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PATHOLOGY OF RESPIRATORY TRACT IN FORMER WORKERS IN MUSTARD GAS FACTORY — A REVIEW —

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Abstract : Mustard Gas (MG), a potent carcinogen, has been elucidated to induce lung cancer, by means of long-term clinical observation in former workers in MG factory on Okunojima, Hiroshima prefecture. On this rare occasion, an opportunity to observe pulmonary carcinogenetic process in human was given. Histological research on bronchial epithelium revealed the presence of preneoplastic lesions including dysplasia and various types of hyperplastic or metaplastic lesions. Furthermore, recent progresses in research techniques on genetic abnormality have been applied to analyze specific genetic changes related to exposure to MG, in order to clarify multistep carcinogenesis of bronchial epithelium. The knowledge obtained through research of former workers in MG factory should prove useful to understand chemical carcinogenesis in humans. (*J Toxicol Pathol* 8 : 343~353, 1995)

Key words : Mustard gas, Lung cancer, Chronic bronchitis, Premeoplastic lesion, p53 gene

Introduction

On Okunojima, a small island in Hiroshima Prefecture, a poisonous gas factory established by the Japanese army had been operated from 1929 until 1945 (Fig. 1). Mustard gas (Bis (2-chloroethyl) sulphide, $\beta\beta'$ -dichloroethyl sulphide,

Schwefel-Lost, S-lost, S mustard, sulphur mustard, sulphur mustard gas, yellow cross liquid, MG) was the main product. Also produced had been lewisite (chlorvinylarsine), diphenylcyanarsine (sneezing gas), hydrocyanic acid, phosgene, and chloracetophenone. This factory had a maximum ability to produce MG of 450 tons per month (Fig.

Fig. 1. Location of Okunojima in Hiroshima Prefecture.

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2). In the factory, most of the workers were protective cloth and mask, but accidentally attached poison gases and repeatedly aspirated very small amounts of poisonous gas that could not be protected. Many workers were suffering from acute injuries including erosion and bulla formation of skin, and acute inflammation of respiratory tract^{1,2} (Fig. 3).

The history of MG as a chemical weapon is long. During World War I, MG was first used in the battle field on the Western Front in 1914-1918 war on July 12-13, 1917. In this battle, about 160,000 British soldiers were treated for gas poi-

soning. The clinical study reported that 80% of victims of MG poisoning had chronic bronchitis and significant excess of deaths attributed to cancer of the lung and pleura³. However, it was reported that among US veterans exposed to MG, there was no significant increase of lung cancer mortality, compared with the controls⁴. After World War I, the use of poisonous gas including MG had been forbidden by an international treaty, but the production of poisonous gas had been continued in many countries.

The epidemiologic study on the workers at British MG factory at Cherine, England, during

Fig. 2. Mustard gas factory on Okunojima, photographed after the end of World War II (Offered by Dr. Yukutake, the Director of Tadanoumi Hospital).

Fig. 3. Skin lesions of MG worker (Offered by Dr. Yukutake, the Director of Tadanoumi Hospital).

World War II, showed strong evidences that exposure to MG could cause lung cancer and non-malignant respiratory disease as well as cancers of esophagus and stomach⁵. In the case of German MG factory worker, it was reported that malignant tumor, especially bronchial carcinoma, bladder carcinoma, and leukemia occurred with significant rate of increase⁶.

After World War II, most of the countries had abandoned to produce and keep poisonous gas, but a possibility of using poisonous gas at local wars still remains. During the Iran-Iraq war (1980–1988), Iraq used MG as a chemical weapon against Iran. In a clinical study of 535 Iranian patients exposed to MG, skin lesions (erythema, bulla, purpura, pigmentation, desquamation), and lesions of the eyes, lung, and gastrointestinal tract were demonstrated⁷. Eisenmenger *et al.* also reported the same clinical findings of Iranian victims transferred to Germany. Somani and Babu described the delayed disorders of MG on the respiratory tract among Iranian victims including chronic bronchitis, asthma, rhinopharyngitis, tracheobronchitis, laryngitis, recurrent pneumonia, and bronchiectasis⁸. Furthermore, Iraq armed forces also began a major military offensive against the Kurds on August 25, 1988, and on the occasion, MG gas was used in northern Iraq⁹.

