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SPIN TRAPPING OF SUPEROXIDE RELEASED BY OPSONIZED ASBESTOS FROM HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE, HL60

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Summary: By ESR using 5,5-dimethyl-1-pyrroline-1-oxide as a spin trap, superoxide(O_2^-) production was proved upon stimulation of dimethyl sulfoxide-differentiated HL60 by crocidolite opsonized with fresh or refrigerated serum, as well as by phorbol myristate acetate(PMA). Crocidolite, unopsonized or opsonized with frozen-thawed or heat-inactivated serum, did not induce O_2^- release. Addition of iron chelators or superoxide dismutase inhibited O_2^- release completely. Neither undifferentiated nor PMA-differentiated HL60 released O_2^- upon stimulation with opsonized crocidolite.

Asbestos is a well known carcinogen for lung cancer and mesothelioma, however, its carcinogenic mechanisms are unknown(1). Asbestos is a commercial term for a group of fibrous hydrated silicates and the various types of asbestos fibers differ in their chemical and physical properties(1,2). Recently DNA damages induced by oxygen radicals are considered to be important for carcinogenesis(3,4). Asbestos has been

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Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO-OH, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl; DMPO-OOH, 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl; DTPA, diethylenetriaminepentaacetic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; PBS, phosphate buffered saline without Mg^{++} and Ca^{++}; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

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reported to induce release of oxygen radicals by chemiluminescence method, especially O_2^- from neutrophils and macrophages(5-9). ESR spin trapping is rather direct and useful assay for free radicals(10-12). Although 5,5-dimethyl-1-pyrroline-1-oxide(DMPO) is the most reliable to detect O_2^- by spin trapping, no report has demonstrated formation of O_2^- spin trapped adduct of DMPO(DMPO-OOH) by the reaction between asbestos and these cells. The mechanism and the difference among asbestos fibers to induce O_2^- release have not been elucidated neither. Most studies on human systems used neutrophils or monocytes prepared from blood(6,7,9), therefore, comprehensive and repetitive studies seem to be hard to conduct because sufficient number and repeated preparations of cells are not easily available.

A human promyelocytic leukemia cell line, HL60, can be induced to differentiate into neutrophils or macrophages by dimethyl sulfoxide(Me_2SO) or PMA, respectively(13-15). The differentiated HL60s have similar functions to their respective counterparts. One report has described the effect of asbestos on the differentiation of HL60(16), however, no report has elucidated the mechanism of O_2^- release by asbestos with HL60.

Here we used HL60 as target cells and detected O_2^- release by crocidolite, one of potent carcinogenic asbestos fibers(1), by the use of ESR with DMPO as a spin trap. We also studied the mechanisms of O_2^- release induced by crocidolite.

Materials and Methods

Materials: Crocidolite was the UICC Reference Standard sample and kindly supplied by National Centre for Occupational Health, South Africa. Crocidolite was suspended in PBS, autoclaved and sonicated. Opsonized crocidolite was prepared by incubation of crocidolite with filtrated normal human serum at 37 °C for 30 min under shaking, then kept on ice until use. Purified DMPO was obtained from Labotec, Japan. Deferoxamine mesylate was purchased from Sigma, U.S.A.
Cell: HL60(kindly supplied by Japanese Cancer Research Resources Bank, Japan) was grown in RPMI1640(Nikken, Japan) supplemented with 20 % heat-inactivated fetal calf serum(Bocknek, Canada), penicillin(100 units/ml) and streptomycin(100 μg/ml). HL60 was induced to differentiate by 1.3 % Me_2SO for 9 days or 300 nM PMA for 20 h, then washed and suspended to $1X10^7$/ml in Hanks' balanced salt solution containing 10 mM HEPES-NaOH pH 7.2.
Spin trapping: Cells were mixed with DMPO(0.1 M), and the reaction was started with the addition of crocidolite(50 μg/ml) or PMA(1 μM). ESR spectra were recorded at 25 °C with

Varian E12 ESR spectrometer. ESR settings were microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.8 G; response time, 1 s; gain, $10X10^3$; sweep time, 12.5 G/min.

