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Similar Pattern of Fourier-Transformed Infrared Spectrum of Bond Shift Shown in Human Cervical Cancer Cells and Rat Splenocytes Exposed to Colchicine and Methomyl

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ABSTRACT *: Apoptosis is the normal physiological process of cell death essential for the maintenance of homeostasis. The function of nicotinamide adenine dinucleotide (NAD) and adenine diphosphate (ADP) ribosylation (transfer of ADP-ribose to proteins) reactions in modifying apoptosis have recently been of great interest. Recently, CD38, a type 2 transmembrane glycoprotein expressed in hematopoietic and non hematopoietic cell lines, has been reported to possess NAD glycohydrolase activity (Han, 1999) and PC-1 and CD38 NADase regulates T cells by inhibition of phosphodiesterase/pyrophosphatase activity of PC-1 by its association with glycosaminoglycan (Hozada et al., 1999). Sindhuphak et al. (2000) has reported that cervical cancer cells can be differentiated from normal cells by using FTIR (Fourier-Transformed Infrared) technique, which has characterized shifts to be due to the phosphodiester bond in nucleic acid, protein amide I&II, carbohydrate and glycogen bands. Mechanisms how phosphodiester bond shift in cervical cancer cells as compared to control cells remain to be elucidated. Suramana et al. (2000) as well as Lohitnavy and Sinhaseni (1998) have studied methomyl and colchicine effects in rat splenocytes. Lactate Dehydrogenase Isozymes 3 (LDH3) and LDH4 were observed to increase transiently and subsided in plasma of rats exposed to 6~8 mg/kg methomyl after 48 hours. Phosphodiester bond shift of nucleic acid, detected by FTIR, was also reported (Suramana et al., 2000). We report here, after analysis of bond shift patterns, a similar bond shifts detected by FTIR spectrum observed in human cervical cells and splenocytes of rats exposed orally to 2~8 mg/kg methomyl as well as rats exposed to colchicine 2~6 mg/kg orally.*

I. INTRODUCTION

Cellular energy metabolism is an important part of the system which ensures proper immune function to combat tumor and infections.

Methomyl, a methyl carbamate insecticide, widely used in many countries around the world can be partly metabolized to acetonitrile. Lohitnavy and Sinhaseni (1999) reported increased in lactate dehydrogenase isozymes 3 and 4 in rats exposed to 6 mg/kg orally to methomyl which are indicative of splenocytes and/or endothelial cell injury in rats. N-Acetyl Cysteine can blocked this effect.

We report here, a similar bond shifts pattern detected by Fourier-Transformed Infrared (FTIR) spectrum observed in human cervical cells and bond shifts found in splenocytes of rats exposed to 2~8 mg/kg methomyl as well as rats exposed to colchicine 2~6 mg/kg orally. Colchicine is a cytoskeleton disruptor and a known anti-inflammatory drug commonly used in patients with gout. These statistically significant bond shifts found in colchicine- and methomyl-exposed rats were detected in a dose-response manner.

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II. METHODS

1. FTIR Technique

FTIR spectroscopy normally used for studying in biomolecular structures such as protein secondary structure, nucleic acid and their compounds, carbohydrates, and lipid including membrane.

FTIR technology for cervical screening has been re-

cently developed and applied widely to detect various human cancers and other diseases. This technique is based on the Fourier-Transform Infrared spectroscopy (FTIR). It detects the cell abnormalities at molecular levels which occur before the change in morphology seen under the light microscope. The advance of the FTIR technology make it possible to detect inflammatory and precancerous stages as well as stages of dysplasia, providing patients chances of recovery.

2. Cervical Cancer Cell Screening

1) Cervical cell specimens collection and preparation

Three hundred and sixty-eight cervical cell specimens were received from patients undergoing hysterectomy at the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn Hospital, Sample collection was taken by scraping with wood spatula from ectocervical part. The spatula was placed in 10 m*l* of normal saline and can be kept at 20°C for few days before preparation.

The cervical cell specimens were centrifuged to provide a small pellet of cells for FTIR analysis and then stored at 80°C until the spectral analysis was carried out. The whole procedure was shown in Fig. 1.

