Protective mechanism of curcumin against *Vibrio vulnificus* infection

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Abstract

Curcumin, a natural polyphenolic flavonoid extracted from the rhizome of *Curcuma longa* L., has many beneficial biological activities. However, there are relatively few reports of the effects of curcumin on pathogen infections. This study examined the effect of curcumin on a *Vibrio vulnificus* infection. The cytotoxicity of *V. vulnificus* to HeLa cells was significantly inhibited by curcumin (at 10 or 30 μM). To further examine the inhibitory mechanism of curcumin against *V. vulnificus*-mediated cytotoxicity, the level of bacterial growth, bacterial motility, cell adhesion, RTX toxin expression and host cell reactions were evaluated. Curcumin inhibited *V. vulnificus* growth in HI broth. Curcumin inhibited both bacterial adhesion and RTX toxin binding to the host cells, which can be considered the major protective mechanisms for the decrease in *V. vulnificus* cytotoxicity. Curcumin also inhibited host cell rounding and actin aggregation, which are the early features of cell death caused by *V. vulnificus*. In addition, curcumin decreased the *V. vulnificus*-induced NF-κB translocation in HeLa cells. Finally, curcumin protected mice from *V. vulnificus*-induced septicemia. In conclusion, curcumin may be an alternative antimicrobial agent against fatal bacterial infections.

Introduction

Pathogenic bacteria cause infectious diseases that may be treated with antibiotics, either bactericidal or bacteriostatic. The widespread use of antibiotics in treating human disease and in promoting animal growth has been attributed to the rapid development of antibiotic resistance in bacterial populations (Angulo et al., 2004).

*Vibrioaceae* is a diverse family of bacteria that includes more than 80 species (Thompson et al., 2004). Vibrios are gram-negative rods that normally inhabit aquatic habitats, often in association with eukaryotes. This large family includes important human pathogens, such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Tantillo et al., 2004). *Vibrio vulnificus*, a halophilic estuarine bacterium, causes fatal septicemia and necrotic wound infections (Oliver, 2005). This pathogen might be a good model organism of bacterial septicemia because the bacterial infection shows a wide pathogenic spectrum, which has a fulminating course with high mortality (> 50%) within days (Tacket et al., 1984).

Curcumin was found to inhibit *V. vulnificus* infection. Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], an important polyphenolic flavonoid extracted from the rhizome of the plant *Curcuma longa* L. (turmeric) has a range of pharmacological effects. Curcumin was reported to arrest *Helicobacter pylori* growth (De et al., 2009) and is considered to be a potential chemopreventive candidate against *H. pylori*-related gastric carcinogenesis (Zaidi et al., 2009; Sintara et al., 2010). Curcumin also reduced the virulence factors of *Pseudomonas aeruginosa*, such as biofilm formation, pyocyanin biosynthesis, elastase/protease activity and acyl homoserine lactone production (Rudrappa & Bais, 2008).
In an acute lung injury mouse model induced by *Klebsiella pneumoniae*, curcumin protected the host from pulmonary inflammation (Bansal & Chhibber, 2010).

This study examined the effects of curcumin on *V. vulnificus* infection using a variety of methods including bacterial growth, motility, toxin production, adhesion to host cell, cytotoxicity and host responses.

**Materials and methods**

**Bacterial strains and reagents**

MO6-24/O is a clinical isolate of *V. vulnificus* (Reddy et al., 1992) and CMM770 is MO6-24/O with a deletion mutation in the *rtxA1* gene (Kim et al., 2008). *Vibrio vulnificus* strains were grown in 2.5% NaCl heart infusion (HI) medium at 37 °C shaking incubator. To prepare the log phase bacterial cells, overnight cultures of *V. vulnificus* were diluted 200-fold in fresh HI broth and grown at 37 °C and 200 rpm for 4 h. The cultured bacterial suspension was harvested by centrifugation and washed three times with PBS (pH 7.2).

Curcumin (> 98% purity, FW 368.38) was purchased from Sigma-Aldrich (USA), and dissolved in dimethyl sulfoxide (DMSO, Sigma) to produce a 100 mM stock solution. All other reagents were purchased from commercial sources.