Toxicodynamics and Experimental Animal Carcinogenicity Study of Mustard Gas

Chemical and physical properties of MG are described as follows; Chemical formula of MG is $C_4H_8Cl_2S$ (Fig. 4). MG is colorless when pure, but normally a yellow oily liquid and forms prisms on cooling. It has a slight garlic-type odor. Molecular weight is 159.1. Melting point is 13–

14°C and boiling point 215–217°C as normal atmospheric pressure. MG is sparingly soluble in water, but soluble in fat, fat solvents, and other common organic solvents¹⁰.

The toxic effects of MG include inhibition of mitosis, nicotinamide adenine dinucleotide (NAD) depletion, decreased tissue respiration, and finally cell death. Most of the toxic effects are related to alkylation of DNA. Mustard gas is also selective in their accumulation in fat tissue, however, the effects of MG poisoning may be local, systemic, or both, depending on environmental conditions, exposed organs, and extent and duration of exposure¹¹.

MG has also been reported to be a potent carcinogen as well as monofunctional and bifunctional DNA alkylating agent. It also alkylates RNA and proteins and produces DNA lesions, which may be repaired only at low doses. The sulfur mustards also alkylate the O⁶-position of guanine. DNA alkylation is likely to be primarily responsible for the mutagenic consequences of cellular exposure. The sulfur mustards induced a wide variety of genetic changes in many types of mammalian cells *in vitro* in a dose-related fashion including *in vitro* in peripheral blood lymphocyte at low dose¹². O⁶-alkylguanine is repaired by O⁶-alkylguanine DNA alkyltransferase¹³, however, O⁶-ethylthioethyl guanine induced by MG was shown to be a poor substrate for this repair system¹⁴. Possible toxicodynamics of MG are shown in figure 4. In contrast to mustard gas, the genetic toxicology of lewisite has not been fully studied. So far, there are no carcinogenicity, mutagenicity, and teratogenicity¹⁵.

As for animal carcinogenesis, nitrogen mustard, methyl bis (2-chloroethyl) amine hydrochloride had been mainly used experimentally instead of MG, because of its similar action. However, there are a few animal experiments using MG. MG, when injected subcutaneously into strains C3H, C3Hf, and A mice, induced neoplasms at the site of injection, as well as nitrogen mustard, injected subcutaneously into C3H and C3Hf mice. The neoplasms occurring at the site of injection were reported to be 11 sarcomas including fibrosarcoma, neurogenic sarcoma, and rhabdomyosarcoma, 3 papillomas, one squamous cell carcinoma, one

Fig. 4. Chemical structure of MG (I.A.R.C monograph, 1975).

Fig. 5. Possible toxicodynamics of MG (modified from Somani and Babu, 1989).

hemangioendothelioma, and one breast carcinoma. Significant increase of lung tumors in C3H and C3Hf mice injected subcutaneously with nitrogen mustard compared with controls was reported¹⁶. Intravenous injection of MG to strain A mice resulted in significant increase of lung tumors, compared with the control¹⁷. Induction of skin malignancy in rat by chamber test has been also

reported¹⁸.

Clinical Research on Former Workers in Mustard Gas Factory

Wada *et al.* described a summary observed among MG-exposed workers as follows. That is, among the 495 MG-exposed workers followed for

Table 1. Number of Malignant Neoplasms Observed in Former Workers in MG Factory (1952-1981)

Group	A group	B group	C group	Total
Number of survivors in 1952	761 (100)	705 (100)	602 (100)	2068 (100)
Total number of malignant neoplasms from 1952 to 1981	140* (18.4)**	103 (14.6)	44 (7.3)	287 (13.9)
Respiratory system	64 (8.4)	41 (5.8)	12 (2)	117 (5.7)
Digestive system	71 (9.3)	51 (7.2)	28 (4.7)	150 (7.3)
Others	16 (2.1)	14 (2)	5 (0.8)	35 (1.7)