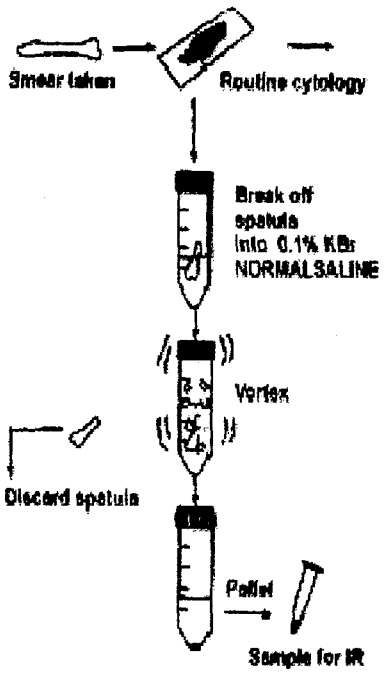

Fig. 1. Diagram of sample collection and preparation.

2) FTIR analysis

The prepared cervical cell pellet was placed on a disposable sample holder, blown with cold hair-dryer. Infrared spectra were obtained on Nicolet Prote'ge' 460 Fourier- transformed infrared spectrometer provided with DTGS KBr detector. For each spectrum, a total of 100 scans at 4 cm^{-1} resolution were applied.

Spectral parameter of each spectrum, such as peak frequency shifts and intensity ratios of various infrared bands, which were the criteria for the characterization among the normal and abnormal cervical cell specimens were calculated using software developed by Professor Patrick Wong in Ottawa, Canada.

3) Diagnostic criteria

The diagnostic criteria are the changes of various spectral parameters such as peak positions and intensity ratios, which are the results of changes in structural properties at the molecular level in cervical cells. Spectral parameters of each spectrum were calculated using Professor Patrick Wong's software developed by Professor Patrick Wong in Ottawa, Canada.

3. Rat Spenocyte Isolation

1) Determination of FTIR spectra from the methomyl- and colchicine-exposed group

After 7-day acclimatization, 42 rats were divided into 7 groups. All chemicals were diluted separately in distilled water. The control animals were dosed with distilled water by gavage. Rats were given methomyl by gavage at 2, 6 and 8 mg/kg in single doses. Colchicine was given to rats at 2, 4 and 6 mg/kg in single doses. Then 24 hours after dosing, six rats of each group were killed by diethyl ether. The spleens were removed and washed in 0.1% KBr in normal saline solution. They were weighted indivdually and then kept in 0.1% KBr in normal saline solution at -20°C for further spleen cell preparation.

2) Procedure for spleen cell specimen collection

Samples were collected by scraping with a wooden spatula from individually throughout whole spleen, and then the spatula was placed in 10 m*l* of 0.1% KBr in normal saline solution and kept at -20°C for further preparation.

3) Procedure for spleen cell preparation (Sindhuphak *et al.*, 1997)

Spleen cell specimens were thawed at room temperature and vortex stirred for 1 minute for resuspending the spleen cells, and then the wood spatula was removed from the tube. After centrifugation at 3600 rpm and 4°C for 15 minutes, the supernatant was discarded by decantation. After resuspending the spleen cells again by adding 0.8~1.0 *ml* of 0.1% KBr in normal saline solution, the whole spleen cell suspension was transferred to the microcentrifuge tube (1.5 m*l* capacity) by pasteur pipette. After centrifugation at 6000 rpm for 10 minutes, the supernatant was discarded again by pasteur pipette. Finally, the spleen cell pellet was obtained.

4) FTIR analysis (Sindhuphak *et al.*, 1997)

The prepared spleen cell pellet was placed on a disposable sample holder, blown with a cold hair-dryer. Infrared spectra were obtained by a Nicolet Prote'ge' 460 Fourier-transformed infrared spectrometer provided with a DTGS KBr detector. For each spectrum, a total of 100 scans at 4 cm^{-1} resolution were applied. Spectral parameters of each spectrum such as peak frequency shifts and intensity ratios of various infrared bands, which were the criteria for characterization among the control and exposed groups, were calculated using software developed by the National Research Council of Canada (Moffatt, 1986).

4. Statistical Analysis

Results are expressed as mean±S.E. for statistical comparisons of wavenumbers (cm^{-1}) and absorbance ratios. One-way ANOVA is used coupled with Duncan's multiple range test. A probability level (p-value) of < 0.05 is regarded as significant.

III. RESULTS

The wavenumber shift in these regions in both methomyl- and colchicine-exposed groups has a dose-response relationship. Characteristic infrared absorption bonds of peptide linkages at these amide I and amide II regions correspond to alpha-helix protein conformational change (Rice-Evans and Diplock, 1991).

