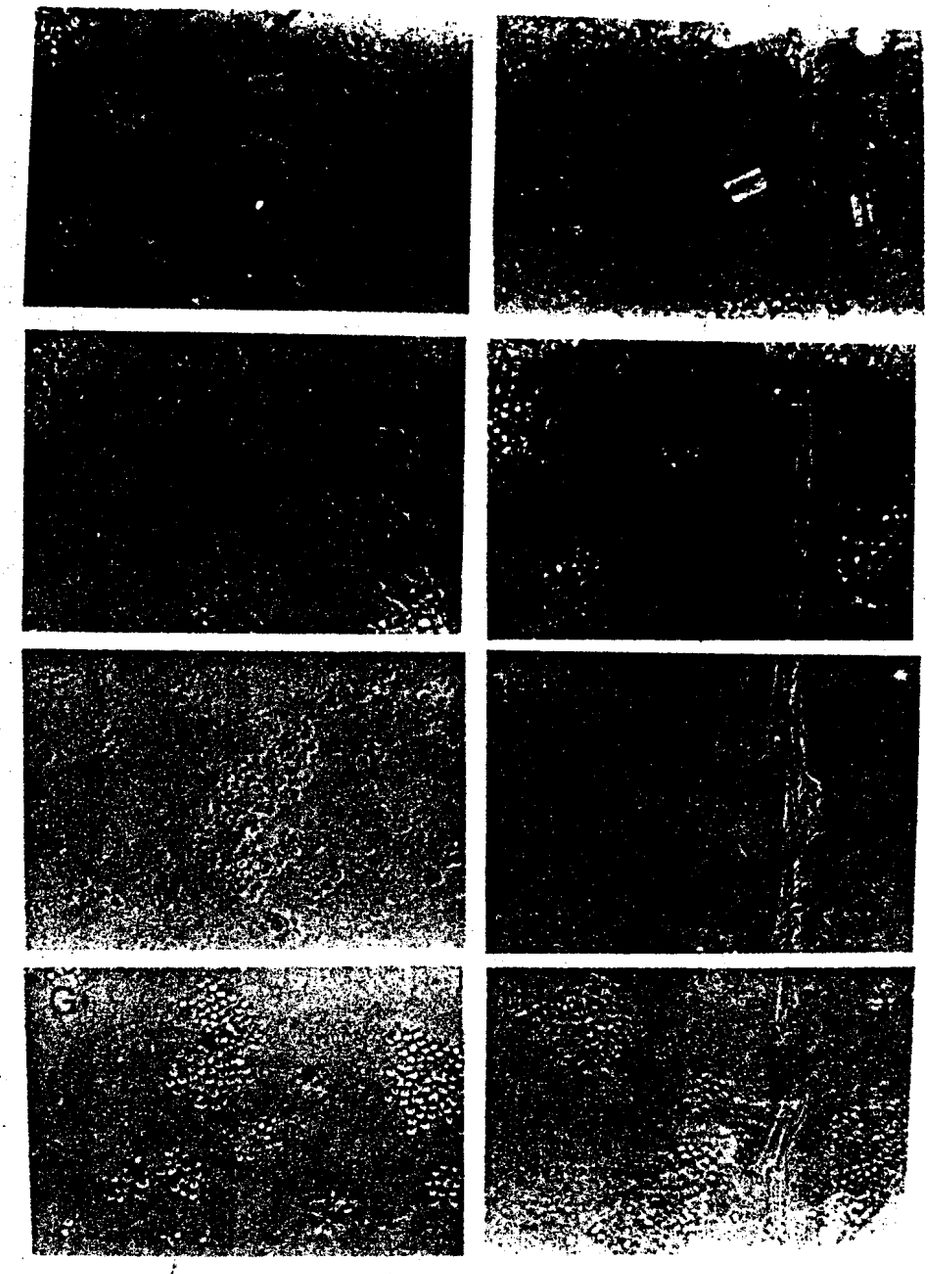


Figure 8. Three day cultured RAW cell with amosite (A), anthophyllite (B), crocidolite (C), chrysotile (D), SiO_2,(E), sepiolite (F), TiO_2, and no fiber and partilce (H). Arrows indicate fiber ingested giant cell.

B. WI38 cell

5, 10, 20 μg의 fiber와 particle을 WI38 cell에 더하여 1, 3, 5 day 후 이들의 독성을 MTT assay로 상호비교하여 보았다. Figure 9 (5 μg), Figure 10 (10 μg), Figure 11 (20 ug)에서 보이는 것과 같이 낮은 농도에서는 일부 fiber나 particle (SiO_2, sepiolite, TiO_2)에 대해 세포들이 배양일이 경과하면서 세포의 vibility를 회복하는 경향을 보여주지만 10 ug 이상의 농도에서는 이런 현상을 보여주지 않는다. 배양일이 경과하면서 급격히 viability의 감소를 볼수 있다 (Figure 10 & 11). Figure 11 에서는 chrysotile, amosite, anthophyllite, sepiolite등이 50% 이하의 viability를 보여주고 있다. Cytotoxicity의 등급은 chrysotile~ amosite~ anthophyllite> sepiolite> crocidolite> SiO_2 > TiO_2 순이다.

위에서 보이는 바와 같이 세포형태에 따라 fibers나 particles에 대한 세포독성의 현격한 차이를 볼수 있다. RAW macrophage는 이런 fiber and particle에 대해 내성이 강한 세포임을 볼수 있고, lung fibroblast는 내성이 약한 것을 볼 수 있다.

3. Tryphan blue exclusion에 의한 test

Fiber나 particle의 독성을 test하는 다른 방법으로 trypan blue exclusion법을 이용하였다. 이 방법은 아주 손쉬운 방법이나 현미경하에서 많은 세포를 계수해야하는 점과 또 육안으로 판단하기 때문에 주관적인 점이 단점이라 하겠다. Figure 12에서 보이는 바와 같이 fiber나 particle을 더한 RAW 세포를 1 day 경과후 trypan blue exclusion법으로 죽은 세포를 계수하여 전체세포에서 죽은 세포의 % 로 나타내었다. 여기에서도 TiO_2는 농도에 상관없이 높은 viability를 보여주고 있다. Toxicity의 등급은 SiO_2~ chrysotile> amosite> crocidolite~ anthophyllite~ sepiolite> TiO_2 순이다. 특기할 것은 위의 결과와 비교해 볼때, MTT assay에서는 가장 낮은 독성을 보여주는 SiO_2 가 가장 높은 독성을 보여주는 것이다. 이는 현미경하에서 SiO_2를 탐식한 세포가 trypan blue에 의해 청색을 띤 세포와 유사하기 때문에 죽은 세포로 계수하여서 이런 결과가 나오지 않았나 생각된다. 이런 결과를 제외하면 이 두가지 방법에 의한 시험에는 결과의 큰 차이는 없는 것으로 보인다.