Results and Discussion

When Me_2SO-differentiated HL60 was stimulated with PMA, an ESR spectrum consisted of DMPO-OOH(a_N = 14.0 G, a_H = 11.3 G, $a_H\gamma$ = 1.4 G) and hydroxyl radical spin trapped adduct, DMPO-OH(a_N =14.7 G, a_H =14.7 G), was detected(Fig.1A, these hyperfine splitting constants are compatible with those of DMPO-OOH and DMPO-OH as reported(11)). This ESR spectrum is similar to that observed when neutrophils prepared from human blood were stimulated with PMA(17). Untreated crocidolite did not(Fig.1B), however, crocidolite opsonized with fresh serum did induce formation of both DMPO-OOH and DMPO-OH(Fig.1C). No

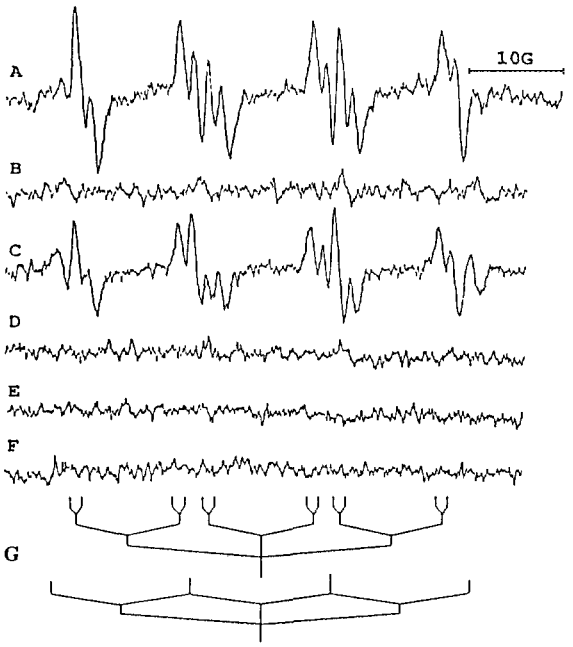

Fig.1. ESR spectra obtained with Me_2SO-differentiated HL60 in the presence of 0.1 M DMPO.
A, cells were stimulated with 1μM PMA. **B**, stimulated with unopsonized crocidolite(50 μg/ml). **C**, stimulated with crocidolite opsonized with fresh serum(50 μg/ml). Serum concentration in the reaction mixture was 0.5 %. **D**, opsonized crocidolite(50 μg/ml) without cells. **E**, stimulated with 0.5 % fresh serum(without crocidolite). **F**, same as **C** with SOD(50 units/ml). SOD was added before stimulation. **G**, stick diagrams for the ESR spectra of DMPO-OOH(upper) and DMPO-OH(lower). ESR spectra were recorded after 12 min(A), 22 min(B,C), and 17 min(D,E,F) of stimulation.

adducts were observed without HL60(Fig.1D) or with the fresh serum alone(without crocidolite, Fig.1E). These data establish that the adducts were formed upon stimulation of Me_2SO-differentiated HL60 with opsonized crocidolite. Addition of SOD(50 units/ml) inhibited both DMPO-OOH and DMPO-OH formation completely(Fig.1F), demonstrating the oxygen radical released from HL60 by opsonized crocidolite was O_2^-. DMPO-OH shown in Fig.1C might be derived from decomposition of DMPO-OOH as reported(18). To our knowledge, detection of DMPO-OOH as shown in Fig.1C is the first direct evidence by ESR spin trapping that asbestos induces O_2^- release from human cells. On the contrary to Me_2SO-differentiated HL60, undifferentiated HL60 did not release any oxygen radicals adducts with opsonized crocidolite(data not shown). PMA-differentiated HL60 formed DMPO-OH without stimulation, however, did not form further DMPO-OH or DMPO-OOH upon stimulation with opsonized crocidolite(data not shown).

