Ultraviolet Light-induced Lipid Peroxidation of Cultured Skin Fibroblast Membrane

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UVA (320-400 nm) light-induced lipid peroxidation was investigated in the cultured human fibroblast membrane. The main reactive oxygen species (ROS) involved in the process were identified as the partially-reduced oxygen species (superoxide radical, hydrogen peroxide and hydroxyl radical) rather than singlet oxygen. It was found that the amount of reactive oxygen species produced by UVA in extracellular matrix was not sufficient to cause membrane lipid peroxidation. The effectiveness for protection of skin from UVA induced damage was found by using scavengers and/or terminators for these oxygen species.

Introduction

Toxicity by oxygen radicals has been suggested as a major cause of cancer, tissue injury and aging[1,2]. Oxygen radicals and other oxidants appear to be toxic, mainly bacause they initiate the chain reaction of lipid peroxidation[3,4]. Lipid peroxidation generates various reactive species, with the capability of causing damage to DNA, RNA, proteins and cellular membranes[5-8]. There are many reports that reactive oxygen species are generated by UV radiation[9-12], and that these oxygen radicals mediate lipid peroxidation in skin[13]. Cunningham *et al.*[14] showed that superoxide anion was produced by photosensitized reactions of sunlight with biomolecules. In addition, Miyachi *et al.*[15] observed that topical application of liposomal SOD to hairless mouse skin could improve acute manifestations of psoralen mediated photosensitized reactions. Ultraviolet radiation can be divided according to its wavelength into UVA (320-400 nm), UVB (290-320 nm), and UVC (less than 290 nm). Among these UV lights, UVB and UVA can reach our skin. In contrast with intensive studies for the phototoxic effect of UVB on skin, the role of UVA in photoging has been thought to be negligible because of its low energy. However, UVA is present in sunlight all day long and in all seasons; the dose in summer sunlight may be 1000 times that of UVB. Several studies demonstrated that UVA may contribute most part of cytotoxic effect of light on the skin[16,17]. Most sunscreens protect only against the UVB or provide very little protection against UVA, and this may result inadvertently in an increased exposure to UVA.

In this report, it has been determined which reactive oxygen species are responsible for UVA-induced membrane lipid peroxidation in human skin fibroblast, through measurement of protective effects of various scavengers. The ressults of this report may give an idea for formulating anti-aging cosmetics and druge.

Experimental

Materials. Superoxide dismutase (SOD), mannitol, α-tocopheryl acetate, quercetin, catechin, β-carotene, TBA (thiobarbituric acid) and cimetidine were obtained from Sigma, USA. Woogon ext., ryokucha ext. and chouji ext. were supplied by Ichimaru Pharcose, Japan. Dulbeco's Modified Eagle's Medium for cell culture was purchased from Flow, USA and all other reagents were supplied by Sigma, USA. Double-distilled water was used in all experiments.

Cell culture. The human fibroblast cultures were established from neonatal foreskin. The cultures were maintained in Dulbeco's Modified Eagle's Medium (DMEM, Flow, USA) supplemented with 10% fetal calf serum (FCS, Biological Industries, Israel), 0.2 mM L-glutamine, 0.2 mM HEPES, sodium bicarbonate (Sigma, USA), 100 IU/ml penicilin, 100 mcs/ml streptomycin, and 25 mcg/ml fungizone (Flow, USA) at 37℃ in 5% CO_2 atmosphere.

The biopsies were kept at 4℃ and used within 24 hours after circumcision. The skin biopsies were rinsed twice with 70% ethanol, and subcutaneous tissues were removed. Then they were sliced in small pieces (1×3 mm) and digested by 0.25% trypsin (Biological Industrie) at 4℃ overnight. In the next day, trypsin solution is discarded and incubated again at 37℃ for 30 min followed by inactivation of trypsin with adding culture medium. After remaining tissue was removed, the cell suspension was centrifugel 1000×g for 5 min and the precipitated cell pellet was resuspended in culture medium and 10^6 cells/ml were seeded in 25 cm^2 and 75 cm^2 culture flasks (Falcon, USA). The cells were fed every other day and passed overy 4 days.