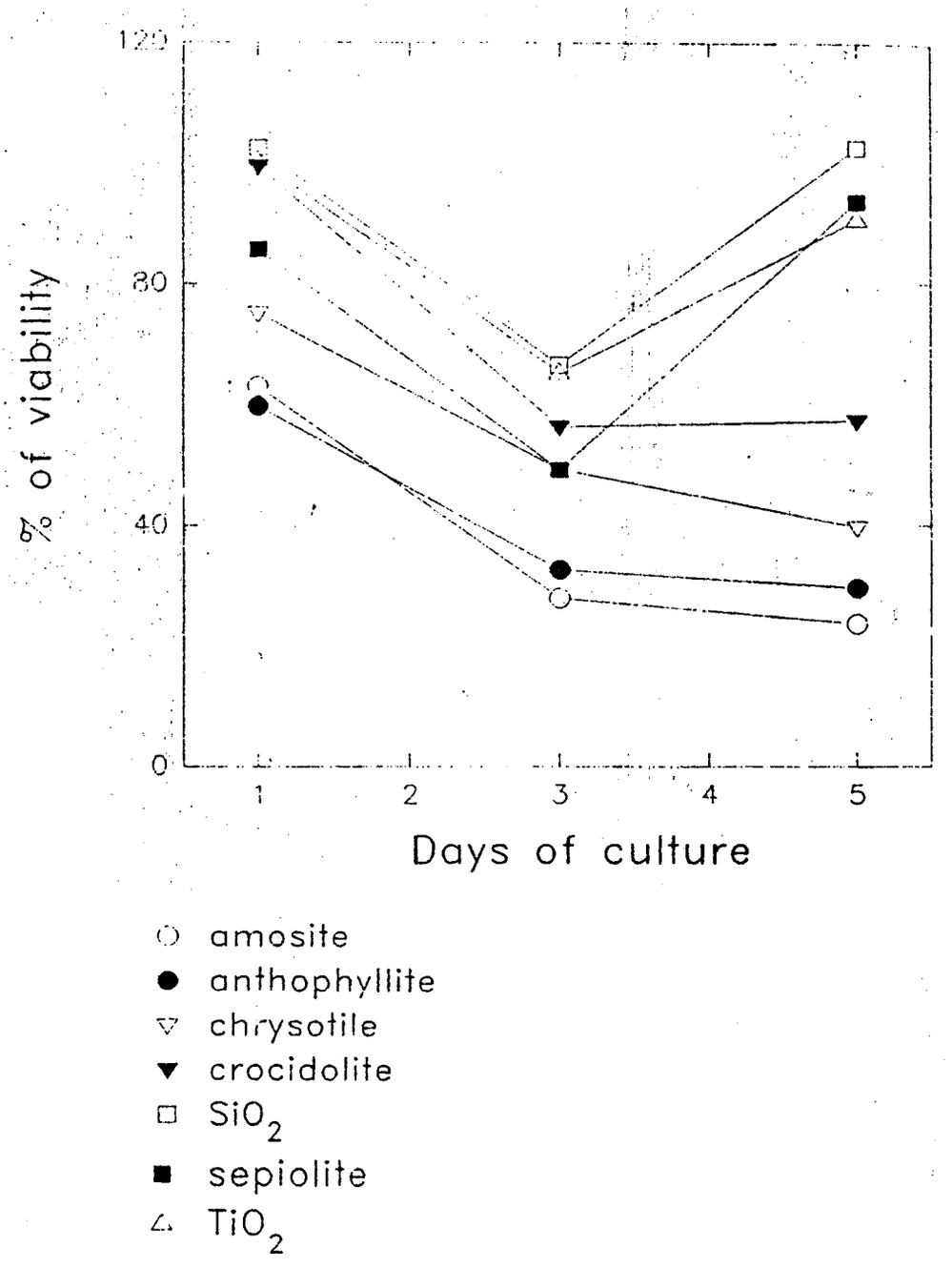


Figure 9. Cytotoxicity of several fibers and particles to WI 38 cells.
MTT assay was used for cytotoxicity test.
Concentrations of fibers and particles were 5 ug.
Results are presented as % of viability.