Table 1. Cervical cancer screening from 368* cases, a comparison of the results between FTIR technique and histologic technique (Gold Standard)

		Histologic Technique	
		Abnormal	Normal
FTIR Technique	Abnormal	104	6
	Normal	4	161

*Ninety three cases analyzed by FTIR technique were excluded : 12 cases (3.3%) were cancer at the other sites, 11 cases (3.0%) were specimens which have been kept at room temperature too long and the cells were deteriorated, 10 cases (2.7%) were inflammation, 11 cases (3.0%) showed very noisy spectra due to instrumental failure and 49 cases (13.3%) were inadequate samples.

Table 2. Average peak frequencies (Mean±SD) of normal and abnormal spectra and major contribution of cervical cells

Peak frequencies (cm^{-1})	Abnormal (n = 110)	Normal (n = 165)	Major contribution
A	1027.16*±4.32	1024.97±1.01	Glycogen
B	1081.60*±1.96	1080.25±0.36	Phosphodiester region of nucleic acid
C	1158.78*±4.91	1155.66±0.96	Carbohydrate
D	1236.86*±1.70	1239.54±1.95	Phosphodiester region of nucleic acid
E	1405.21*±9.42	1414.48±7.72	Protein
F	1455.06*±2.96	1454.37±2.43	Protein
G	1544.47*±4.24	1547.33±2.76	Protein (Amide II)
H	1652.12*±9.41	1647.63±11.84	Protein (Amide I)

*Significant ($p < 0.05$) when compared to normal.

Table 3. Intensity ratios of normal and abnormal spectra of cervical cells, presented as Mean±SD

Peak ratios	Abnormal	Normal
A/B	1.05*±0.39	1.55±0.10
D/C	1.24*±0.99	0.25±0.03
D/G	0.28*±0.16	0.20±0.12
E/F	0.87*±0.19	0.82±0.13
D/F	0.91*±0.28	0.60±0.11

*Significant ($p < 0.05$) when compared to normal.

IV. DISCUSSION

Energy requiring events for immune functions include phospholipid turnover, ionic signals, alteration of cytoskeleton and gene transcription and transduction.

Tubulin, the building block of microtubule is subjected to several post-translational modifications. The importance of this tubulin modification cycle to the

Table 4. Shifts in FTIR spectra of amide bands in methomyl- and colchicine exposed rat splenocytes

Group	Dosage	Amide I (Mean±S.E.)	Amide II (Mean±S.E.)
Control		1653.3776±0.1650	1543.0490±0.1650
Methomyl	2 mg/kg	1653.8993±0.1650	1543.3098±0.2020
Methomyl	6 mg/kg	1654.2906±0.1300*	1543.6648±0.1940*
Methomyl	8 mg/kg	1654.6818±0.1650*	1544.2277±0.1300*
Colchicine	2 mg/kg	1653.8993±0.1650	1543.3098±0.0000
Colchicine	4 mg/kg	1654.0297±0.1750*	1543.7010±0.1750*
Colchicine	6 mg/kg	1654.5514±0.1300*	1544.0923±0.2020*

*Statistical significance compared with the control group (One-way ANOVA, $P < 0.05$).

Table 5. Increase in absorbance ratios at phosphodiester regions in methomyl- and colchicine-exposed rat splenocytes

Group	Dosage	Absorbance ratio
Control		1.3075±0.0537
Methomyl	2 mg/kg	1.4186±0.0104*
Methomyl	6 mg/kg	1.4656±0.0451*
Methomyl	8 mg/kg	1.4413±0.0271*
Colchicine	2 mg/kg	1.4634±0.0146*
Colchicine	4 mg/kg	1.4603±0.0238*
Colchicine	6 mg/kg	1.4520±0.0235*

*Statistical significance compared with the control group (One-way ANOVA, $P < 0.05$).

physiology of the cell has remained elusive.

Recently, Eiserich *et al.* (1999) reported that microtubule dysfunction by posttranslational nitrotyrosination of α-tubulin can be demonstrated as mechanism of cellular injury which are dependent on nitric oxide. Nitric oxide synthase also has recently been reported to be involved in uterine UK cells function.

Incorporation of the modified amino acid nitrotyrosine on to α-tubulin led to altered cell morphology and cellular dysfunction and finally apoptosis. Therefore, a functional cycle of tyrosination/ detyrosination seems important for cell viability.