For the experiments, confluent cultures of 4 through 10 passages in 60 mm culture dishes (Falcon) were used. The viability of the culture was measured by trypan blue dye exclusion method.

Preparation of dermal homogenate. Dermal homogenate was prepared from mouse skin. The abdomen of mouse was shaved with blade, treated with depilatory, and rinsed with distilled water. After the animal was killed, skin was taken and subcutaneous tissues were removed 4.5 ml of phosphate buffered saline (PBS) was added to 0.5 g tissue, and incubated in boiling water bath for 20 min. The tissue was then homogenized with Potter-Elvehjem homogenizer and unhomogenizable part, which was mainly epidermis, was removed. Dermal homogenate was autoclaved and kept frozen until used.

UVA treatment. 150 watts xenon short arc lamp (Solar

Simulator Model 1148, Solar Light Incorporated, USA) was used as UVA source and the light intensity was measured by Radiometer IL1700 with SED038 UVA sensor (International Light Co., USA). The culture medium was replaced with PBS, and the cultures were irradiated with 1.5 J/cm^2 UVA light. After irradiation, PBS was changed with fresh medium containing test materials, and lipid peroxide was determined for each cultures after incubation for 24 hours.

0-32 μg/culture SOD, 0-50 μg/culture catalase, 0-400 μM catechin, 0-100 μl/culture woogon extract, 0-50 μM cimetidine or cimetidine-Cu (Sigma), and 0-100 μM β-carotene or α-tocopherol acetate were used as scavengers. Water insoluble scavengers were dissolved in dimethylsulfoxide (DMSO, Sigma) and diluted in culture medium. In the case of liposome, each vial was irradiated with 1.5 J/cm^2 UVA and incubated at 37℃ for 24 hours followed by lipid peroxide determination. When the effect of dermal homogenate was measured, 0-20% of homogenate was added to PBS.

Determination of lipid peroxide. Lipid peroxide was determined by thiobarbituric acid (TBA) method with some modification[18]. Each culture was treated with 1 m*l* of 0.25% trypsin-EDTA and transferred to a test tube. 2 m*l* of 0.2 M glycine-Cl buffer (pH 3.3) containing 0.4% SLS, 0.1 mM BHT, and 0.5 mM $FeCl_3$, and 2 m*l* of *n*-hexane were added to each tube and vortexed well. Tubes were incubated for 20 min in boiling water bath and 1 m*l* of 1% TBA (Sigma) in 0.08 N NaOH were added. the absorbance at 530 nm was measured after incubation in boiling water bath for 1 hr, and the concentration of malonyl dialdehyde (MDA) was calculated. Tetramethoxypropane (Tokyo Kasei, Japan) was used as standard for MDA[19].

Results and Discussion

Preventive effects of scavengers on lipid peroxidation in cultured human fibroblast. Before the experiments start, optimum dosage of UVA and optimum post-incubation time were determined to be 1.5 J/cm^2 and 24 hrs to get maximum of cell viability and level of MDA, respectively. In addition, scavengers were added after irradiation to rule out the UVA effect on scavenging action. When we take a look at the results shown in Figures 1(A)-(D), tendencies of the protecting effects of various scavengers are revealed to be same except the case of SOD. The ineffectiveness of SOD was observed *in vivo* (Figure 1(A)) in contrast with the one observed *in vitro* (liposome peroxidation) experiments. A suggestable explanation for this phenomenon is that SOD may be membrane impermeable and thus it is difficult to contact with its substrate superoxide radical in the cell. If we want to put SOD into the cell, one possible way will be a cell fusion between the liposome entrapped SOD and the intact cells. Nevertheless, in order to know whether superoxide radical was involved in the lipid peroxidation or not, cimetidine-Cu known to be a good scavenger