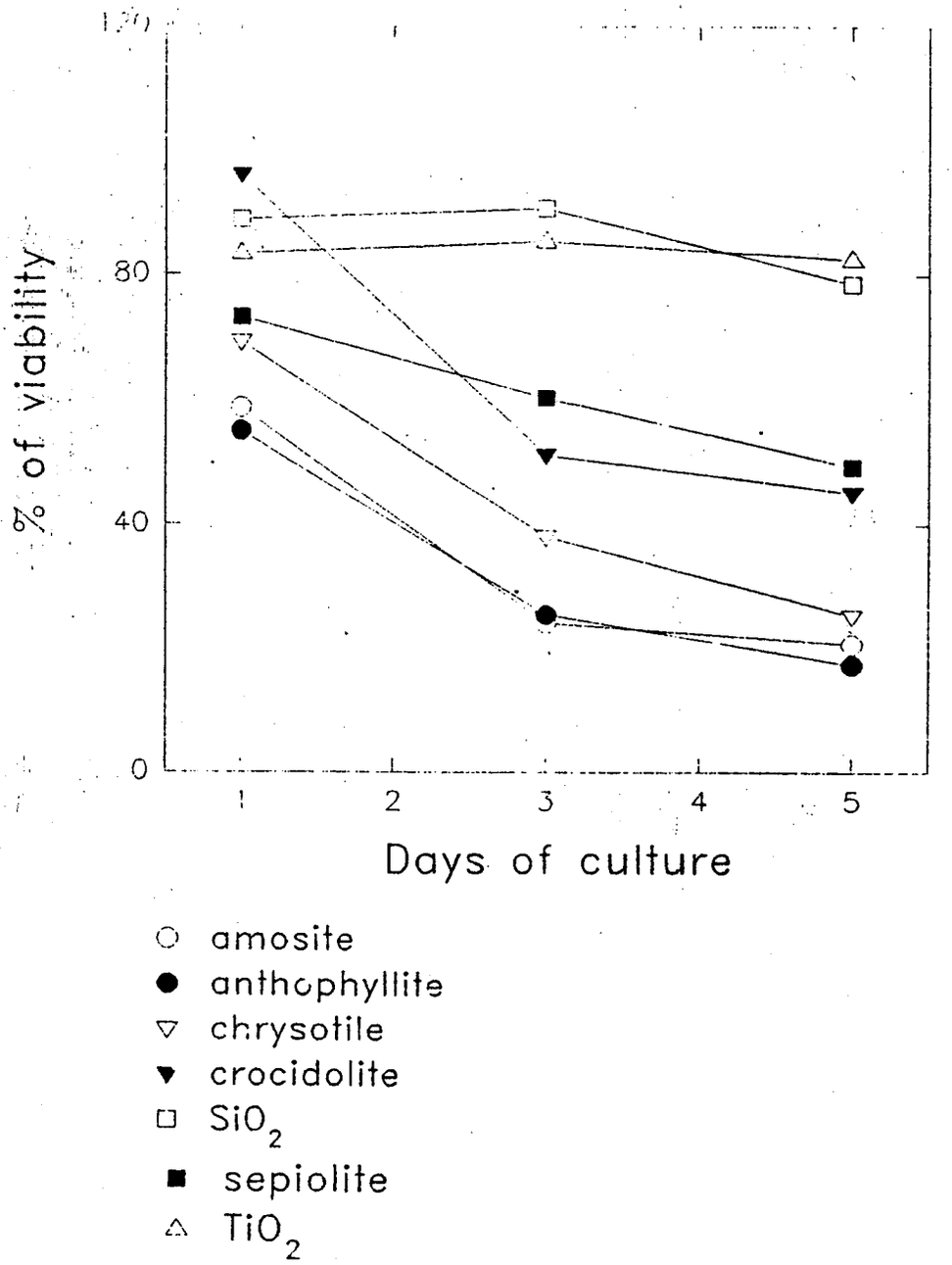


Figure 10. Cytotoxicity of several fibers and particles to RAW cells. MTT assay was used for cytotoxicity test. Concentrations of fibers and partilces were 10 ug. Results are presented as % of viability.

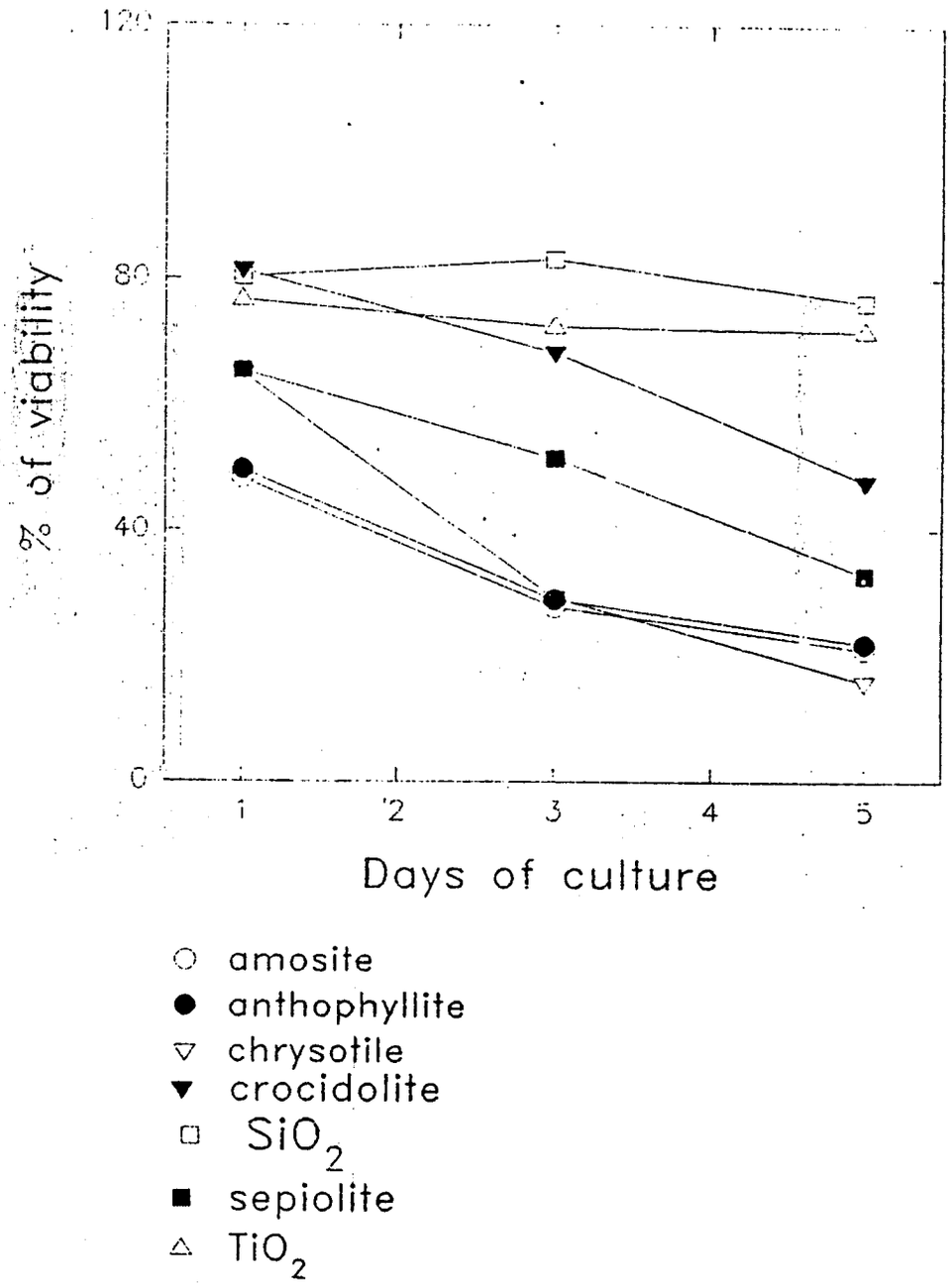
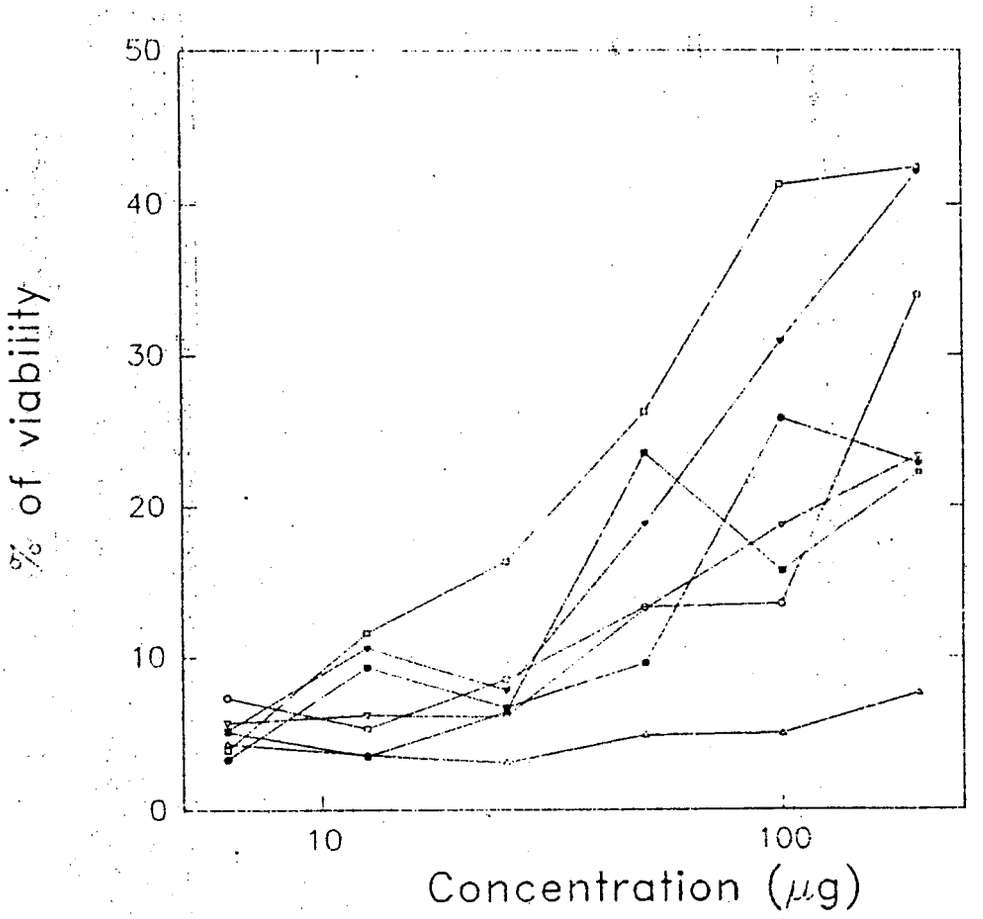


