Effect of Squalene Monohydroperoxide on Cytotoxicity and Cytokine Release in a Three-Dimensional Human Skin Model and Human Epidermal Keratinocytes

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Received October 31, 2001; accepted February 6, 2002

In order to clarify that squalene monohydroperoxide (SQOOH) correlates with changes in morphology through cytotoxicity and establish in vitro evaluation of the cytotoxicity of lipid hydroperoxide, the effect of SQOOH on cytotoxicity and morphology in normal human epidermal keratinocytes (NHEK(B)) and the Gunze three-dimensional cultured human skin model (Vitrolife-skin) was investigated. Additionally, the effect of radical scavengers (mannitol, vitamin C and (+)-catechin) on the cytotoxicity in Vitrolife-skin was studied. The level of lipid hydroperoxide (phosphatidylcholine hydroperoxide: PCOOH) in the cellular membrane was increased with the concentration of SQOOH, and the rise in cytotoxicity in NHEK(B) was associated with changes to the cellular membrane. A concentration-dependent and protective effect on the increase in cytotoxicity and PCOOH content was observed. To clarify the effect of SQOOH on the release of cytokine from cells, IL-2 level from NHEK(B) and Vitrolife-skin were investigated. IL-2 release from the cells was enhanced by SQOOH and increased at a non-cytotoxic dose.

These results suggest that the increase in lipid hydroperoxides resulting from the auto-oxidation of lipids within cellular membranes in the presence of SQOOH correlates with changes in morphology due to cytotoxicity. SQOOH enhanced the release from cells at a non-cytotoxic dose. A method for assessing the protective effect on the cytotoxicity of lipid hydroperoxides using cells would be useful for in vitro evaluation of the cytotoxicity.

Key words squalene monohydroperoxide; cytotoxicity; IL-2; normal human epidermal keratinocytes (NHEK), Vitrolife-skin

Squalene monohydroperoxide (SQOOH) is a primary oxidized lipid produced from squalene by solar UV.1,2) We have already reported on the cytotoxicity and PGE2 release due to skin aging and atopic dermatitis.2,5) Many reports about the have concentrated on the cytotoxicity of effects of hydroperoxides on cytotoxicity in cultured cells model.3,4) Others have reported that SQOOH is a product of presence of a photosensitizer in a three-dimensional human skin model.6–9) (a type of peroxidant of cellular membrane lipids with changes in morphology through cytotoxicity and establish a non-cytotoxic dose.

 MATERIALS AND METHODS

Cells Normal human epidermis keratinocytes (NHEK(B)) and the Gunze three-dimensional cultured human skin model (Vitrolife-skin; a new skin model (sizes, 8 mm) composed of two types of collagen sponge and two types of human skin cells, fibroblasts and keratinocytes, and has two layers dermis and epidermis, like real skin, and keratinocytes which were cultured with an air interface14,15) were purchased from Kurabo Co. (Osaka, Japan) and Gunze Co. (Kyoto, Japan), respectively.

Reagents SQOOH was a gift from Dr. Yoshiyuki Kohno (Shiseido Co.). IL-2 kits were purchased from Funakoshi Co. (Tokyo, Japan). Medium for NHEK(B) (Humedia-KG2) and medium for Vitrolife-skin (DMEM+5% fetal bovine serum (FBS)) were purchased from Kurabo Co. (Osaka, Japan) and Gunze Co. (Kyoto, Japan), respectively. Mannitol (MA) was purchased from Kanto Chemicals (Tokyo, Japan). Other reagents were from Wako Chemicals (Osaka, Japan).