We then investigated kinetics of oxygen radicals adducts formation(Fig.2). PMA-induced DMPO-OOH and DMPO-OH formation started rapidly after the stimulation, and reached the maximum about 10 min. Crocidolite-induced adducts formation took time to be detected and lasted longer. Disappearance of the adducts is considered to be due to the combination of decrease in O_2^- generation, decay of DMPO-OOH, and destruction of DMPO-OH by

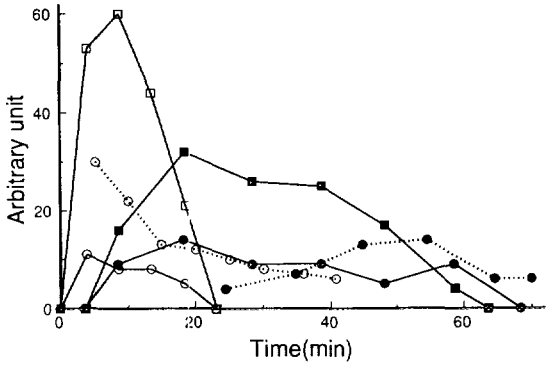

Fig.2. Formation of DMPO-OOH and DMPO-OH as a function of reaction time with Me_2SO-differentiated HL60.
Peak heights of ESR spectra of DMPO-OOH(□,■) and DMPO-OH(○,●) were plotted as a function of reaction time. Cells were stimulated with 1 μM PMA(open symbols) or opsonized crocidolite(closed symbols). SOD(50 units/ml) was added 2 min after stimulation with PMA(·····), or 20 min after stimulation with opsonized crocidolite(·····).

O_2^-. Decay of DMPO-OOH is reported to be very rapid(50 s according to (19)). Elimination of DMPO-OH is also rapid by the reaction with O_2^-(20). Addition of SOD 2 min after stimulation with PMA showed only DMPO-OH formation. Inability to detect DMPO-OOH must be due to rapid decay of DMPO-OOH because at least 2.5 min was required to set the reaction mixture into an ESR spectrometer to be recorded, during that period DMPO-OOH must be decayed out. Contrary to DMPO-OOH, the amount of DMPO-OH was more than that without SOD, probably because of protection of DMPO-OH from destruction by O_2^-. Addition of SOD 20 min after stimulation with opsonized crocidolite showed a different DMPO-OH profile from that with PMA. Same as PMA, DMPO-OOH was not detected, however, the amount of DMPO-OH at 23 min was less than that without SOD, probably because destruction of DMPO-OH by Fe^{2+} (21) was greater than the SOD protection of DMPO-OH decay. Fe^{2+} might have been generated by O_2^- until the addition of SOD, since iron is rich in crocidolite(2). Slight increase of DMPO-OH afterward might be due to generation of hydroxyl radical from H_2O_2, which was accumulated by SOD, and Fe^{2+}. The existence of Fe^{2+} was important for formation of DMPO-OH because addition of SOD at 0 min did not form DMPO-OH(Fig.1F). Estimating from the peak height of DMPO-OOH, the rate of O_2^- release by PMA, a potent tumor promotor, was higher than that by opsonized crocidolite, however, opsonized crocidolite also released enough O_2^- to be detected by DMPO. This may be in accordance with the fact that asbestos is acting not only as a complete carcinogen such as to induce mesothelioma by itself(22) but also as a promotor such as to enhance the tumor development of carcinogens in *in vivo* system(23).