In eukaryotes, posttranslational modifications including tyrosination, phosphorylation, polyglycylation, polyglutamylation and acetylation enable tubulin heterogeneity. The observation that nitrotyrosination altered microtubule structure and function via posttranslational incorporation into α-tubulin as well as the irreversibility of this process may explain pathologic roles for nitric oxide and oxidative stress.

Lymphocytes physiologically rearrange their genes to achieve antigenic diversity. Hypoxia enhanced peritoneal macrophage activity as revealed by enhanced phagocytosis and free radical production. It is proposed that hypoxia imposes an oxidative stress leading to decreased T-cell activity. Collard *et al.* (2000) found that the complement system plays an important role in mediating tissue injury after oxidative stress. iC_{3b} deposition on hypoxia (24 hours; $1\%O_2$) reoxygenated (3 hours; $21\%O_2$) human endothelial cells was attenuated by N-acetyl D-glucosamine or D-mannose, but not L-mannose, in a dose-dependent manner.

Recently, the lymphoid surface antigen CD38 which is basically a NAD^+ glycohydrolase involved in the metabolism of cyclic ADP-ribose, has been shown to be degraded in the presence of reducing agents such as dithiothreitol. Thie enzyme has potential signaling roles in T and B cells. The binding of CD38 ligands to the active site triggers conformational changes (Berthelier *et al.*, 2000). In this study, rat splenocytes exposed to methomyl (2~8 mg/kg orally) and colchicine (2~6 mg/kg orally) also show alpha-helix protein conformational change.

The other bond shifts seen are indicative of phosphodiesterase bond shifts associated with nucleotide (Wong, 1995). Phosphodiesterase bond shift may be related to the unknown action of methomyl non-cholinergic mechanism of action reported earlier since methomyl toxicity is synergized by caffeine, the inhibitor of phosphodiesterase. Methomyl has earlier been reported to affect progesterone reporter gene. Similar findings have been proposed in oxidative stress chemicals on uterine leiomyomas cells *in vitro* (Hodges *et al.*, 2000).

The validity of FTIR screening technique for early detection of cervical cancer is published else where (Sindhuphak *et al.*, 2000). Although the mechanism of toxicity of methomyl is still not fully understood, FTIR detection might serve as a tool to staging of uterine cell differentiation. Mechanisms in relation to modified amino acid nitrotyrosine of α-tubulin is

under further investigation.

REFERENCES

Berthelier, V., Laboureau, J., Boulla, G., Schuber, F. and Deterre, P. (2000): Probing ligand-induced conformational changes of human CD38. *Eur. J. Biochem.*, **267**, 3056-3064.

Collard, C.D., Kev, A., Morrissey, M.A., Agah, A., Rollins, S.A., Reenstra, W.R., Buras, J.A., Meri, S. and Stahl, G.L. (2000): Complement activation after oxidative stress: role of the lectin complement pathway. *Amer J. Path.*, **156**, 1549-1556.

Eiserich, J.P., Estevez, A.G., Bamberg, T.V., Ye, Y.Z., Chumley, P.H., Beckman, J.S. and Freeman, B.A. (1999): Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad Sci. USA*, **96**, 6365-6370.

Hodges, L.C., Bergerson, J.S., Hunter, D.S. and Walker, C.L. (2000): Estrogenic effects of organochlorine pesticide on uterine leiomyomas cells *in vitro*. *Toxicol Sci.*, **54**, 355-364.

Lohitnavy, O. and Sinhaseni, P. (1998): Increase in lactate dehydrogenase isozyme-4 and splenocyte toxicity in methomyl-treated rats. *J. Toxicol. Sci.*, **23**, Suppl 2, 209-213.

Rice-Evans, C.A. and Diplock, A.T. (1991): Technique in Free Radical Research. Elsevier, New York, 207-218.

Sindhuphak, R. *et al.* (1997): Cervical cancer screening by infrared spectrometer technology. *Bull Dept. Med. Serv.*, **23**, 3-9.

Sindhuphak, R., Sindhuphak, S., Issaravanich, S., Udomprasertkul, V., Srisookho, P., Bunbandarnchai, R., Warakamin, S. and Dusitsin, N. (2000): Fourier-transform infrared spectroscopy (FTIR): A new approach to cervical cancer detection in Thai women (in manuscript).

Wong, P.T.T. *et al.* (1995): Characterization of exfoliated cell and tissues from human endocervix and ectoservix by FTIR and ATR/FTIR spectroscopy. *Biospectroscopy*, **1**, 357-364.