Cytotoxicity NHEK(B) cells at 5×10^3/well in 0.1 ml of medium were placed into 96-well plates and incubated at 37 °C, 5% CO2, and a humidity above 90% for 48 h. After the incubation, hydroperoxides (0—100 μM SQOOH dissolved in 0.2 ml of medium containing 1% EtOH) was added and the plates were incubated for 24 h. The viability of NHEK(B) was then determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay1,4,16) and concentrations of IL-2 released as an indicator of the immunoreaction were determined by enzyme immuno assay (EIA).17)

Vitrolife-skin was placed into 24-well plates and pre-incubated at 37 °C, 5% CO2, and a humidity above 90% for 1 h. Then hydroperoxide (0—100 μM SQOOH dissolved in 0.1 ml of medium containing 1% EtOH) was added to the surface of the cells and the incubation continued for 24 h. Fi-
nally, the viability of Vitrolife-skin was determined by an
MTT assay-2 and lactate dehydrogenase (LDH) assay, and
the concentration of IL-2 released was determined by
EIA.

After 1 h pre-incubation, anti-oxidants were added to 0.25 ml
of medium of another set of Vitrolife-skin samples (in 24-
well plate) and incubated at 37 °C, 5% CO2, and a humidity
above 90% for 2 h. After the incubation, 100 µl SQOOH di-
luted with 0.2 ml of squalene (SQ) was added to the surface
of the cells. The cells were again incubated at 37 °C, 5%
CO2, and a humidity above 90% for 2 h. The viability of the
Vitrolife-skin was determined by MTT assay-2, the con-
centration of IL-2 release was determined by EIA and the lev-
els of phosphatidylcholine hydroperoxide (PCOOH) and
phosphatidylethanolamine hydroperoxide (PEOOH) (lipid hy-
droperoxides of the cellular membrane) were determined by
chemiluminescence high-performance liquid chromatogra-
phy (CL-HPLC).

MTT Assay-1 One hundred microliters of MTT solution
(MTT diluted with medium: 2 mg/ml) was added to each
well and NHEK(B) cells were incubated at 37 °C, 5% CO2,
and a humidity above 90% for 2 h. After the incubation,
the cells were washed twice with phosphate-buffered saline
(PBS, pH 7.4) and then 200 µl of MTT extracting solution
(isopropanol containing 0.05 M HCl) was added to each
well and the plate shaken continuously at 60 rpm and room
temperature for 1 h. The absorbance at 540 nm (A540) was
determined spectrophotometrically by a microplate reader (Model

Viability was calculated according to the following equa-
tion:

\[
\text{viability} \% = \frac{(A_{s540} - A_{t540})(A_{c540} - A_{t540})}{100}
\]

\[A_{s540}=\text{absorbance of the extract when SQOOH was applied to NHEK(B)}\]
\[A_{c540}=\text{absorbance of the extract when only medium (without SQOOH)}\]
\[A_{t540}=\text{absorbance of the extract when medium (without cells) was used as a blank at 540 nm}\]

MTT Assay-2 One milliliter of MTT solution (MTT di-
luted with medium: 2 mg/ml) was added to each well and
the Vitrolife-skin incubated at 37 °C, 5% CO2, and a humidity
above 90% for 3 h. After the incubation, the tissues were
washed twice with PBS and holed out with a punch. Then
2 ml of MTT extracting solution and 40 µl of sodium dodecyl
sulfate (SDS; helpful for extracting) were added to each well.
The rest of the procedure was as for the MTT assay-1.

Viability was calculated according to the following equa-
tion:

\[
\text{viability} \% = \frac{(A_{s540} - A_{t540})(A_{c540} - A_{t540})}{100}
\]

\[A_{s540}=\text{absorbance of the extract when SQOOH was applied to Vitrolife-skin at 540 nm}\]
\[A_{t540}=\text{absorbance of the extract when only medium containing 1% EtOH was applied to Vitrolife-skin (control) or SQ was applied to Vitro-
life-skin (negative control) at 540 nm}\]
\[A_{c540}=\text{absorbance of the extract when collagen sponge without cells was used as a blank at 540 nm}\]

LDH Assay Fifty microliters of medium and reagent
(nitro blue tetrazolium at 0.74 mg/ml, diaphorase and NAD+ dissolved with Lithium DL-lactate buffer at 50 mg/ml) was
added to the wells and then the Vitrolife-skin was incubated
at room temperature for 45 min. After the incubation, 100 µl
of stop reagent (0.5 M HCl) was added and then the ab-
sorbance at 570 nm was determined. The absorbance of the
sample was calculated according to the following equation:

\[A_{OD570}=A_{s570} - A_{t570}\]

\[A_{s570}=\text{absorbance of the medium when SQOOH was applied to Vitro-
life-skin at 570 nm}\]
\[A_{t570}=\text{absorbance of the medium when only medium containing 1% EtOH was applied to Vitrolife-skin at 570 nm}\]