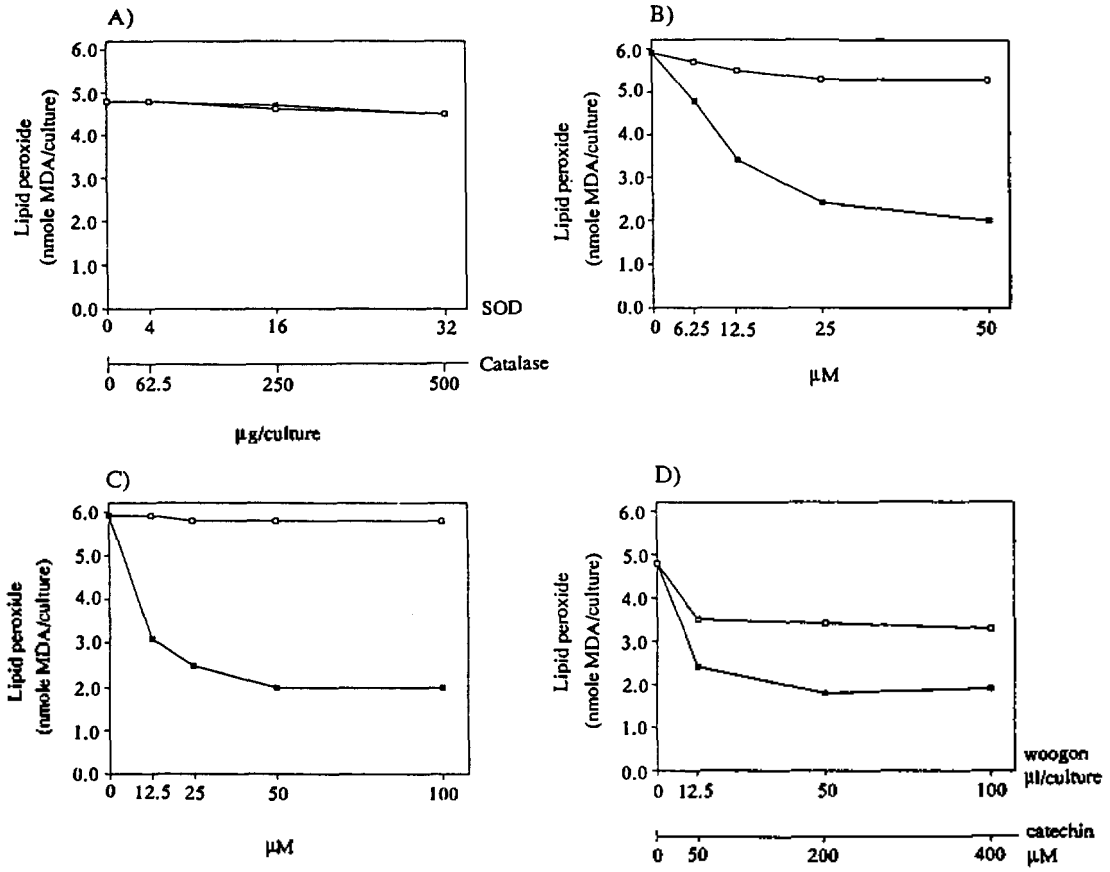

Figure 1. Effect of various scavengers for ROS on fibroblast membrane lipid peroxidation. Peroxidation of lipid was measured as a MDA level and scavenging effect was appeared as a reduction of MDA level. A) ■—■, SOD; □—□, catalase. B) □—□, cimetidine; ■—■, cimetidine-Cu. C) □—□, β-carotene; ■—■, α-tocopheryl acetate. B) □—□, catechin; ■—■, woogon extract.

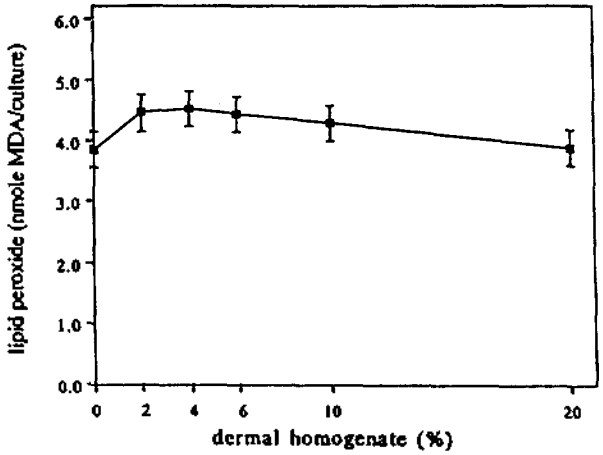

Figure 2. Effect of dermal homogenate on fibroblast membrane lipid peroxidation. Dermal homogenate (% concentration) was added to the culture treated with 1 ml of 0.25% trypsin-EDTA solution.

