OBSTETRICS

Hydroxylated fullerene: a potential antiinflammatory and antioxidant agent for preventing mouse preterm birth

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OBJECTIVE: Intrauterine infection such as by *Escherichia coli* and *Ureaplasma* spp induce placental inflammation and are one of the leading causes of preterm birth. Here we evaluated hydroxylated fullerene ($C_{60}[OH]_{44}$) for its in vitro antiinflammatory and antioxidant effects against host cellular responses to the ureaplasma toll-like receptor 2 (TLR2) ligand, UPM-1. In addition, we investigated the preventative effects of $C_{60}(OH)_{44}$ in vivo in a mouse preterm birth model that used UPM-1.

STUDY DESIGN: TLR2-overexpressing cell lines and the primary cultures of mouse peritoneal macrophages were pretreated with $C_{60}(OH)_{44}$. After UPM-1 addition to the cell lines, the activation of the nuclear factor kappa-light chain-enhancer of activated B cells (NF-kappaB) signaling cascade and the production of reactive oxygen species were monitored. The levels of expression of inflammatory cytokines of interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, and the production of reactive oxygen species were quantified after stimulation with UPM-1. The in vivo preventative effects of $C_{60}(OH)_{44}$ on mice preterm birth were evaluated by analyzing the preterm birth rates and fetal survival rates in the preterm birth mouse model with placental histological analyses.

RESULTS: Pretreatment with $C_{60}(OH)_{44}$ significantly suppressed UPM-1-induced NF-kappaB activation and reactive oxygen species production in TLR2-overexpressing cell lines. In the primary culture of mouse peritoneal macrophages, UPM-1-induced production of reactive oxygen species and the expression of inflammatory cytokines such as IL-6, IL-1β, and TNF-α were significantly reduced by pretreatment with $C_{60}(OH)_{44}$. In the UPM-1-induced preterm birth mouse model, the preterm birth rate decreased from 72.7% to 18.2% after an injection of $C_{60}(OH)_{44}$. Placental examinations of the group injected with $C_{60}(OH)_{44}$ reduced the damage of the spongiotrophoblast layer and reduced infiltration of neutrophils.

CONCLUSION: $C_{60}(OH)_{44}$ was effective as a preventative agent of preterm birth in mice.

Key words: hydroxylated fullerene, nuclear factor-kappaB, preterm birth mouse, reactive oxygen species, ureaplasma membrane-1

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The World Health Organization has defined preterm birth as birth that occurs prior to the 37th gestational week.[1] The estimated number of preterm births worldwide was 15 million in 2010 (11.1%).[2] Preterm birth is a risk factor in more than 50% of all incidents of perinatal mortality and infantile respiratory failure, and it results in long-term neurologic morbidity.[2,3]

Babies born before 28 gestational weeks have a risk of disease during childhood and a risk of cerebrovascular and ischemic heart disease in early adulthood.[4] The development of perinatal care has raised the survival rate of infants with extremely low birthweights,[5] which resulted in the recent

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observation that females born preterm have an increased risk of reproductive difficulties.[6] Considerable efforts have been focused on the control of preterm birth; however, the preterm birth rate is still increasing.[7]

It is widely accepted that intrauterine bacterial infections are one of the most important causes of preterm birth.[8] Lipopolysaccharide (LPS) from *Escherichia coli*, which is a ligand of the toll-like receptor (TLR) 4, activates downstream inflammatory responses.[9,10] LPS has often been used in mammalian models of preterm birth.[11-13]

There is growing evidence that *Ureaplasma* spp, belonging to the family *Mycoplasmataceae*, has been a common causative bacteria of chorioamnionitis (CAM) and, consequently, preterm birth.[14-16] Patients with threatened preterm birth and intact membranes who test positive on polymerase chain reaction (PCR) for *U urealyticum* in the amniotic fluid are at risk of going into preterm birth with adverse perinatal outcomes.[17]

We analyzed 151 preterm and stillbirth placentas, and 63 (42%) of them were positive for *Ureaplasma* spp.[18] Although *Ureaplasma* spp has been considered to be involved in the pathobiology of preterm birth for more than half a century, the mechanism of its involvement remains unclear. Synthesized diacylated 21 N-terminal amino acids of the ureaplasmal outer membrane lipoprotein (UPM-1) activates the signaling cascade of nuclear factor-kappa—light-chain enhancer of activated B cells (NF-kappaB) through TLR2. UPM-1 has been shown to induce preterm birth and intrauterine fetal death in C3H/HeN mice.[19]