**Effect of curcumin on bacterial cytotoxicity**

The cytotoxicity of *V. vulnificus* to HeLa cells was measured using a CytoTox96 Non-Radioactive cytotoxicity assay kit (Promega, Madison, WI), as described elsewhere (Kim & Rhee, 2003). The HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS (GIBCO® Invitrogen), in a 37 °C incubator with 5% CO₂. HeLa cells were seeded into each well of a 48-well cell culture plate (5 × 10⁴ cells per well) and cultured overnight. The cells were then washed with serum-free DMEM. The HeLa cells were inoculated with the bacteria at a multiplicity of infection (MOI) of 100. To assay the effect of curcumin, it was added to HeLa cells 2 h before the bacteria. After 100 min incubation at 37 °C, the lactate dehydrogenase (LDH) released in the supernatant was assayed as a marker of cytotoxicity according to the manufacturer’s protocol.

**Cell staining and fluorescence microscopy**

HeLa cells were seeded on an eight-well chambered cover glass of #1 German borosilicate (Nalge Nunc International) according to the method described elsewhere (Dhakal et al., 2006). The HeLa cells were preincubated in serum-free DMEM with or without curcumin for 2 h and *V. vulnificus* was added at an MOI of 100 for 50 min. To obtain live cell images, the HeLa cells were stained with Hoechst (Molecular Probes Invitrogen) and Alexa Fluor 594-conjugated wheat germ agglutinin (WGA) (Molecular Probes Invitrogen) to visualize the nucleic acid and plasma membrane, respectively. Fluorescence images were acquired by fluorescence microscopy with a digital camera (DXM1200C, Nikon).

**Effect of curcumin on bacterial growth**

The effect of curcumin on the viability and growth of *V. vulnificus* was tested by diluting bacterial suspensions cultured overnight 200-fold in fresh broth containing various concentrations of curcumin. After culturing in a 37 °C incubator with or without shaking at 200 rpm for 24 h, the level of bacterial growth was determined by measuring A₆₀₀nm using a biophotometer (Eppendorf, Germany). The live bacterial numbers were counted after plating 10 μL of the diluted culture media on HI agar and cultured overnight.

**Swarming motility test**

To test the effect of curcumin on the *V. vulnificus* swarming motility, 5 μL of the bacterial culture was inoculated onto semisolid agar plates containing 0.3% bactoagar with or without curcumin, as described previously (Kim & Rhee, 2003). The distance of swarming migration was measured approximately 7 h after inoculation.

**Assay of bacterial adherence to HeLa cells**

Bacterial adhesion was assayed as described previously (Kim & Rhee, 2003). The HeLa cells seeded into four-well Lab Tec chamber slides (Nunc, Inc., Naperville, IL) were preincubated in serum-free DMEM with or without curcumin for 1 h, and the HeLa cells were infected with *V. vulnificus* at an MOI of 250. After 30 min incubation at 37 °C, the HeLa cells were washed thoroughly three times with prewarmed DMEM and stained with a Giemsa solution (Merck, Darmstadt, Germany). The bacterial cells adhered to 90 HeLa cells were counted and the results are reported as the average number of adhered bacteria per HeLa cell.

**Western blot analysis of RtxA1 protein**

A polyclonal antibody corresponding to the RtxA1 amino acids 4080-4701 was produced (Kim et al., unpublished data) and the C-terminal portion of RtxA1 was detected by Western blot analysis in accordance with the method described previously (Kim et al., 2008). *Vibrio vulnificus*
was cultured in 2.5% NaCl HI with or without curcumin and the bacterial culture supernatant (400 μL) was precipitated using cold acetone.

**Immunostaining of RtxA1 protein and confocal microscopy**

HeLa cells in an 8-well glass chamber plate (Nalge Nunc International) were infected with wild-type V. vulnificus at an MOI of 100. The V. vulnificus RtxA1 protein was immunostained according to the method described elsewhere (Kim et al., 2008). After fixation, permeabilization and blocking, the HeLa cells were stained with the thoroughly adsorbed polyclonal anti-RtxA1 antibody, followed by the Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, OR). Confocal images of the specimens mounted with a ProLong® gold antifade reagent (Molecular Probes) were acquired using laser scanning confocal microscopy (EZ-C1, Nikon).