A group: MG production, MG and Lewisite production, Lewisite production

B group: Laboratory, repair, inspection, incineration, and guard

C group: Tear gas, Sneezing gas and other gas production, desk work, medical doctor, and nurse

* Actual number, ** Incidence (%)

(Nishimoto, *et al.*, 1983)

Fig. 6. Changes of standardized mortality ratio (SMR) of lung cancer in former workers in MG factory (1952-80) (Maeda, *et al.*, 1994).

16 years, 33 deaths from cancer of the respiratory tract were observed, compared with the expected number of 0.9¹. Furthermore, Nishimoto *et al.* presumed that the cancer of respiratory tract among MG-exposed workers was induced by exposure to MG and possible influence of smoking might be negligible¹⁹ (Table 1). In addition, it was reported that clinical symptoms of chronic bronchitis at heavily-MG-exposed workers were more severe than that at less-exposed workers and unexposed coworkers²⁰, and 55.5% of these workers were suffering from chronic bronchitis²¹.

The latent period of lung cancer induced by exposure to MG was thought to be 10-33 years with an average of 22 years²². Recently, Maeda *et al.* analyzed the clinicopathological features of 45 autopsied cases with lung cancer from 1952 to 1990. The lung cancer mortality rate until 1977 was significantly higher than that among the general population of Hiroshima prefecture, after adjusting the background mortality rate for sex,

age, and year. However, this fact was not observed among the cases autopsied after 1977²³ (Fig. 6). From these facts, it is suggested that lung cancers occurring until 1977 were only those induced by exposure to MG.

Pathological Research on Former Workers in Mustard Gas Factory

The comprehensive report by Yamada summarized the autopsy and biopsy cases examined pathologically during 1952-1974²⁴. There were 68 cancer cases among 102 MG-exposed workers examined (including 94 autopsy cases and 8 biopsy cases). Among cancer cases, 44 cases had cancers of the respiratory tract. The characteristic features of respiratory tract cancer in these 44 cases are as follows;

1. Most of carcinomas were located at the central portion of the lung, as well as upper airway such as paranasal sinus, larynx, pharynx, and

Table 2. Autopsy Cases with Malignant Neoplasm among Former Workers in MG Factory (1952-1974)

Total number autopsied	102
Cases with malignant neoplasm	68 (66.7%)
Cases with multiple malignant neoplasm	8 (7.8%)
Number of cases with malignant neoplasm	
Respiratory system	
Lung	27
Pharynx	3
Larynx	8
Sinus	3
Trachea	2
Total	44 (43.1%)
Digestive system	
Stomach	9
Liver	2
Colon	2
Esophagus	3
Tongue	1
Total	17 (16.7%)
Urinary bladder	1 (0.9%)
Thyroid gland	2 (1.9%)
Skin	2 (1.9%)
Lymph node	2 (4.3%)

(Yamada, 1974)

trachea.

2. As to histopathological type of carcinoma, squamous cell carcinoma and small cell undifferentiated carcinoma occupied 70.3%.

3. There were 8 cases with multiple cancers (Table 2).

On the other hand, Makiyara conducted a pathoanatomical study on chronic bronchitis in MG-exposed workers²⁵. He observed multiple sections of tracheo-bronchial trees (19 sections from each cases) from 25 MG-exposed cases (including 11 lung cancer cases and 14 non-lung cancer cases) and 53 non-MG-exposed cases (including 13 lung cancer cases and 40 non-lung cancer cases). As the result, respiratory tracts in all of the MG-exposed cases showed pathological features of chronic bronchitis including bronchial gland hypertrophy, thickening of basement membrane and inflammatory cell infiltration, congestion, edema, fibrosis, and thickening of elastic and muscle layer in the lamina propria, and the degrees

among MG-exposed cases were more intense than that of non-exposed cases.