Figure 11. Cytotoxicity of several fibers and particles to RAW cells. MTT assay was used for cytotoxicity test. Concentrations of fibers and particles were 20 ug. Results are presented as % of viability.



- amosite
- anthophyllite
- crocidolite
- chrysotile
- SiO_2
- sepiolite
- TiO_2

Figure 12. Cytotoxicity of several fibers and particles to RAW cells. trypan blue exclusion assay was used for cytotoxicity test. Concentrations of fibers and particles were ranged form 200, 100, 50, 25, 12.5 to 6.3 ug. Results are presented as % of viability.

IV. 고 찰

In vitro fiber toxicity test에 최근 약이나 대사물의 cytotoxicity를 측정하기 위하여 쓰이고 있는 MTT assay 방법과 또 예전부터 널리 쓰이고 있는 tryphan blue exclusion 방법을 사용하여 석면과 particle의 toxicity test에 적용하였다. 이런 방법외에도 적혈구의 용혈을 측정하는 방법 (MacNab & Harington, 1967; Harington et al., 1971)과 세포막의 투과성을 측정하는 LDH (lactate dehydrogenase) release (Tikes & Beck, 1980, 1983), beta-glucuronidase (Brown et al., 1979; Tikes and Beck, 1983; Davis, 1980) 와 beta-galactosidase (Dunnigan, 1984)와 세포의 분열능력을 측정하는 colony forming efficiency (Brown et al., 1986; Chamberlain et al., 1980; Haugan et al., 1982; Hart et al., 1991) 방법등이 사용되고 있다. 본 연구에서 사용한 MTT방법은 Denizot & Lang (1986)의 방법으로서 아주 실험실에서 colorimetric assay로 방사성 동위원소의 사용이 필요없이 정량적으로 측정할 수 있는 방법이다.

이런 in vitro fiber toxicity test에서는 본 연구가 제시하듯이 세포독성은 세가지의 요인에 의해 영향을 받는 것을 볼 수 있다: 세포형태 (cell type), 배양기간 (duration of culture), fiber의 종류 (fiber type)에 따라 많은 차이를 보여준다. Macrophage, epithelial cell, fibrobalst 각각의 세포에서 각각의 fiber에 대한 dose-response와 세포독성을 보여주고 있다. 본 연구에서는 macrophage가 lung fibroblast나 epithelial cell 보다는 높은 dose-response를 보여주는 것을 볼 수 있다. 그리고 세포배양의 기간, fiber type에 의해 dose-response가 다른 것을 보여주고 있다. 다음의 연구에서는 이런점을 염두에 두고 여러가지 형태의 세포의 조합적인 배양 즉 Macrophage + lung fibroblast의 조합적인 배양 같은 것을 시도해야 될 것이다. 또 이런 particle을 탐식한 세포의 viability 조사에서만 연구가 그칠 것이 아니라 기능적인 역할의 검정 (functional activity assessment)이 필요하리라 생각된다.

본 연구가 추구하는 in vitro법에 의한 fiber toxicity test는 7개의 fiber와 particle의 독성을 연구하는데 그쳤지만, 앞으로의 연구에서는 좀더 다른 in vitro toxicity방법이 개발되어야 할 것이고, 석면대체물질쪽으로 관심을 가져 이런 대체물질의 동일한 길이로 fiber를 만드는 방법이 개발되어야 할 것이고 이런 대체물질의 in vitro toxicicity test가 행해져야 할 것이라고 생각된다.

V. 결론 및 요약

실험동물을 이용한 석면과 대체물질의 독성학적 평가의 문제점을 개선하기 위해 단기간의 In vitro시험 방법을 통하여 석면과 대체물질의 독성학적평가를 시도하였다. 세포독성을 측정하기 위하여 MTT 분석방법과 Trypan blue exclusion 방법을 사용하여 석면과 분진들의 세포독성을 비교평가 하였다. Amosite, anthophyllite, chrysotile, crodicodlite, sepiolite등의 석면과, SiO_2, TiO_2 등의 분진들이 in vitro 시험방법을 이용하여 평가되었다. 거식세포에서는 amosite> anthophyllite> chrysotile> crocidolite> SiO_2> TiO_2 순으로 독성이 나타났고, Fibroblast에서는 Chrysotile~amostite~anthophyllite~> sepiolite> crocidolite> SiO_2> TiO_2 순으로 독성이 나타났다. 세포의 형태, 석면과 분진의 종류, 또 배양기간에 따라 독성의 차이를 보여주는 것을 볼 수 있었다. 비교적 거식세포(macrophage)가 fibroblast보다는 섬유나 분진에 비해 강한 것을 볼 수 있었다.

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'94 Toxicological Studies on Asbestos and Man-made Mineral Fibers

-Analysis of Mineral Fibers from Stone-kettle

-Growth and Differentiation of Macrophages by Asbestos

-In vitro testing for Asbestos and Particles

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Industrial Health Research Institute

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Analysis of Mineral Fibers from Stone-Kettle

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Abstract

To analyze mineral fibers found in serpentine which is used for stone kettle manufacturing and to protect workers in this factory, samples obtained from the factory were analyzed for their metal contents using an electron microscope equipped with energy dispersive X-ray analyzer. In addition, the metal contents were compared with other known asbestos. Although many kinds of fibers similar to asbestos were observed, any known asbestos fibers were not found. These fiber may have similar toxicity to known asbestos even if their toxicities are not known at this time. It is suggested that pulmonary function test and chest X-ray are needed for the protection of stone-kettle manufacturing workers. Further epidemiological study is also required.

I. Introduction

Korean asbestos rocks are classified three types: serpentine, amphibole and fiber cluster from lime stone like rocks presumably sepiolite (Kim, 1987). Chrysotile was distributed in Hong-sung of Chung-nam province, and Ga-pyung of Kyung-gi province, and sepiolite was distributed in Moon-kyung of Kyung-buk province, and Je-won of Chung-buk province, and amphibole was produced form Chin-an and Chang-su of Chun-buk province.