IL-2 Assay Each well in a 96-well assay plate was rinsed
immediately with 0.5 ml/l of Tween 20 in PBS (the Wash
Buffer) and then 50 µl of culture medium, with either 100 µl
of acetylcholinesterase: IL-2 Fab’conjugate solution, was
added to each well. After incubation at 4 °C for 18 h, the
wells were rinsed five times with the Wash Buffer and
then 50 µl of Ellman’s Reagent was added. The absorbance at 405 nm
was then determined and the concentrations of IL-2 was cal-
culated by comparison with the absorbance of standards.

Determination of PCOOH and PEOOH Lipids in the
Vitrolife-skin were extracted twice with a cooled mixture of
chloroform:2-propanol (1 : 1, v/v) containing 10 ppm butyl-
hydroxytoluene. The lower layer was collected and concen-
trated with a rotary evaporator under reduced pressure and
the residue was dissolved with Lithium DL-lactate buffer at 50 mg/ml) was
sampled and after 4 h, the increase in IL-2 reached a plateau (Fig. 2).

Significant differences from the con-
matic assay-1. The IC50 of SQOOH against NHEK(B) was 630
mM, which was (Fig. 2).

The time course of the release of IL-2 from NHEK(B) was
investigated. After 1 h incubation, the IL-2 release signifi-
cantly increased depending on the concentration of SQOOH
and after 4 h, the increase in IL-2 reached a plateau (Fig. 2). In
the presence of 100 µM SQOOH, a significant decrease in
viability was not observed, but a significant increase in IL-2
was (Fig. 2).

SQOOH was added to the Vitrolife-skin (considered more
useful for estimating the harmful effects of lipid hydroperox-
ide on the human body than NHEK(B)), the system incu-
bated for 24 h at 37 °C, and the viability assessed (Fig. 3).
The viability of Vitrolife-skin decreased significantly de-
pending on the concentration of SQOOH. Therefore, the re-
lease of LDH from Vitrolife-skin in the presence of 100 mM SQOOH (the viability of the cells at 24 h after addition of SQOOH was reduced more than 50%) was investigated. The LDH release from Vitrolife-skin increased after 4 h and reached a peak after 8 h (Fig. 4). In addition, Vitrolife-skin stained with HE in the presence of SQOOH was investigated for 24 h. First, swelling of the stratum corneum was observed after 2 h. After 4 h, the cells in the epidermis shrunk and curled, and then shrinking, curling and vacuolization of the cells in the dermis was observed after 8 and 24 h (Fig. 5). The control after 24 h was the same as in the presence of SQOOH after 0 h (Fig. 5a). The amount of IL-2 release at 24 h after the addition of SQOOH was investigated. A significant increase in the amount released was observed depending on the concentration of SQOOH (Fig. 6).

**Effect of Anti-oxidants on Cytotoxicity, Cytokine Release and PCOOH and PEOOH Contents** To clarify the cytotoxic contribution of SQOOH to auto-oxidation, the effect of anti-oxidants on the cytotoxicity of SQOOH in Vitro-life-skin was investigated at 24 h. In the presence of MA (OH

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**Fig. 1. Effect of SQOOH on Cytotoxicity in NHEK(B)**
NHEK(B) cells were incubated for 24 h after the addition of SQOOH. Data are means±S.D. (n=3–4). Significantly different from control (only medium was applied to NHEK(B)), *p<0.05, **p<0.01, ***p<0.001.

**Fig. 2. Effect of SQOOH Concentration on IL-2 Release from NHEK(B)**
Data are means±S.D. (n=3–5). Significantly different from control (only medium was applied to NHEK(B)), *p<0.05, ***p<0.001.