Kou analyzed goblet cell metaplasia of tracheo-bronchial epithelium among MG-exposed and non-MG-exposed autopsy cases²⁶. The frequency of goblet cell metaplasia was the highest in the group of MG exposed non-lung cancer group, although the frequency of goblet cell metaplasia were not correlated with Reid index in any of the groups.

Inai *et al.* examined mucus glycoprotein of goblet cells in tracheo-bronchial epithelium of MG-exposed cases compared to non-MG-exposed ones²⁷. As the result, the proportion of case positive for PNA and SBA lectin binding in MG-exposed and/or lung cancer cases had a significant difference from non-MG-exposed non-lung cancer cases. This observation suggested a large heterogeneity in oligosaccharide chains of mucus glycoprotein, which resulted from its incomplete or abnormal synthesis. This abnormality was most likely to be related to the previous exposure carcinogen, including MG.

Detection of Preneoplastic Lesion in Bronchial Epithelium

By the fact that MG-exposed cases have a high risk of lung cancer, it has been supposed that histological findings of various steps on pulmonary carcinogenesis can be observed on the bronchial epithelium of MG exposed cases. Therefore, Tokuoka *et al.* performed total histological observation of bronchial tree using step-cut sections²⁸. Using 66 male autopsy cases, composed of the group of 19 MG-exposed cases with lung cancer (MGLC), 17 MG-exposed cases with no lung cancer (MGNLC), 10 non-MG-exposed cases with lung cancer (LC), and 20 non-MG-exposed cases with no lung cancer (NLC). The tracheo-bronchial tree from distal trachea to third branch was serially cut transversely every 3-4 mm and all of the sections were prepared for histological observation. As the result, foci of moderate or severe atypical cellular lesions, that is, dysplasia, and carcinoma-*in-situ* (CIS) in total slides of each group, were counted as 146 in 3485 of MGLC, 72 in 2226 of MGNLC, 70 in 3797 of LC, and 18 in

Table 3. Frequency of Atypical Lesions in the Tracheo-bronchial Epithelium in 66 Autopsied Cases

Group	No. of tissue slides	No. of atypical lesions	Rate (%)	Test (MG vs. non-MG)
MG-exposed with lung cancer	3485	146	4.2	p<0.10
Non-MG exposed with lung cancer	2226	72	3.2	
MG-exposed with no lung cancer	3797	70	1.8	p<0.001
non-MG exposed with no lung cancer	4611	18	0.4	

(Tokuoka, *et al.*, 1986)

4611 of NLC, respectively (Table 3). The frequency of moderate or severe dysplasia and CIS in MGLC was the highest, and on the comparison between MG and non-MG cases showed significantly higher frequency in MG cases. A multivariate analysis revealed a significant correlation between the frequency of dysplasia and presence of MG-exposed history, though the frequency of dysplasia was also influenced significantly by age, smoking history, and pathological diagnosis of chronic bronchitis. Furthermore, the frequency of dysplasia was significantly higher in the

cases of squamous cell carcinoma than those of other histological types, particularly small cell carcinoma. Also these foci of dysplasia were frequently observed around squamous cell carcinoma-*in-situ* and microinvasive squamous cell carcinoma²⁹. Consequently, it was suggested that atypical cellular lesion (dysplasia) may be a preneoplastic lesion of squamous cell carcinoma.

A histological study was also performed to examine various types of hyperplastic lesions in the tracheo-bronchial epithelium of MG-exposed cases, using the same sections mentioned above, selected from 10 MG-exposed cases with no lung cancer and 15 non-MG-exposed cases with no lung cancer. Hyperplastic lesions of the tracheo-bronchial epithelium were divided into basal cell hyperplasia (polygonal cell type and spindle cell type), intermediated cell hyperplasia, and transitional metaplasia. In addition, two unique lesions, called as "intraepithelial cyst" and "flat-unheaved columnar epithelial hyperplasia" by us, were observed. The frequency of all of these hyperplastic lesions in MG exposed cases with no lung cancer were significantly higher than that in non-MG-exposed cases with no lung cancer. One lesion called as "flat-unheaved columnar epithelial hyperplasia" was observed in the case of MG-exposed case with non-lung cancer. A similar