Our study was aimed to verify the identities of fibrous materials produced from rocks used in stone utensil factories and to confirm whether they are asbestos related materials. This result will provide basic epidemiological data for the protection of stone utensil manufacturing workers.

II. Materials and Methods

Dusts from the stone grinding factory were sampled into plastic test tube and transported. Fragments of the stone kettle were also sampled into plastic bags and transported. The samples were filtered through nuclear pore filter (0.2 um diameter) into a flask, after sonicating with 100 ml of distilled water. Approximately 3 mm^2 square of the filter paper was placed on EM grid (200 mesh, Ni) treated with carbon. The filter was solubilized and removed with chloroform vapor. The analysis was done using a KEVEX-7000Q Energy Dispersive X-ray Analyzer analysis Equipment (EDX) (Sakai et al, 1993).

III. Results

As shown in Table 1, 4 samples were obtained from the factory. Sample A was dusts obtained from grinding of stone kettle. Sample B was fragments from blackish stone. Sample C was fragments from another blackish stone, and sample D was fragments from whitish stone. Sample B, C, and D were shown in Figure 2. When sample A was analyzed, several types of fibers were observed (Figure 3, A, B, & C). These fibers were analyzed for their mineral contents by EDX. Two types of EDX were observed: Si, Al, Ca (type 1, Figure 4A) and Si Mg, and Fe (type 3, Figure 4 B).

Sample B also showed fibers (Figure 5 A, B, C, and D). Five types of EDX were observed; Si, Al, and Ca (type 1, Figure 6A), Si, Al, and Fe (type 2, Figure 6B), Si, Mg, and Fe (type 3, Figure 6C), Si, Al, Mg, and Fe (type 4, Figure 6D), and Si, Al, Mg, Fe, and K (type 5, figure 6 E).

Sample C was examined for the presence of fibers. As shown in Figure 7, many fibers were found in several samples.
The results of EDX indicated that there are 4 types of fibers; Si, Al, and Fe (type 2, Figure 7 A), Si, Mg, and Fe (type 3, Figure 7B,), Si, Al, Mg, Fe, and K (type 5, figure 7C) and Si, Ca, Al, Fe, and Mg (type 6, Figure 7D).

When sample C was examined under EM, several fibers were also found (Figure 8 A & B). Three types of EDX were observed; Si, Al, and Fe (type 2, Figure 9 A), type 5 (Figure 9B), and type 6 (figure 9 C) major component is Si and some Al, Ca, or Fe were found.

There was no known asbestos from 4 samples (Table II). As shown in Si and Al were major components of the mineral fibers. By using EDX, 6 kinds of fibers were determined: Type 1 for Si, Al, and Ca; Type 2 for Si, Al, and Fe; Type 3 for Si Mg and Fe; Type 4 for Si, Al, and Mg; Type 5 for Si, Al, Mg, K, and Fe; Type 6 for Si, Al, Ca, and Fe. The analysis of chrysotile fiber showed that it contained mainly Si, and Mg (Figure 10 A & B). In addition, amosite contained Si, Fe, Mg, and Mn (Figure 11 A & B). Type 3 has some similarities with chrysotile but do not have tubular structure found in chrysotile fiber. In addition, the ratio of Mg/Si was also small compared to chrysotile (Mg/Si= nearly 1). It was suggested that it is not talc due to 10% content of Fe. Comparison of these fibers with chrysotile, amosite and other asbestos (Table III) indicated that the fibers from stone kettle showed somewhat different composition from other known fibers (Figure 12 A & B and Figure 13).

IV. Discussion

In this paper, we demonstrated that the fibers from stone kettles are different from other known asbestos. Initially we intended to survey the east of Chon-buk province to find out the presence of asbestos fibers. We expected that this fiber may include serpentine with some tremolites. Our results showed that there are no known asbestos or tremolited produced from stone kettle. The toxicological data of these fibers are completely unknown at this time. Although hazard of these fibers is uncertain, fiber is generally known to be more hazardous than particles. Intake of fibers through oral route have been implicated in several carcinoma including Stomac carcinoma, and others (Moran, 1992). The working condition of stone kettle manufacturing factory was so poor. The workers in the factory had no proper protective equipments or ventilation. Further investigation including chest X-ray and epidemiological study is needed.

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Growth and Differentiation of Macrophages by Asbestos

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Abstract

Asbestos has been implicated in lung fibrosis and mesothelioma. The disruption of cell division cycle has been suggested for one of mechanisms for disease process. To understand the possible role of asbestos fibers on the cell division cycle, RAW macrophage and Chinese hamster lung cell (CHL) were cultured 6 days with 200 ug/ml of chrysotile fibers (average size 4 um). In each day, the duplicate cultures were processed for histochemistry and immunohistochemistry. The histopathology RAW macrophage treated with asbestos fibers showed continual cellular differentiation from the third day. From fourth day, the macrophages were developed into multinuclear cells and the asbestos fibers were completely covered with cellular materials. In contrast, macrophage without asbestos fibers showed continual cell division. The histopathology of CHL cell treated with asbestos showed massive cell death from the beginning, while CHL cell without asbestos fiber showed fiber showed continual cell division. Antibody against p34cdc2 kinase, an essential enzyme for cell division cycle in eukaryotic cells, and p34cdc2 kinase specific peptide were employed to monitor the changes of p34cdc2 kinase level. The macrophages differentiated by asbestos showed significant decreases on p34cdc2 kinase activity level. Immunoblotting of p34cdc2 kinase also coincided with the results of the activity assay. Taken together, our results indicated that asbestos fiber induces cellular differentiation for macrophages by suppressing cell division cycle.

I. Introduction

It is widely known that asbestos causes asbestosis and mesothelioma ultimately. The mechanisms of asbestosis and mesothelioma become slowly disclosed with the development of immunology and molecular biology.

Asbestosis is characterized by the disruption of lung structure and an overgrowth of connective tissue, inducing to diffuse interstitial fibrosis and small airway fibrosis. The overgrowth of connective tissue, particularly fibroblasts, by asbestos exposure could be occurred by overproduction of cytokines, tumor necrosis factor (TNF), and interleukin 1 (IL-1) from bronchoalveloar (BAL) leukocytes. In rats instilled intratracheally with amosite asbestos, there was increased secretion of both IL-1 and TNF by BAL macrophages (Brown et al., 1991). Secretion of TNF was greater with a long fiber sample than a short fiber sample of amosite. BAL macrophages from rats dosed intratracheally with chrysotile asbestos released a factor that stimulated fibroblast proliferation and that these animals went on to develop lung fibrosis (Lemaire et al., 1986). In addition, BAL macrophages from sheep exposed to asbestos consistently secrete enhanced levels of fibronectin compared with controls (Begin et al., 1986).