Fullerene, a nanomaterial with radical-scavenging activity, is a molecule that comprises carbon in the form of a hollow sphere.[20-24] These observations suggest fullerene is an antioxidant agent. In our previous study, we demonstrated injections of the 70 nm silica nanoparticle restricted fetal growth and induced abortion in pregnant mice. However, fullerene did not cause any pregnancy-associated complications at the dose used in our study.[25]

In this study, we aimed to clarify the antiinflammatory and antioxidant effects of water-soluble hydroxylated fullerene ($C_{60}[OH]_{44}$) on TLR2-mediated inflammation in vitro. We found that $C_{60}(OH)_{44}$ suppressed the inflammation and the production of reactive oxygen species in cultured cells. Furthermore, $C_{60}(OH)_{44}$ reduced preterm birth and fetal loss in our UPM-1—induced preterm mouse model, suggesting $C_{60}(OH)_{44}$ has potential as a therapeutic agent for preterm birth in mice.

Materials and Methods

Cell culture and $C_{60}(OH)_{44}$ cytotoxicity

Hydroxylated fullerene [$C_{60}(OH)_{44}$], which has an average molecular formula of $C_{60}(OH)_{44}\cdot 8H_2O$, was synthesized with hydrogen peroxide, according to a previously reported method.[26]

HeLa and HEK293T cells lines were purchased from RIKEN (Saitama, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co LLC, St. Louis, MO) containing 10% fetal calf serum, 100 U/mL of penicillin G, and 100 mg/mL of streptomycin under the condition of 5% CO_2 in humid air at 37°C. Both cell lines (6.0×10^3) were cultured overnight and exposed to various concentrations of $C_{60}(OH)_{44}$ (5, 10, 25, or 50 μM).

Cell viability was assayed with a cell-counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) at different time points (24, 48, or 72 hours). In brief, we added 10 μL of the cell-counting kit-8 reagent at different time points and incubated for 2 hours. The optical density at a wavelength of 450 nm (OD_{450}) as absorbance was calculated with a microplate reader (Bio-Rad model 3550; Bio-Rad Laboratories, Hercules, CA). Cell viability was calculated as follows: [{(OD_{450}) of sample − (OD_{450}) of blank}/{(OD_{450}) of control − (OD_{450}) of blank}] × 100(%).

NF-kappaB reporter assay

An NF-kappaB reporter assay was conducted as previously described.[19] In brief, after an overnight culture of HeLa cells or HEK293T cells (2.4×10^4), 0.3 μg of pNF-kappaB-luciferase (Agilent Technologies, Santa Clara, CA), 0.1 μg of pFLAG-TLR2 and 0.1 μg of pRL-tk (Promega Corp, Madison, WI) were cotransfected with the lipofection reagent, FuGENE 6 (Promega Corp). After 48 hours of transfection, we added 0—50 μM of $C_{60}(OH)_{44}$. One hour after adding the reagents, the transfected cells were stimulated with 27.2 nM of UPM-1. After a further 8 hour exposure to UPM-1, the activated cells were lysed and a luciferase assay was performed with a dual-luciferase reporter assay system (Promega Corp) and quantified with a Luminescence Reader BLR-301 (Hitachi Aloka Medical, Ltd, Tokyo, Japan).

Flow cytometry for the detection of reactive oxygen species production in HEK293T cells

After the overnight culture of HEK293T cells (2.4×10^4), the cells were transfected with 0.4 μg of pF-TLR2 with FuGENE 6 (Promega Corp). Forty-eight hours after transfection, 50 μM of $C_{60}(OH)_{44}$ was added to the cells. One hour after adding the fullerene, the cells were stimulated with 27.2 nM of UPM-1. Eight hours after stimulation, 5 μM of the cellROX green reagent (Life Technologies, Carlsbad, CA) was added to the culture medium, and the relative fluorescence units (RFUs) of every 10,000 cells were measured with BD FACScalibur HG (Becton, Dickinson and Co, Franklin Lakes, NJ). The mean fluorescence was calculated with CellQuest Pro software (Becton, Dickson and Co).

Reactive oxygen species production of mouse peritoneal macrophages

C57BL/6J mice were from Japan SLC, Inc (Hamamatsu, Japan). The experimental protocols of the animal studies were approved by the animal experiment committee of Osaka Medical Center and the Research Institute for Maternal and Child Health.

Peritoneal macrophages were collected according to the method of Zhang et al[27] with a slight modification. In brief, 1—3 mL of 3% sterilized fluid thioglycollate medium II (Eiken Chemical Co, Ltd, Tokyo, Japan) was injected into the intraperitoneal cavities of 8—13 week old nonpregnant female mice.