Table 4. Frequency of Hyperplastic or Metaplastic Lesions in the Tracheo-bronchial Epithelium of 30 Autopsied Cases

Lesions	Degree	Numbers of lesions (%)			
		MG group (2284 specimens)		Non-MG group (3197 specimens)	
Basal cell hyperplasia					
Polygonal cell type	+	875	(38.3)**	700	(21.9)
	+	203	(8.9)**	97	(3.1)
	+	29	(1.3)**	7	(0.2)
Spindle cell type	+	491	(21.5)*	569	(17.8)
	+	241	(10.6)**	49	(1.5)
	+	13	(0.6)**	4	(0.1)
Intermediate cell hyperplasia		343	(12)**	77	(2.4)
Transitional metaplasia		192	(8)**	123	(3.8)
Intraepithelial cyst		52	(2.2)**	28	(0.9)
“Flat-unheaved columnar epithelial hyperplasia”		1	(0.04)	0	(0)

* P<0.05, ** P<0.01

(Takeshima, *et al.*, 1990)

Table 5. p53 Mutation in Lung Cancer Related to MG Exposure

Case no.	Age ¹⁾	Histology ²⁾	Exposure history ³⁾	Smoking history ⁴⁾	Codon change	DNA base change ⁵⁾	Amino acid change
MG-exposed cases							
MG 1	48	Sm	3×35	20	252	CTC>CTT	Silent
					254	ATC>ATT	Silent
MG 2	62	Sm	3×26	24.8	Wild type		
MG 3	83	Sm	3×77	37	244	GGC>TGC	Gly>Cys
MG 4	65	Sm	1×20	45	Wild type		
MG 5	67	Ad	2×11	41	Wild type		
MG 6	74	Ad	1×15	0.25	Wild type		
MG 7	59	Adsq	1× 2	80	263	AAT>AGT	Asn>Ser
MG 8	83	Adsq	1×63	13.3	Wild type		
MG 9	80	Sq	1×80	0	262	GGT>AGT	Gly>Ser
					274	GTT>ATT	Val>Ile
MG10	57	Sq	4×35	53	199-200	GGAAAT>GGAAT	Frameshift
					(1 base deletion)		
MG11	59	Sq	2×45	40	Wild type		
MG12	60	Sq	1×24	28.5	274-275	GTTTGT>GTTGT	Frameshift
					(1 base deletion)		
Non-exposed cases							
MN 1	87	Sm	—	36	Wild type		
MN 2	72	Sm	—	9	Wild type		
MN 3	78	Sm	—	28	173	GTG>ATG	Val>Met
MN 4	63	Sm	—	51	159	GCC>CCC	Ala>Pro
MN 5	63	Ad	—	?	Wild type		
MN 6	58	Sq	—	?	Wild type		
MN 7	66	Adsq	—	?	Wild type		
MN 8	88	Ad	—	?	235	AAC>GAC	Asn>Asp
MN 9	74	Ad	—	?	249	AGG>AGT	Arg>Ser
MN10	58	Sq	—	114	293-294	GGGGAG>GGGAG	Frameshift
					(1 base deletion)		
MN11	71	Sq	—	45	245	GGC>AGC	Gly>Ser
MN12	63	Sq	—	56	Wild type		

¹⁾ Age at diagnosis²⁾ Histology of lung cancer: sm, small cell carcinoma; sq, squamous cell carcinoma; ad, adenocarcinoma; adsq, adenosquamous carcinoma³⁾ Type of work×working duration (month). Type of work: 1, Yperite (MG) production; 2, Lewisite production; 3, Laboratory, incineration, repair; 4, Sneezing gas production⁴⁾ Pack-Years of cigarette smoking⁵⁾ Mutations on the non-transcribed strand are shown.(Takeshima, *et al.*, 1994)

result was obtained when the frequency was calculated at every branching number of respiratory tract. These findings suggest that MG played a role in forming hyperplastic lesions of tracheo-bronchial epithelium³⁰ (Table 4). So far, it remains unclear whether these bronchial epithelial hyperplastic lesions are the early stage of dysplasia

and carcinoma or not, but from the facts that in the MG-exposed cases, epithelial hyperplasia, dysplasia, and lung cancer were frequently observed in a similar distribution of bronchial tree, the sequential change from hyperplasia to dysplasia and carcinoma is highly suspected.