Recently studies on growth factor regulation of the cell division cycle have been investigated extensively. Such as platelet derived growth factor (PDGF), IL-1, insulin like growth factor (IGF), transforming growth factor (TGF) are known to play a role in the progress of cells through the cell cycle. Alveolar macrophages (AL) synthesize and secrete PDGF, fibronectin, TNF-alpha, FGF, EGF, and TGF-beta (Kovacs, 1991). Asbestos fibers were shown to stimulate production of a PDGF homologue by AM which is mitogenic for rat lung fibroblasts in vitro (Kumar et al., 1988). Furthermore, lavage AMs from asbestotic patients were found to produce high levels of IGF-1, although normal levels of IGF-1 mRNA were found (Rom et al., 1988). In rats, Asbestos exposure induces AMs to secrete a FGF, or also called as MDGF from 1 week to 24 weeks after exposure (Lemaire et al., 1986). No fibroblast proliferation activity could be found in the culture supernatants of cells lavaged from rats instilled long fibers, despite significant pathological effects (Adamson & Bowden, 1990). In contrast, short fibers produced no significant pathological effects, but significant fibroblast proliferation activity was found in cell culture supernatants from rats exposed to these fibers.

Although studies on growth factors, induced by exposure to asbestos, were done rather extensively, little is known about major regulatory proteins. P34cdc2 kinase, a main enzyme for cell cycle regulation, plays an important role in eukaryotic cell cycle progression. It is consisted of regulatory subunit called cyclin and catalytic subunit called p34cdc2. The activity of p34cdc2 kinase is high at M phase of cell cycle, and very low in non dividing cells.

In this study, we are aimed to study the effect asbestos on cell cycle progression in fibroblasts and macrophages. We used RAW macrophage cell line and CHL fibroblast (chinese hamster lung cell) as a model system. Although mature macrophages are seldom dividing, we used for its readiness at any time, and its similarity to macrophages. In this study, we demonstrated histopathological features of asbestos ingested cells, and investigated molecular biochemical effect of asbestos on the cell cycle.

II. Material and Methods

Fibers

UICC standard of chrysotile (Canada, average size 4 um) was used.

Tissue culture

Cultured cells (CHL, and RAW) were grown in 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 supplemented with penicillin and streptomycin (100 units/ml), and 10% (v/v) heat inactivated fetal bovine serum (RPMI-FCSHI) at 37°C.

Histopathology

1 x 10^5 cells per 0.1 ml were cultured for overnight at 37 C to glass coverslips (12 mm) which were placed in 24 well dishes. Fibers were added directly to the coverslips. The concentrations of fibers were ranged from 200 ug to 0.1 ug. After addition of fibers, the duplicate cultures were processed for histopathology by a modified Wright staining (Leukostat, Fisher) and examined under a Olympus microscope.

Protein Kinase Assay

The peptide ADAQHATPPKKKRKVEDPKDF (CSH103) which is based on SV40 LTAg (Marshak et al., 1991) was used as $p34^{cdc2}$ kinase specific substrate. Protein kinase activity was assayed as described (Yu et al, 1991). $p34^{cdc2}$ activity was determined by measuring the incorporation of[$^{32}PO_4$] into $p34^{cdc2}$ specific peptide substrate, as follows. Sample (5 μl) were incubated in a final volume of 30 μl at 37°C for 30 min in presence of $p34^{cdc2}$ buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) with their respective peptide (1 mM) in presence of 100 μM [$-^{32}P$]-ATP (1000-3000 cpm/pmol, Amersham corp.). Reactions were started by adding radioactive ATP. Assays using the peptide were stopped by adding trichloroacetic acid to a final concentration of 10% (w/v) and phosphate incorporation was determined by adsorption to phosphocellulose paper according to previously described protocols (Kuenzel & Krebs, 1985; Marshak & Carroll, 1991).

Gel Electrophoresis and Immunoblotting

Electrophoresis was performed on 12.5% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulfate using the buffer system of Laemmli (1970). Proteins were transferred electrophoretically to nitrocellulose (Towbin et al., 1979 & 1984). The nitrocellulose was blocked with 3% BSA (w/v) in PBS and then incubated with antiserum at desired dilutions in the same buffer. goat anti-rabbit IgG alkaline phosphatase labelled was used as secondary antibody.

III. Results

Different cellular response was observed in chrysotile treated cells, depending on cell types. Untreated control CHL cells grown from day 1 to day 6, as shown in Figure 1 A (1 d), C (2 d), E (3 d), G (4 d), I (5 d), and K (6 d), were continually growing and filled whole culture dish spaces. In contrast, treated CHL cells with chrysotile fibers, seen in Figure 1 B (1 d), D (2 d), F (3 d), H (4 d), J (5 d), and L (6 d), showed cell death from 1 day and no live cells were observed from 2 day. It indicated that CHL cells were sensitive to the chrysotile fibers, thus induced cell death. It also demonstrated that chrysotile fiber inhibited cell's ability of growing with attachment to the substrate.

RAW cells (mouse macrophage cell line) showed cellular differentiation upon treatment of chrysotile fibers. In Figure 2 A (1 d), C (2 d), E (3 d), G (4 d), I (5 d), and K (6 d), RAW cells without chrysotile fibers showed a round cellular morphology with rapid cell division, and filled culture dish in the 6 day. In contrast, RAW cells with chrysotile fibers, shown in Figure 2 B (1 d), D (2 d), F (3 d), H (4 d), J (5 d), and L (6 d), showed an arresting of cell division cycle and formed multinucleated cells from 2 day and developed into multinucleated giant cell by 4, 5, 6 day. Many cells ingested chrysotile were also observed.

To verify the arresting of cell division cycle by asbestos, the activity and amount of p34cdc2 kinase were examined. As shown in Figure 3, the activity of p34cdc2 kinase was not changed in cells having no chrysotile fibers (open circles). RAW cells with chrysotile fibers showed gradual decreasing of p34cdc2 kinase activity with increasing of culture days (closed circles). In addition, the amount of p34cdc2 examined by immunoblotting showed no significant changes of its amount in cells with no asbestos (Figure 4, lane A, B, C). RAW cells with chrysotile fibers showed gradual decreasing of p34cdc2 kinase amount with increasing of culture days (Figure 4, lane D, E, F).

IV. Discussion

This report investigated effects of asbestos on cell growth and differentiation. Fibroblast and macrophage showed quite different response to asbestos. CHL fibroblast induced rapid cell death and RAW macrophage stopped its cell cycle division and began cellular differentiation. Although the mechanism of arresting cell cycle division is not known, it is suggested that signal transduction pathway of asbestos ingested fibroblast and macrophage is completely different. CHL fibroblast seems to have a signal transduction pathway leading to rapid cell death. As seen in this report, RAW macrophage seems to have a signal transduction pathway leading to cellular differentiation following arresting of cell division cycle. Although antioncogene p53 known to be involved in apoptosis has not been investigated in this report, if roles of p53 relating to p34cdc2 in asbestos ingested cells become known, the mechanism of cell death and cellular differentiation could be more clear.