of superoxide radical[20] was used. It showed an excellent peventive effect (Figure 1(B)), demonstrating superoxide radical could be one of the most responsible reactive oxygen species for membrane lipid peroxidation. α-Tocopheryl acetate showed an excellent preventive effect, and woogon and catechin had moderate effects (Figures 1(C), (D)). Theses results imply that free radical quenchers can prevent membrane peroxidation effectively. However, the singlet oxgen quencher, β-carotene, did not prevent the lipid peroxidation in the cultured cells (Figures 1(C)). Data presented here suggest that the partially reduced oxygen species play the critical roles for lipid peroxidation in the intact cell, while singlet oxygen has no or little effect on this process.

Effect of dermal homogenate. Some investigators reported that SOD could prevent UV-induced damage *in vivo* when used topically or intracutaneously[1,21]. Skin has very complex tissue components. There are various cells including keratinocytes, fibroblast, Langerhans' cells, macrophages, and other cells. The most part of the dermis is filled with extracellular matrix materials, such as collagen, elastin and various proteoglycans. The skin also has blood vessels and leucocytes and lymphocytes within blood. Reactive oxygen species may be produced in extracellular matrix by UV light. Morever there are many evidences that, during active phagocytosis, leucocytes and macrophages produce large amount of superoxide radical when they are stimulated by UV-induced inflammation[22,23]. If this is true, topically applied enzyme scavengers may be able to prevent skin from quenching superoxide anion produced in extracellular matrix or blood cells. In this regard the effect of extracellular matrix was also studied.

Dermal homogenate was used to determine the action of extracellular matrix to produce reactive oxygen species by light. The MDA level was not changed by dermal extract when cells were treated with UVA light (Figure 2). It was concluded that even if the reactive oxygen species were produced by UVA light in extracellular matrix, they were not sufficient to cause membrane lipid peroxidation. The other possible source of reactive oxygen species *in vivo* is hemopoietic cell lines, such as leukocytes, lymphocytes and macrophages[24,25]. To determine the effect of scavengers on reactive oxygen species produced within whole skin, the experiments using the system including those cells are further needed.

Our results suggest that scavengers of partially reduced oxygen species and peroxide radicals can prevent skin from UVA-induced damage when they are used topically.

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Solid State Reactions of Iridium-1,5-Cyclooctadiene Compounds with Hydrogen and Carbon Monoxide

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Solid-gas reactions of $[Ir(COD)(PhCN)L]ClO_4$ (**1**), $[Ir(COD)L_2]ClO_4$ (**2**), $[Ir(H)_2(COD)(PhCN)L]ClO_4$ (**3**), $[Ir(H)_2(COD)L_2]ClO_4$ (**4**), $[Ir(COD)(CO)_2L]ClO_4$ (**5**), $[Ir(COD)(CO)L_2]ClO_4$ (**6**) and $[Ir(COD)(PhCN)_2]ClO_4$ (**7**) (COD=1,5-cyclooctadiene; L=PPh_3 (a), $AsPh_3$ (b)) with H_2 and CO have been investigated to find the differences in reactivities from those in solution. $Ir(H)_n$ moiety and cyclooctene (COE) are detected in the solid-gas reactions of **1** and **2** with H_2 while they are not observed in the reactions in solution. Complexes, **3** and **4** lose H_2 in the solid state while they undergo hydride transfer to COD in solution to produce COE and cyclooctane (COA). Solid-gas reaction of **5** and **6** with H_2 produce only COE at 25℃ while their reactions in solution produce COA. The reaction of CO with **3a** in the solid state gives quantitative amount of **5a** while in solution it gives only unknown product. Both reactions of **7** in the solid state and in solution with CO give unidentified brown solid which further reacts with CO/H_2 to give $Ir_4(CO)_{12}$.