**NF-κB staining**

To determine NF-κB translocation, HeLa cells on an eight-well glass chamber plate (Nalge Nunc International) were infected with V. vulnificus. The HeLa cells were pretreated with curcumin 2 h before the pathogen infection. The NF-κB p65 protein was detected by immunostaining using a polyclonal-anti-NF-κB p65 antibody (Santa Cruz Biotechnology, Inc.) and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Molecular Probes Invitrogen). F actin was visualized by Alexa Fluor 594-conjugated Phalloidin (Molecular Probes). Confocal images of the specimens stained according to the method described elsewhere (Kim et al., 2008). After fixation, permeabilization and blocking, the HeLa cells infected with V. vulnificus were stained with Alexa Fluor 594-conjugated WGA. Curcumin dramatically blocked the host cell rounding caused by the pathogen (Fig. 1b).

**Lethality in mice caused by V. vulnificus**

Log phase V. vulnificus cells [2 × 10⁶ colony forming unit (CFU) per mouse] were administered to 8-week-old CD-1 mice via the intra-peritoneal route. The mice were pre-treated with curcumin (5 mg kg⁻¹) twice by intra-peritoneal route, 1 day and 2 h before the V. vulnificus infection. Five mice were tested for each group, and the infected mice were observed for 60 h. All animal procedures were carried out in accordance with the guidelines of the Animal Care and Use Committee of Chonnam National University.

**Statistical analysis**

All values are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were made using a Student’s t-test. All experiments were repeated three times and the results from a representative experiment are shown.

**Results**

**Curcumin inhibits the cytotoxicity of V. vulnificus**

Live V. vulnificus is highly cytotoxic to host cells. Several natural compounds were screened to identify agents that could protect HeLa cells from V. vulnificus cytotoxicity using an LDH assay (data not shown). HeLa cells were pretreated with curcumin 2 h before the V. vulnificus infection. The HeLa cells infected with V. vulnificus at an MOI of 100 showed abrupt cell death within 90 min. Curcumin (at 10 or 30 μM) inhibited the V. vulnificus cytotoxicity significantly in a dose-dependent manner (Fig. 1a).

Vibrio vulnificus causes cytoskeletal rearrangement and rounding of the host cells (Kim et al., 2008). To examine the morphological changes, the HeLa cells treated for 50 min with V. vulnificus were stained with Alexa Fluor 594-conjugated WGA. Curcumin dramatically blocked the host cell rounding caused by the pathogen (Fig. 1b).

**Curcumin inhibits the growth of V. vulnificus**

The effects of curcumin (at 10 or 30 μM) on V. vulnificus growth were examined by detecting the absorbance of the bacterial culture broth using biophotometer at 600 nm. Curcumin partly inhibited V. vulnificus growth in the HI broth (Fig. 2) and had a more significant effect in the shaking culture (Fig. 2a) than the standing culture (Fig. 2b). On the other hand, when viable cells were examined, curcumin did not have any bactericidal effect on V. vulnificus, even at 300 μM (data not shown). This suggests that curcumin has bacteriostatic activity to V. vulnificus but not bactericidal activity.

**The effect of curcumin on the motility of V. vulnificus**

The effect of curcumin on the V. vulnificus swarming motility was examined. Curcumin at 30 μM slightly inhibited the swarming motility of V. vulnificus on the semisolid agar plates (Fig. 3).

**Curcumin inhibits the adhesion of V. vulnificus to host cells**

Effect of curcumin on bacterial adherence to host cells was tested by Giemsa staining. The number of bacterial cells adhered to at least 90 HeLa cells were counted and the average number of bacteria adhered per HeLa cell was...
calculated. Curcumin (at 10 or 30 μM) significantly inhibited adhesion of *V. vulnificus* to host cells (Fig. 4).