Considering this result with in vivo situation, inhaled asbestos fibers are mostly ingested by alveolar macrophages or interstitial macrophages, and small amount of fibers may reach into interstitial fibroblasts. If asbestos fibers are ingested by interstitial fibroblasts, it may induce rapid cell death of interstitial fibroblast and finally be ingested by macrophages. Then asbestos fiber ingested macrophages may further differentiate to secrete fibrogenic factors to stimulate fibrosis.

Factors secreted by asbestos ingested macrophages were not investigated in this report. Further researches on the stimulation of fibroblast cell division by addition of culture supernatants from asbestos fiber ingested macrophage using ^{3}H-thymidine should be done. Researches on presence of fibroblast growth factor such as PDGF, fibronectin, TNF-alpha, INF-gamma, EGF, FGF in culture supernatants should be included in future.

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In vitro toxicity testing for asbestos and particles

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Abstract

To substitute inhalation toxicology which requires experimental animal and high cost, intensive labor, and time, in vitro toxicology has been attempted to test asbestos and man-made mineral fibers. To measure cytotoxicity, asbestos (amosite, anthophyllite, chrysotile, crocidolite, and sepiolite) and particles (SiO_2 and TiO_2) were evaluated using MTT assay and trypan blue exclusion assay. Toxicity degree to macrophage showed amosite> anthophyllite> chrysotile> crocidolite> SiO_2> sepiolite> TiO_2. Toxicity degree to fibroblast showed chrysotile~ amosite~ anthophyllite> sepiolite> crocidolite> SiO_2> TiO_2. It is suggested that in vitro fiber toxicity test was influenced by three factors: cell type, duration of culture, and fiber type. Generally, macrophage showed more strength to fibers or particles than that of fibroblast.

I. Introduction

Fiber toxicology in asbestos and man made mineral fibers (MMMF) requires high technology and various experiences. Especially, fiber inhalation toxicology demands more technology than gas inhalation or mist inhalation toxicology. To do fiber inhalation toxicology, desired size of fibers should be prepared. This is not an easy task. Electron microscopic analysis should be followed to examine the desired size. In addition, to make fibers, well protected and restricted place are required to protect general people. Maintaining consistent concentration to expose experimental animal al so requires high technology and experience. Especially, fiber inhalation toxicology is known to high cost research. Carcinogenic study may cost significant amount of money.

To substitute inhalation toxicology which requires experimental animal, high cost, intensive labor, and time, in vitro toxicology is getting recognized. Two objectives of in vitro toxicity studies have been to increase understanding of mechanisms of fiber injury at the cellular level, and to find in vitro correlates of in vivo toxicity which can be used to screen fibers for their toxicologic potential. The second objective can be seen as a logical progression from the first, leading to a greater understanding of the mechanisms involved they by enhancing the predictive accuracy of any tests that are developed. With an awareness of the cause-effect relationships between specific fiber characteristics and cellular responses, the product developer will know which direction to take in the formulation of safe fibrous materials (Hesterberg et al., 1993). In vitro testing has limitations as well as strengths. Several important in vivo determinants of fiber toxicity are difficult or impossible to assess with cell culture alone, such as lung deposition and clearance, fiber durability and dissolution, and immunogenic cellular interactions. Therefore, an approach utilizing a battery of different short-term tests has been suggested (Hesterberg in Warheit and Johnson, 1990).

In vitro fiber toxicity testing uses cell culture systems. Mossman and Sesko (1990) suggested a series of three different assays, each with a different target cell type: rat alveolar macrophages, hamster tracheal epithelial cells, and rat lung fibroblasts. Results from such a series, which utilizes an assortment of cell types, may provide a fiber toxicity profile that correlates more closely with in vivo results than data from one in vitro system. Another approach combined short-term in vivo exposure with in vitro assessment effects. In this approach, animals were exposed to fibers by instillation or inhalation. Cells were recovered from the animals by lavage and assessed in vitro (Warheit et al., 1991). Other in vitro test systems have combined two different cell types to study fiber induced cell-cell interactions, such as the effect of fiber exposed macrophages on fibroblasts (Bauman, 1990), macrophage production of neutrophil chemotactic factors (Hayes et al., 1990), macrophage cytotoxicity to tumor cells (Bissonnette et al., 1990), in vitro organ cultures, such as hamster trachea (Mossman and Craighead, 1980).

In spite of inherent limitations of cell culture systems, many reports have an established role in mechanistic studies. The use of in vitro approaches for short term toxicity screening tests becomes increasingly feasible as researchers continue develop new methods and add to the body of data on fiber-cell interactions.

To conduct fiber inhalation toxicology in the future, it was attempted to undertake asbestos and MMMF toxicity test using in vitro methods which would be possible in our level and equipments. A large number of different compositions of MMMF and man-made organic fibers are currently in use and more are under development. (list of MMMF in Japan can be seen at Table 1). Very few have been tested, thus it is important to develop in vitro toxicity screen methods.

II. Materials and Methods

Cells

Cultured cells (HeLa, CHL, and RAW) were grown in 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 supplemented with penicillin and streptomycin (100 units/ml), and 10% (v/v) heat inactivated fetal bovine serum (RPMI-FCSHI) at 37°C.

For fiber toxicity experiments, test cells (10,000 cells/well) were cultured in 96 well plates with DMEM or RPMI 1640 supplemented with 1% (w/v) penicillin and streptomycin, and 10% (v/v) fetal bovine serum at 37°C. Test cells were exposed to desired concentrations of fibers in next day and subjected to be assayed by several toxicity tests.

Fibers

UICC standard of chrysotile (Canada), crocidolite (South Africa), amosite (South Africa), and anthophyllite (Finland) and sepiolite (Kuzup, Tochigi, Japan) were used. All these fiber was provided by Dr. Hisanaga. SiO_2 TiO_2 were purchased from Sigma.

Mononuclear cell preparation

Normal human blood was collected from healthy volunteers by venipuncture in to phosphate saline buffer (PBS) containing 3.8% Na citrate + 50 mM EDTA in a ratio of 9:1 (v/v). Mononuclear cells were separated by layering the blood on Ficoll Hypaque (Pharmacia), and centrifuged at 350 g for 35 min at RT (Boyum). Mononuclear cell accumulated at the interface of the PBS and Ficoll Hypaque solution was collected, washed three times, and resuspended in PBS. To remove platelets, the mononuclear cells were layered on to 100 % FCSHI and centrifuged at 100 g for 8 min at RT. The mononuclear cells were washed twice with PBS and resuspended in RPMI-1640 supplemented with 10 % FCSHI (RPMI-FCSHI) at a concentration of 10^6 mononuclear cells/ml. 10^5 cells were added into each well of 96 well plate.

Viability test

Trypan blue Exclusion:

2.5% Trypan blue stock solution were made into distilled water. 20 ul of trypan blue solution were added into 200 ul of cell culture and suspended well with pipetting. 20 ul of solution were taken and counted for cell viability with hemocytometer. Viable cell has no blue staining of nucleus, and dead cells has blue staining of nucleus (Metcalf et al, 1986).