**Fig. 3. Effect of SQOOH on Cytotoxicity in Vitrolife-Skin**
Vitrolife-skin was incubated for 24 h after the addition of SQOOH. Data are means±S.D. (n=3–4). Significantly different from control (only medium containing 1% EtOH was applied to Vitrolife-skin), *p<0.05, ***p<0.001.

**Fig. 4. Effect of SQOOH on LDH Release from Vitrolife-Skin**
Data are means±S.D. (n=3–4). Significantly different from control (incubation time 0 h), ***p<0.001.
radical scavenger) or ascorbic acid (VC, radical scavenger and reducer), the cytotoxicity decreased significantly compared to that in the SQOOH-treated group (Fig. 7-1). CATC, VC and MA also significantly inhibited the production of IL-2 at 24 h (Fig. 7-2). In the absence of SQOOH, there was no significant effect of CATC, VC and MA on viability and IL-2 release (data not shown).

To clarify the contribution of the oxidation of membrane lipids caused by the auto-oxidation of SQOOH to the cytotoxicity, the effect of CATC on the amount of PCOOH and PEOOH (hydroperoxides of cell membrane lipids) in Vitrolife-skin 24 h after addition of 100 mM SQOOH was investigated. The PCOOH content of Vitrolife-skin increased significantly on addition of SQOOH. But in the presence of CATC, this increase was significantly inhibited and the PCOOH content was no different than in the control (without SQOOH) group (Fig. 8). PEOOH was not detected.

DISCUSSION

Kohno and Takahashi\textsuperscript{2)} reported that when Chinese hamster lung fibroblasts were incubated in DMSO with SQOOH for 48 h, the IC\textsubscript{50} of SQOOH was about 230 \mu M. We obtained a slightly higher value than theirs in the case of NHEK(B), but in the case of Vitrolife-skin, the IC\textsubscript{50} (about 70 mM) was over 100 times higher. So we considered that the stratum corneum in Vitrolife-skin might protect cells from damage...
due to SQOOH.

Kohno and Takahashi\(^{20}\) reported that no cytotoxicity for TESTSKINTM (three-dimensional cultured human skin model) on addition of 2100 mM SQOOH was observed after 4 h, but that cytotoxicity was strong after 24 h. So they considered that the mechanisms of cytotoxicity result from the penetration by SQOOH of the epidermis and dermis of TESTSKINTM and proposed that SQOOH damaged the membrane lipids in cells through auto-oxidation. Chiba et al.\(^{21}\) reported that repeated topical application of 10 mM SQOOH to hairless mice for 3 weeks induced slight hyperkeratosis, moderate epidermal thickening and slight hyperplasia of sebaceous glands. In the present study, cytotoxicity against cells in the epidermis was induced 4 h after the addition of SQOOH, and at 8 h strong cytotoxicity was observed. CATC, VC and MA as radical scavengers significantly protected cells from the cytotoxicity by attenuating the activity of SQOOH. SQOOH increased the amount of PCOOH in cellular membrane but CATC significantly protected the cells from this increase by preventing the peroxidation of cellular membrane lipids due to SQOOH. It was not clear whether PEOOH was present, but it is conceivable that a lack of detection of PEOOH may have caused the difference between PE (inner-cellular membrane) and PC (surface cellular membrane). As reported by Kohno and Takahashi, it is conceivable that SQOOH caused the peroxidation of cellular membrane lipids within the dermis and epidermis of Vitrolife-skin due to auto-oxidation and consequently the cytotoxic effects appeared after 4 h.

These results suggest that the increase in lipid hydroperoxides resulting from the auto-oxidation of lipids within cellular membranes in the presence of SQOOH correlates with changes in morphology through cytotoxicity. And SQOOH enhanced IL-2 release from the cells at a non-cytotoxic dose. A method for assessing the protective effect on cytotoxicity and cytokine release of lipid hydroperoxides using cells would be useful for the in vitro evaluation of the cytotoxicity of lipid hydroperoxides.

### Acknowledgements
The authors would like to thank Dr. Yoshiyuki Kohno with Shiseido Company for providing SQOOH.
REFERENCES