**Effect of curcumin on the RtxA1 toxin of *V. vulnificus***

*Vibrio vulnificus* is highly cytotoxic to host cells and RtxA1, a RTX toxin, plays essential key roles in this cytotoxicity (Kim et al., 2008). The effect of curcumin on RtxA1 expression was examined by Western blot analysis. Curcumin did not have any inhibitory effect on *V. vulnificus* RtxA1 expression in HI broth culture (Fig. 5). To examine the effect of curcumin on the RtxA1 cytotoxicity to host cells, wild-type *V. vulnificus*-infected HeLa cells were immunostained with an RtxA1 antibody. The binding of RtxA1 protein to HeLa cells was decreased by the curcumin treatment (Fig. 6, green). F actin was visualized by Alexa Fluor 594-conjugated Phalloidin. Curcumin inhibited the actin aggregation caused by the RtxA1 toxin (Fig. 6, red).
Curcumin inhibits the V. vulnificus-induced translocation of NF-κB from the cytosol to the nucleus

The effect of curcumin on V. vulnificus-induced NF-κB pathway was tested. V. vulnificus caused the translocation of NF-κB from the cytosol to the nucleus, which was inhibited by the curcumin treatment (Fig. 7).

Curcumin protects mice from V. vulnificus-induced septicemia

The effect of curcumin on mice lethality from a V. vulnificus infection was studied. V. vulnificus was administered to 8-week-old CD-1 mice via the intra-peritoneal route. Some mice were pretreated with curcumin twice via the intra-peritoneal route; 1 day and 2 h before the V. vulnificus injection. The V. vulnificus-infected mice showed difficulties in breathing, bristled fur and decreased activity. The symptoms were much milder in the mice injected with curcumin. V. vulnificus caused acute lethality, which was ameliorated by curcumin (5 mg kg⁻¹) (Fig. 8).
Discussion

Curcumin was reported to have a range of pharmacological effects including antimicrobial activity against *H. pylori* (De et al., 2009), *P. aeruginosa* (Rudrappa & Bais, 2008) and *K. pneumoniae* (Bansal & Chhibber, 2010). The present study showed that curcumin protects host cells from *V. vulnificus* cytotoxicity and infection (Figs 1 and 8). The inhibitory mechanism was examined by analyzing the effects of curcumin on both bacteria and host cells. Curcumin showed many activities on both eukaryotic cells and a pathogen *V. vulnificus*. First, curcumin inhibited host cell rounding (Fig. 1b) and actin aggregation (Fig. 6), which are the early features of cell death caused by *V. vulnificus* (Kim et al., 2008). In addition, curcumin decreased significantly the *V. vulnificus*-induced NF-κB translocation in HeLa cells (Fig. 7). Curcumin inhibited both the cell death and the induction of the pro-inflammatory response caused by the bacterial infection.

The effects of curcumin on bacterial growth, bacterial motility, toxin production and adhesion to host cells were tested to determine the direct influence of curcumin on *V. vulnificus*. Curcumin inhibited *V. vulnificus* growth in a shaking culture, but had no significant effect in the standing culture (Fig. 2a and b). In addition, curcumin did not have any bactericidal activity on *V. vulnificus* at the concentration tested (data not shown). Interestingly, curcumin significantly delayed bacterial growth in the shaking culture compared to that in the standing culture, suggesting that the bacterial metabolism may have been compromised by curcumin. Curcumin might have inhibited *V. vulnificus* pathogenesis by interfering with bacterial growth, metabolism, etc. This hypothesis should be tested in further studies. The effect of curcumin on RtxA1 toxin expression of *V. vulnificus* was examined. The RTX toxin is one of major toxins that mediates host cell death and the toxin expression increased after contact with the host cells (Kim et al., 2008). Curcumin did not affect the RTX toxin expression of *V. vulnificus* cultured in a HI broth (Fig. 5).