Cytotoxicity Assay

To measure cytotoxicity of fibers, MTT assay was used. The principal of MTT assay was similar to the [^{3}H] thymidine incorporation. MTT assay was measuring MTT in to a blue colored product (formazan) by the mitochondrial enzyme succinate-dehydrogenase. This assay was widely used for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of cells present (Denizot and Lang, 1986)

MTT ((1-(4,5-dimethylthiazol-2yl]-3,5-diphenyl formazan) was purchased from Sigma. MTT was dissolved in PBS to make a 5 mg/ml solution and filtered to sterilize and to removed any insoluble residue. MTT assay was carried out by adding 10 ul MTT/PBS solution to each well of 96 well plate (containing 100 ul of cells) and by incubating cells 2 hr in a 37 C with 5% CO_2 humidified incubator. After 2 hr of incubation, 100 ul of 10% SDS in 0.01N HCl were added to each well and the plate was further incubated at 37 C for 2 hr. Aliquots of each well were taken and measured at 570 nm.

III. Results

1. Cell type dependent toxicity of chrysotile

A. RAW cell with chrysotile

RAW cell, a mouse macrophage cell line, has many characteristics like normal macrophage. To measure cytotoxicity of chrysotile fibers, 10,000 cells/well were cultured for overnight and 200, 100, 50, 20, 10, and 1 ug of chrysotile were added into each well then assayed using MTT after 1, 3, and 5 days. As seen in Figure 1, cytotoxicity was increased with increasing concentrations of chrysotile fibers. Similar results were observed in 1 day, 3 day, and 5 day cultured cells. In addition, culture duration with fibers affected cytotoxicity thus 3 day and 5 day showed higher cytotoxicity. Fifty percent of cytotoxicity were reached at 25 ug.

B. WI 38 lung fibroblast with chrysotile

WI 38 cells were isolated from fetal lung fibroblasts. Same method as above was used to measure cytotoxicity of chrysotile fibers. As seen in Figure 2, cytotoxicity of chrysotile fibers was increased with increasing concentrations of chrysotile fibers similar to the results observed in RAW cells. Fifty percent of cytotoxicity were reached at 1 ug. In addition, culture duration with fibers also affected fiber cytotoxicity, thus 3 day and 5 day showed higher cytotoxicity.

C. HeLa cell with chrysotile

HeLa cells were isolated from cervical carcinoma and is a type of epithelial cell. Same method was applied to measure cytotoxicity of chrysotile fibers. Concentrations were ranged from 20, 10, 1, to 0.1 ug. As seen in Figure 3, cytotoxicity was determined by concentration dependent manner. Fifty percent of cytotoxicity was reached at 10 ug.

D. HeLa cell and TiO_2

Cytotoxicity induced by TiO_2 was measured by using MTT. As seen in Figure 4, cytotoxicity of TiO_2 was not dependent on particle concentration. It was suggested that TiO_2 could be used for control particle.

2. Comparison of cytotoxicity among fibers and particles

A. RAW cell

After addition of fibers and particles into RAW cell culture from 1 to 5 day, cytotoxicity was compared using MTT. In Figure 5 (5 ug), Figure 6 (10 ug), and Figure 7 (20 ug), below 50% of viability were observed with 5 ug of amosite and anthophyllite in 1 day cultured cells. In contrast, lower than 40% of viability were observed with 20 ug of crocidolite and sepiolite. Although amosite

showed consistent toxicity with culture days, other fibers and particles showed increasing their viability with increasing culture days in RAW cells. In addition, TiO_2 showed no significant changes of cytotoxicity. The order of toxicity is amosite> anthophyllite> chrysotile> crocidolite> SiO_2> sepiolite> TiO_2. Figure 8 shows RAW cells with amosite (A), anthophyllite (B), crocidolite (C), chrysotile (D), sepiolite (E), SiO_2 (F), TiO_2 and no treatment (H). Arrows indicate fiber of particle ingested giant macrophages.

B. WI 38 cell

As shown in Figure 9 (5 ug), Figure 10 (10 ug), and Figure 11 (20 ug), low concentrations of fibers or particles inclined to show the recovery of their viability with increasing culture days. Over the 10 ug of concentrations, the recovery was not observed with increasing culture days. Rapid decline of viability was seen with increasing culture days (Figure 10 & 11). In Figure 11, less than 50% of viability were observed with chrysotile, amosite, anthophyllite, and sepiolite. The order of toxicity is chrysotile~ amosite~ anthophyllite> sepiolite> crocidolite> SiO_2> TiO_2.

RAW macrophage showed persistence to fibers and particles, but WI 38 fibroblast showed weakness to fibers and particles.

3. Trypan blue exclusion test

Another method called trypan blue exclusion was adopted to test toxicity of fibers and particles. This method is easy but has limits such as counting of many numbers of cell under microscope with naked eyes. As shown in Figure 12, RAW cells with particles and fibers were counted with trypan blue exclusion after 1 day. Data were presented as percents of dead cells/total cells counted. TiO_2 showed high viability as seen above. The order of toxicity is SiO_2~ chrysotile> amosite> crocidolite~ anthophyllite~ sepiolite> TiO_2. Compared with MTT data, one thing needs to mention is that SiO_2 shows highest toxicity. Presumably, the similarity of SiO_2 ingested cells with trypan blue stained cells may contribute to this result. Besides this result, there is no significant difference between two types of toxicity testing.

IV. Discussion

MTT assay which is used for drug or metabolite toxicity testing recently and trypan blue exclusion method were used to test fibers and particles in vitro. Besides these method, other options include measurement of hemolysis of red blood cells (MacNab and Harington, 1967; Harington et al., 1971), measurement of membrane permeability by quantifying various releasates lactate dehydrogenase (LDH) (Tikes & Beck, 1980, 1983), beta glucuronidase (Brown et al., 1979; Tikes and Beck 1983; Davis 1980), and beta galactosidase (Dunnigan, 1984) and measurement of cell proliferation by colony forming efficiency (Brown et al., 1986; Chamberlain et al., 1980; Haugen et al., 1982; Hart et al., 1991). The MTT method used in this report was developed by Denizot & Lang

(1986). This method is rapid colorimetric assay for cell growth and survival without using conventional radioisotope.

As suggested by this report, in vitro fiber toxicity test was influenced by three factors: cell type, duration of culture, and fiber type. Our results demonstrated dose response and cytotoxicity to each fiber type and each cell type. Macrophages showed rather high dose response compared to lung fibroblasts or epithelial cells. Furthermore, dose-response showed different features depending on the duration of culture and fiber type. Different combinations of cell types such as combining macrophages with lung fibroblasts culture should be attempted in next experiment. Investigations including evaluating cellular viability for particle or fiber ingested cells as well as functional activity assessment of these cells are required for next series of experiments.

Although only 7 fibers and particles were evaluated for their toxicities in this report using in vitro methods, other methods should be developed for next in vitro testing. Sizing of MMMF and in vitro toxicity testing for MMMF should be done in the future.

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