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論　文　名	Effects of anethole on the growth and virulence expression of toxigenic *Vibrio cholerae* and its therapeutic effects in animal models （アネトールがコレラ菌の増殖及び病原因子の発現に及ぼす影響とその動物モデルでの治療効果）
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論文要旨

Introduction

Antimicrobial agents have been playing a pivotal role in controlling bacterial infections since their discovery. However, the current status of global emergence of multidrug resistant (MDR) bacteria demands either development of new antimicrobial agents or alternative approaches to combat them. Like other bacterial pathogens, multidrug resistance in toxigenic *Vibrio cholerae* the causative agent of cholera pandemics is a growing concern.

Cholera toxin (CT, encoded by the *ctxAB* genes) is the major virulence factor in *V. cholerae* and is mostly responsible for the profuse watery diarrhea. Although >200 'O' serogroups have been reported in *V. cholerae*, only O1 (El Tor and classical biotypes) and O139 are responsible for cholera. The O1 El Tor biotype is responsible for the ongoing 7th cholera pandemic, and this biotype replaced the classical biotype strain which caused the 6th cholera pandemic. Recently emerged *V. cholerae* O1 El Tor variant strains (possess some attributes of classical biotype including *ctxB* gene allele) produce more CT and cause more severe diarrhea than prototype El Tor. Along with CT, by using another virulence factor

toxin-coregulated pilus (TCP, encoded by the *tcpA* gene) *V. cholerae* causes pathogenesis to human host. Although the virulence regulon in toxigenic *V. cholerae* has historically been known as the ToxR regulon, ToxT is the direct transcriptional activator of the genes encoding CT and TCP. Indeed, activation of *toxT* occurs via synergistic coupling of two membrane-localized heterodimers ToxR/ToxS and TcpP/TcpH.

Much effort and attention have been paid to search effective antimicrobial agents from natural sources against *V. cholerae*. Alternatively, use of natural compounds as antivirulence drugs, that suppresses virulence of bacterial pathogen could be a novel therapeutic approach. As CT is the major virulence factor in *V. cholerae*, much attention have been paid to search suitable antivirulence drug candidates against CT. However, there is still very limited information regarding the effect of natural products on virulence regulation in *V. cholerae*. Previously, our research group showed that sub-bactericidal concentration of methanol extract of some spices such as red chili, sweet fennel and star anise seed can effectively inhibit CT in *V. cholerae*. Recently, it has been reported that capsaicin, the major component of methanol extract of red chili suppressed CT production *in vitro* in a *toxT*-dependent manner by upregulating *hns* (basal repressor of *ctxA*), but failed to show such activity *in vivo*.

In this study, we have evaluated trans-anethole (the major component of sweet fennel and star anise seed extracts) as a potential antimicrobial and antivirulence drug candidate against MDR toxigenic *V. cholerae*. Furthermore, the possible molecular mechanisms behind anethole (trans-anethole)-mediated virulence-gene inhibition in *V. cholerae* were also investigated.

Chapter 1. Anethole suppresses the growth and virulence potential of toxigenic *V. cholerae* strains–

We tested the effect of anethole on the growth of various toxigenic *V. cholerae* strains in AKI medium (0.5% NaCl, 0.4% Yeast extract and 1.5% Bactopeptone) supplemented with 0.3% $NaHCO_3$ (pH 7.4) at 37˚C under stationary condition and found that ≥150 µg/ml has significant growth inhibitory effect. Moreover, ≥200 µg/ml anethole exerted complete bactericidal effect within 15 minutes against toxigenic *V. cholerae*. But ≤100 µg/ml of anethole did not show any detectable effect on the growth of the tested strains.

The effects of anethole on the production of CT and TCP by *V. cholerae* were tested in presence of 50 µg/ml anethole. O1 El Tor strains (predominant strains in our study) produce maximum CT in AKI medium supplemented with 0.3% $NaHCO_3$ (pH 7.4), when cultures were kept under stationary condition for 4 h followed by a shaking growth phase. So, we maintained these conditions for optimum CT and TCP production by *V. cholerae*. CT production in the culture supernatant fluid was estimated using Bead-ELISA, whereas TCP production was assessed by western blotting. We found that anethole inhibited CT production in the tested strains irrespective of their serogroups or biotypes. Dose dependent effect of anethole on CT and TCP production was analyzed in a representative MDR, high CT-producing O1 El Tor variant strain CRC41. We found that although CT production was inhibited in the presence of anethole (≤100 µg/ml) in a dose dependent manner, bacterial

viability was not affected. Since CT expression is coordinately regulated the expression of TCP in toxigenic *V. cholerae*, expression of TCP was also examined by western blotting. TCP expression was reduced and well correlated with CT inhibition by anethole. Furthermore, we denied a possibility that anethole or its solvent methanol directly acts on CT to cause alteration of its immunological property under our experimental conditions, indicating that anethole might affect virulence regulatory cascade in *V. cholerae.*

It is evident that initial 4 h of stationary condition is crucial for the initiation of CT production by O1 El Tor strains in AKI medium (0.3% $NaHCO_3$). To analyze anethole-mediated CT inhibition trend in the strain CRC41, a time-course assay of CT production was conducted with initial 4 h stationary and followed by various length of shaking conditions. Although CT production in the absence of anethole peaked at 2 h of shaking following initial stationary condition, a high amount of CT was induced at the end of 4 h stationary phase. However, addition of anethole inhibited CT production under these conditions.