![Fig. 5. Effects of curcumin on RtxA1 expression of *Vibrio vulnificus* cultured in HI broth. *V. vulnificus* overnight culture was diluted and cultured in 2.5% NaCl HI broth with or without curcumin at 200 rpm, and the bacterial culture supernatant (400 μL) was precipitated with cold acetone. The C-terminal portion of RtxA1 toxin was detected by Western blot analysis using a polyclonal antibody corresponding to the RtxA1 amino acids 4080-4701. Curcumin did not affect the expression of RtxA1 toxin in the HI broth culture.](https://academic.oup.com/femspd/article-abstract/63/3/355/439248)


**Fig. 5.** Effects of curcumin on RtxA1 expression of *Vibrio vulnificus* cultured in HI broth. *V. vulnificus* overnight culture was diluted and cultured in 2.5% NaCl HI broth with or without curcumin at 200 rpm, and the bacterial culture supernatant (400 μL) was precipitated with cold acetone. The C-terminal portion of RtxA1 toxin was detected by Western blot analysis using a polyclonal antibody corresponding to the RtxA1 amino acids 4080-4701. Curcumin did not affect the expression of RtxA1 toxin in the HI broth culture.

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![RTX Actin](https://academic.oup.com/femspd/article-abstract/63/3/355/439248)

**Fig. 6.** Effects of curcumin on RtxA1 toxin and actin aggregation. Wild-type *Vibrio vulnificus*-infected HeLa cells were immunostained with the RtxA1 antibody. Curcumin decreased the binding of RtxA1 protein to HeLa cells (green color). F actin was visualized by Alexa Fluor 594-conjugated Phalloidin. Wild-type *V. vulnificus* causes actin aggregation, which was inhibited by curcumin (red color).
The effect of curcumin on the host–parasite interaction was determined by examining the effect of curcumin on *V. vulnificus* adhesion to HeLa cells. In a *V. vulnificus* infection, direct contact and adhesion to the host cell are essential for inducing host cell death efficiently (Kim et al., 2008). Curcumin inhibited bacterial adhesion as well as RTX binding to the host cells (Figs 4 and 6), which can be considered the major protective mechanisms for the decrease in *V. vulnificus* cytotoxicity. Curcumin was reported to modulate the bilayer material properties and may have an altered membrane protein function by modulating the lipid bilayer properties (Ingolfsson et al., 2007). Modulation of the bacterial lipid bilayer may be one of the reasons for the metabolism modification observed during bacterial growth. It may also have hindered the bacteria from sensing direct host cell contact. On the other hand, the modulated host membrane may have prevented the bacteria from recognizing contact with the host and further inhibited adhesion. This hypothesis should also be tested in further studies.

In this study, curcumin showed various activities on both eukaryotic cells and *V. vulnificus*. Curcumin partly inhibited *V. vulnificus* growth (Fig. 2), which is likely to result in defects seen in motility, adhesion, cytotoxicity and other virulence-related factors of the pathogen (Figs 1, 3 and 4). In addition, curcumin showed protective effects on host cell damage caused by *V. vulnificus* such as actin aggregation and NF-κB translocation (Figs 6 and 7). Interestingly, curcumin protected mice from *V. vulnificus*-induced septicemia (Fig. 8). The inhibitory effects of curcumin have been suggested to be mediated by a range of factors including inhibition of the inflammatory response and other host protective mechanisms. Curcumin was reported to ameliorate the lung inflammation induced by *K. pneumoniae* in an acute lung injury mouse model without decreasing the bacterial load in the lung.
tissue (Bansal & Chhibber, 2010). Curcumin also showed the inhibition of NF-κB activation induced by H. pylori (Foryst-Ludwig et al., 2004). Various virulence factors of P. aeruginosa were reduced by curcumin (Rudrappa & Bais, 2008). In a Neisseria gonorrhoeae infection model, curcumin reduced the adhesion of the bacteria to the host epithelial cells in a late infection (Wessler et al., 2005).

In conclusion, curcumin had an excellent protective effect against V. vulnificus infection (Fig. 8). Curcumin was reported to have beneficial effects on the host cells including antioxidant activity (Rosello et al., 2008), anti-inflammatory activity (Weisberg et al., 2008), anti-allergic activity (Lee et al., 2008) and anticancer (Bar-Sela et al., 2010). These results suggest that a combination therapy of curcumin and other antibiotics may also assist in the efficient treatment of bacterial infectious diseases.

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Authors’ contribution

H.S.N. and M.H.C. contributed equally to this work.

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