Introduction

Heterogeneous reactions of solid transition metal complexes with gaseous molecules in the absence of a solvent have been attracted by some chemists since they often occur *via* different reaction pathways from those of homogeneous ones and provide synthetic methods for new compounds that can not be readily prepared from the reactions in solution.[1,2] We have recently prepared new iridium(I) complexes, $[Ir(COD)(CO)_2L]ClO_4$ and $[Ir(COD)(CO)L_2]ClO_4$ (COD=1,5-cyclooctadiene; L=PPh_3, $AsPh_3$) from the reactions of $[Ir(COD)(PhCN)L]ClO_4$ and $[Ir(COD)L_2]ClO_4$ with CO, respectively in the absence of a solvent[3] which are not prepared from the reactions in solution. We now wish to report solid-gas reactions of some Ir-COD complexes with H_2 and CO including some unique reactions that are not observed for reactions in solution.

Results and Discussion

Reactions of $[Ir(COD)(PhCN)L]Cl_4$ (1) and $[Ir(COD)L_2]ClO_4$ (2) (L=PPh_3 (a), $AsPh_3$ (b)) with H_2. Cyclooctane (COA) is quantitatively produced from the reactions of **1** and **2** with H_2 at 25℃ in the absence of a solvent. Solid samples obtained at the early stage of these reactions showed strong infrared absorptions at 850-950 and 2100-2250 cm^{-1} due to ρ(Ir-H) and ν(Ir-H), respectively which slowly disappeared as the reactions proceed. On the other hand, ^{1}H-NMR spectral measurements confirmed a considerable amount of the initial hydrogenation product, cyclooctene (COE) at the early stage of the reactions which was further hydrogenated to cyclooctane (COA). Neither $Ir(H)_n$ moiety nor COE is detected by IR and ^{1}H-NMR measurements during the reactions of **1** and **2** with H_2 (1 atm) in solution at 25℃ where the conversion of the coordinated COD to COA is rapid. Dihydridoiridium(III) complex, $[Ir(H)_2(COD)(PhCN)(PPh_3)]ClO_4$ (**3a**),[4] $[Ir(H)_2(COD)(PhCN)(AsPh_3)]ClO_4$ (**3b**),[5] $[Ir(H)_2(COD)(PPh_3)_2]ClO_4$ (**4a**)[6] and $[Ir(H)_2(COD)(AsPh_3)_2]ClO_4$ (**4b**)[5] have been prepared from the reaction of **1** and **2** with H_2 in solution at low temperature where the corresponding solid-gas reactions do not occur. Complex **4b** could be obtained in good purity from the solid-gas reaction of **2b** with H_2 at room temperature when the reaction is stopped at the early stage (within 5 minutes) (Eq. 1) while none of other dihydrides (**3a**, **3b**, **4a**) were isolated from the solid-gas reactions at room temperature. These observations suggest that the production of COA from the solid-gas reactions of **1** and **2** with H_2 may also occur through the well-known reaction pathways (*via* either metal-hydride or metal-olefin route) established from the homogeneous catalytic hydrogenation of olefins with metal complexes.[7] A similar observation was previoulsly reported: the solid-gas reaction of $[Ir(COD)(PPh_3)_2]_3PW_{12}O_{40}$ with D_2 gives COA containing up to 16 deuterium atoms, which was explained to occur *via* $(H)_2Ir$-COE and $(H)_3Ir$-$^3\eta$-cyclooctenyl species.[1c]

$$[Ir(COD)(AsPh_3)_2]ClO_4 + H_2 \longrightarrow \underset{\textbf{4b}}{[Ir(H)_2(COD)(AsPh_3)_2]ClO_4}$$
$$\textbf{2b} \qquad \text{(within 5 min.)}$$
$$\xrightarrow{x\ H_2} \text{"}Ir(H)_n(COE)(AsPh_3)_2\text{"} \xrightarrow{y\ H_2} COA + \text{unknown iridium compound(s)} \quad (1)$$