Chapter 2. Anethole might affect virulence regulatory cascade in *V. cholerae* via cyclic AMP (cAMP)-cAMP receptor protein (CRP) signaling system

To determine at which point of the virulence regulatory cascade anethole affects CT and TCP expression, various regulatory genes including *toxT, toxR, toxS, tcpP, tcpH* and *hns* were analyzed via qRT-PCR analysis. Based upon the trend of CT production by the strain CRC41, a culture condition with 4 h stationary which resembles the environment of host small intestine and followed by 2 h of shaking (at which point CT production by CRC41 was found maximum) were chosen for transcriptional analysis of the mentioned genes, in presence of 50 µg/ml anethole.

We found that anethole drastically suppressed the transcription of *ctxA* and *tcpA* genes compared to those of untreated controls. Transcription of other virulence regulatory genes was also significantly repressed by anethole, namely, *toxT*, *tcpP* and *tcpH*. On the other hand, along with housekeeping gene *recA*, transcription of *toxR*, *toxS* and *hns* were not affected significantly by anethole in either culture conditions. Analysis by western blotting also revealed that TcpP expression was suppressed by anethole and well correlated with that of transcriptional analyses by qRT-PCR. Taken together, these data suggested that anethole suppressed virulence expression in *V. cholerae* in a *toxT*-dependent but *toxR/toxS*-independent manner and through the repression of *tcpP*/*tcpH*.

tcpP/*tcpH* are overlapping operons in *V. cholerae* genome. Since there are also upstream regulatory genes for *tcpPH,* we also analyzed the effect of anethole on the transcriptions of possible regulators of *tcpPH*, including *cyaA, crp, hapR, aphA* and *aphB.* We found that after 4 h stationary condition the relative transcription of *cyaA* (1.5 fold), *crp* (2.4 fold) and *hapR* (1.8 fold) increased but *aphA* (2.8 fold) decreased significantly in the presence of anethole compared to anethole-untreated control. Although we observed certain variation in expression of these genes under stationary and shaking conditions, the transcription of *crp* remained consistently elevated in presence of anethole irrespective of the culture conditions. Taken

altogether, a hypothesis can be raised that anethole initiates inhibition of *tcpPH* transcriptions as well as CT through cAMP-CRP complex-mediated signal.

Chapter 3. Effects of anethole on the pathogenesis of *V. cholerae* in animal models

Rabbit ileal loop (RIL) assay is one of the most extensively used assays to determine the CT-mediated enterotoxic potency of toxigenic *V. cholerae*. To test whether anethole can effectively suppress expression of CT *in vivo*, we used the adult RIL assay and monitored fluid accumulation. Ligated segments of RILs were inoculated with fresh CRC41 culture (10^8 cfu per loop), in the presence (0.08 – 10 mg per loop) or absence of anethole and incubated for 6 h. Fluid accumulation (FA) ratio (mL of fluid per cm of the loop), total recovered bacteria and amount of CT produced in intestinal fluid of each loop were then monitored. We found a marked reduction in fluid accumulation when various sub-lethal doses of anethole were administered together with the strain CRC41 as compared to loop in which bacteria were inoculated without anethole. Moreover, no significant difference was observed in bacterial counts in the ileal loop fluids recovered from assays with sub-lethal doses of anethole compared to loop without anethole, suggesting that reduced fluid accumulation was due to direct effect by anethole but not bacterial growth inhibition by anethole. As the expression of TCP was found to repress by anethole *in vitro*, it is speculated that anethole might have effect on *V. cholerae* colonization. To evaluate the effect of anethole on *V. cholerae* colonization, mice (BALB/c) experiment is ongoing.

Conclusions

In this study, dual beneficiary effects of natural compound anethole have been evaluated against the pathogen *V. cholerae*. We found that ≥150 μg/ml anethole potentially inhibited the growth and sub-bactericidal concentrations (≤100 μg/ml) showed potential antivirulence activities against toxigenic *V. cholerae*. We have given evidences that anethole inhibited CT and TCP production by affecting TcpP at transcriptional level, remaining *toxR* transcription being unaffected. We here propose a mechanism that anethole might suppress TcpP in *V. cholerae* by activating cAMP-CRP complex mediated signal, which is also well conserved in other bacterial pathogens. So, it might be possible to observe cAMP-CRP signaling system mediated virulence regulation in them by anethole. Finally, the ability of suppression of CT-mediated fluid accumulation in RIL demonstrates its potentiality as a future therapeutic candidate against MDR toxigenic *V. cholerae*. Although further studies are needed, we believe that daily intake of sweet fennel seeds containing anethole could be an alternative approach to prevent enteric infections including cholera.