We tried to elucidate factors that affect O_2^- release from Me_2SO-differentiated HL60 by opsonized crocidolite(Table1). Crocidolite opsonized with fresh or refrigerated serum induced O_2^- release as shown in Fig.1C. However, crocidolite opsonized with frozen-thawed or heat-inactivated serum did not induce O_2^- release. Repeated experiment confirmed inability of the frozen-thawed serum to induce O_2^- release. So far we could not think of any factors in serum those are affected by freezing. The result with heat-inactivated serum is in good agreement with a report that heat-inactivation of serum to opsonize asbestos markedly suppressed O_2^- release from normal

Table1. Effects of various treatments on O_2^- release from Me_2SO-differentiated HL60 by crocidolite

Treatments	O_2^- release
Unopsonized crocidolite	−
Crocidolite opsonized with fresh serum	+
refrigerated serum[a]	+
frozen-thawed serum[b]	−
heat-inactivated serum[c]	−
addition of DTPA[d]	−
Deferoxamine[d]	−
Opsonized after soak in DTPA[e]	−
Deferoxamine[e]	−
Washed twice with PBS after opsonization	−

Cells were mixed with DMPO and the reaction was started by the addition of crocidolite. O_2^- release was recorded by ESR. "+" indicates that both DMPO-OOH and DMPO-OH were detected as Fig.1C, while "−" indicates no adducts were detected as Fig.1B.

[a]Serum was stored at 4 °C for 2 weeks.
[b]Serum was stored at -80 °C and thawed before use.
[c]Fresh serum was heat-inactivated at 56 °C for 30 min.
[d]DTPA or Deferoxamine were added before stimulation with crocidolite at 0.1 mM.
[e]Crocidolite was soaked in 1 mM DTPA or Deferoxamine, washed 6 times with sufficient volume of PBS, then opsonized with fresh serum.

neutrophils assayed by chemiluminescence(6). Although immunoglobulins are reported to enhance asbestos to release O_2^- as determined by chemiluminescence(24,25), our data and others(6) suggest that several serum factors other than immunoglobulins are also required to activate crocidolite to induce O_2^- release. In the course of study we found difference between sera in activation of crocidolite. Some were potent and some were weak to activate crocidolite. Further studies are required to identify these factors and to study the interaction of these factors with asbestos.

Iron is one of major components of crocidolite(2), and reported to be necessary for crocidolite to induce cellular damages(26,27). To evaluate the importance of iron on O_2^- release by crocidolite, we studied the effects of iron chelators. Iron chelators such as DTPA or deferoxamine inhibited O_2^- release completely(Table1). These chelators did

not inhibit O_2^- release by PMA(data not shown), indicating that the inhibition of O_2^- release was not due to the cytotoxic effect of these chelators. Soak of crocidolite in iron chelators with extensive washing before opsonization also inhibited O_2^- release. Addition of $FeCl_3$ to DTPA soaked crocidolite before opsonization made the crocidolite aggregated, suggesting tight interaction between DTPA and crocidolite, and did not restore the ability of crocidolite to release O_2^- from HL60. Addition of $FeCl_3$ to the soaked and opsonized crocidolite(Table 1) neither restored the ability. These data suggest that iron is an important factor for O_2^- release by making crocidolite more adsorbable to opsonin. The interaction between crocidolite and opsonin might be weak, because twice washes of opsonized crocidolite with PBS abolished the spin trapped adducts formation completely.

We demonstrated that asbestos induced O_2^- release from Me_2SO-differentiated HL60 by ESR with DMPO as a spin trap, and pointed out the importance of serum factors and iron for the O_2^- release. Parts of the data obtained in the present ESR experiments were compatible with data obtained by chemiluminescence method with neutrophils prepared from blood. Because HL60 grows rapidly and preparation of Me_2SO-differentiated cells is simple, the assay method presented here may be useful for repetitive studies to investigate the mechanism and to elucidate the difference among asbestos fibers to induce oxygen radicals release from human cells. Furthermore this method could be a model to investigate oxidative DNA damage caused by inflammation as well as asbestos, and these studies are now in progress.

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