Genetic Abnormality in Lung Cancer of MG-exposed Cases

Recently, research techniques on molecular biology have developed especially in the field of detection of abnormalities on oncogenes and suppressor oncogenes. The application of polymerase chain reaction (PCR) method has made a great contribution to this advance, especially when archival paraffin embedded materials are used. Mutation of the p53 suppressor oncogene is known to be detected in approximately half of all human cancers³¹. This allows comparison of the mutational spectra of various cancer type. *In vitro* studies have showed that chemicals can produce distinctive mutational spectra, suggesting 'fingerprinting' of genetic damage. Molecular epidemiological studies have generated hypothesis linking environmental carcinogen exposure to human cancer. For example, a mutational hot spot in the third base of codon 249 was detected in hepatocellular carcinoma which is observed in the specific area with high incidence of liver cancer. In that area, people are infected with hepatitis B viruses and are exposed to high levels of dietary aflatoxin B₁^{32,33}. However, this type of mutation was uncommon in low-incidence areas where people are exposed to low level of aflatoxin B₁. A second example is the linkage between mutations at pyrimidine sites and UV radiation. The characteristics of p53 mutation at this type of damage have been found in human skin cancer³⁴⁻³⁶. And it was reported that the mutational spectra at p53 gene of smoker's lung cancers were significantly different from those of non-smokers and probably related to carcinogens included in tobacco smoke^{31,37,38}. These observations suggest that analyses of p53 gene mutational spectra provide us a clue to cancer etiology³⁹⁻⁴¹.

p53 gene mutation was analyzed on 12 primary lung cancers from MG-exposed cases at Okunojima and 12 lung cancers as controls. Genomic DNA was isolated from archival paraffin-embedded tissues. Exon 5-8 were amplified by PCR using p53-specific primers, and sequenced by dideoxy termination methods. Six out of 12 lung cancers from MG workers contained a total of eight somatic point mutations: two cases

had double G : C to A : T transitions; one had a G : C to T : A transversion; one had an A : T to G : C transition; and two cases had single base deletions. Four of six mutations occurred on the non-transcribed, DNA coding strand. Out of 12 unexposed cases, there were six single base mutations in six cancers, and no double mutations was noted. The p53 mutational frequency in the MG-exposed case was similar to that in the non-exposed controls as well as the usual smoking-related lung cancers reported previously. However, the distinctive double mutations (G : C to A : T transition) observed in two cases were unusual and might be related to exposure to MG⁴².

It has been elucidated that the process of carcinogenesis is highly related to the accumulation of genetic abnormality. From the fact that a number of preneoplastic epithelial lesion was observed in relation with exposure to MG, it is expected that a specific abnormality of gene exists in the preneoplastic lesions. The methods included PCR-SSCP analysis and direct sequencing to detect p53 gene mutation and analysis of genomic instability using microsatellite polymorphism (CA repeat polymorphism) of 17p13 and 3p21.3. As the result, a part of columnar cell hyperplasia and severe hyperplasia showed genomic instability, although p53 mutation could not be detected. These facts suggest that genetic abnormality may occur at the early stage of pulmonary carcinogenesis by MG (unpublished data).

Conclusion

By means of long-term clinical observation on former workers in MG factory at Okunojima, occurrence of lung cancer with significantly higher rate, especially squamous cell carcinoma and small cell carcinoma, was demonstrated. Histological observation of carcinogenetic process revealed the presence of a preneoplastic lesion. Furthermore, analysis of specific genetic changes related to exposure to MG was performed. The knowledge obtained from these studies should prove useful for understanding chemical carcinogenesis in human.

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