審査結果の要旨

コレラ菌はO抗原の違いに基づき現在200種以上のO群血清型に分類されている。これらの中で、いわゆるコレラの原因となるのはコレラ毒素（CT）産生性のO1とO139の血清型である。さらにO1コレラ菌は生物学的性状の違いに基づき、第一次から第六次の世界大流行に関わる病原性の強い古典型と1961年から始まった第7次世界大流行の原因菌で環境中での安定性の高いエルトール型の2種類がある。しかし、近年、エルトール型であるが古典型のCTを産生するO1エルトールバリアントと呼ばれる流行株が出現し、南アジア、アフリカ、さらにはハイチで猛威を振るっている。

一方、多剤耐性コレラ菌、多剤耐性緑膿菌、NDM-I産生菌、ESBL産生菌など様々な多剤耐性菌が出現し世界的に大きな問題となっている。近年、世界流行を引き起こしているO1エルトールバリアントの多くは多剤耐性菌であり、抗菌薬治療が困難となっている。これらの耐性菌に対して新たな抗菌薬の開発が望まれているが、抗菌活性を示さず病原因子の発現を抑制する物質が耐性菌を生まない新たな感染症治療薬の候補として期待されている。コレラの流行地で日常的によく使われているスパイスにその可能性が潜んでいないかを調べている過程でウイキョウの種のメタノール抽出物にCTの産生を抑制する活性を見いだした。そこで、本研究では、ウイキョウの種に含まれるトランスアネトール（以下アネトール）が、1)コレラ菌の増殖と病原因子の発現に及ぼす影響を*in vitro*で調べ、2) *in vitro*で見られた病原因子の発現抑制機構を解析し、3)さらに*in vivo*におけるコレラ菌の増殖と病原因子の発現に及ぼす影響について調べた。

第1章では、アネトールがコレラ菌の増殖と病原因子の発現に与える影響について、様々な濃度のアネトール存在下（50、100、150及び200 μg/mL）で、種々のコレラ菌を培養し調べた。その結果、アネトールが100 μg/mL以下ではコレラ菌の増殖には影響がなかったが、150 μg/mLで増殖が抑制され、200 μg/mLでは完全に抑制された。しかも200 μg/mLのアネトール存在下では、20分以内にコレラ菌が死滅していた。さらに、菌の増殖に影響を与えない50 μg/mL濃度のアネトール存在下でCT及びTCP産生の影響について調べたところ、CT、TCPとも発現が抑制されていた。以上の結果よりアネトールはコレラ菌に対する殺菌作用と病原因子の発現抑制活性の両方を持つことを明らかとした。

第2章では、アネトールのコレラ菌の病原因子発現抑制機構について解析した。CT及びTCPの発現はToxRレギュロンによって制御されていることが知られている。そこで、50 μg/mLのアネトール存在下で培養したコレラ菌を用いて、*ctx*遺伝子と*tcpA*遺伝子及びその上流に存在する調節遺伝子のmRNA量をqRT-PCRで測定した。その結果、*ctxA*と*tcpA*遺伝子の発現は著しく抑制され、*toxT*、*tcpP*や*tcpH*の発現も有意に抑制されていた。しかし、*toxR*、*toxS*や*hns*の発現にはほとんど影響を与えていなかった。*tcpP*/*tcpH*の発現が抑制されていたことから、さらなる上流の遺伝子についても調べた。その結果、*cyaA*と*hapR*の発現が促進し、特に*crp*の発現が有意に促進されていた。以上の結果より、アネトールのCT及びTCPの発現抑制はcAMP-CRPの系を介して、さらに*tcpP*/*tcpH*の抑制に基づくものであると考えられた。

第3章では、ウサギとマウスを用いてアネトールのコレラ菌の病原因子発現抑制活性について評価した。種々の濃度のアネトール（0.08～10 mg/loop）とコレラ菌をウサギの腸管ループに投与し、6時間後にルー

プ内の液体貯留量、コレラ菌数及び CT 量を測定した。その結果、アネトールの濃度依存的に液体貯留量、コレラ菌数、CT 量が減少した。興味深いことに、0.156 mg のアネトールではコレラ菌に対して殺菌作用はみられなかったが、液体貯留量と CT 量を減少させていた。マウスにアネトールを投与した群としない群にそれぞれコレラ菌を経口投与し、排菌数及び排菌期間を調べた結果、アネトールを投与した群ではコレラ菌の排菌期間が有意に短縮した。以上の結果よりアネトールは *in vivo* においてもコレラ菌の病原因子の発現を抑制することを明らかとした。

以上の結果は、アネトールがコレラ菌の増殖を抑制するだけでなく、増殖に影響を与えない低濃度でも CT や TCP の発現を *in vitro* のみならず *in vivo* でも抑制すること、また、その抑制機構は cAMP-CRP 複合体を介したものであることを明らかとした。これらの成果はウイキョウ由来のアネトールがコレラの予防のみならず治療にも使える可能性を示すものであり、獣医学領域のみならず医学領域においても多大な貢献をするものと考えられる。従って、最終試験の結果と併せて博士（獣医学）の学位を授与することを適当と認める。