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学 位 論 文 要 旨

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題　　目：**Studies on the functions of *Haemaphysalis longicornis* ferritins and their potential as target molecules for tick control**
(フタトゲチマダニ由来フェリチンの機能とマダニコントロールへの
標的分子としての可能性に関する研究)

論文要旨：

Ticks are notorious parasitic arthropods, known for their completely host-blood-dependent lifestyle. Female hard ticks (Acari: Ixodidae) can feed for several days and ingest blood more than a hundred times their unfed weight. Their blood-feeding habit facilitates the transmission of various pathogens. It is remarkable how ticks cope with the toxic nature of their blood meal, which contains several molecules that can promote oxidative stress, including iron. While it is required in several physiological processes, high amounts of iron can be dangerous because iron can also participate in the formation of free radicals that may cause cellular damage and death. Therefore, iron-binding proteins involved in iron metabolism must be very crucial for tick survival. The functions of the iron-storage protein ferritin and iron metabolism in ticks need to be further elucidated. Here, I studied the functions a newly identified secreted ferritin from the hard tick *Haemaphysalis longicornis* (HlFER2), together with the previously identified intracellular ferritin (HlFER1) and evaluated their potential as target molecules for tick control.

In Chapter 1, I characterized HlFER1 and HlFER2 and evaluated their importance in blood feeding and reproduction through RNA interference (RNAi). Gene and protein expressions in developmental stages and different organs were investigated through RT-PCR and Western blot analysis, respectively. The localization of HlFERs in different organs was also demonstrated through an indirect immunofluorescent antibody test. The effect of RNAi on midgut function and expression of vitellogenin genes (*HlVg*s) was also examined. RT-PCR showed differences in gene expression in some organs and developmental stages. Interestingly, only HlFER2 was detected in the ovary during oviposition and in egg despite the low mRNA transcript. RNAi induced reduced post-blood meal body weight, high mortality, and decreased fecundity. Abnormalities in digestive cells, including disrupted microvilli, and alteration of digestive activity were also observed. The expression of *HlVg*s was also affected by silencing *Hlfer*s. These results showed that the iron storage HlFERs are critical to successful blood feeding and reproduction of *H. longicornis*.

In Chapter 2, the role of ferritin in protecting the hard ticks from oxidative stress was demonstrated. Evaluation of oxidative stress in *Hlfer*-silenced ticks was performed after blood feeding or injection of ferric ammonium citrate (FAC) through detection of the lipid peroxidation

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product, malondialdehyde (MDA) and protein oxidation product, protein carbonyl. FAC injection in *Hlfer*-silenced ticks resulted in high mortality. Higher levels of MDA and protein carbonyl were detected in *Hlfer*-silenced ticks compared to *Luciferase*-injected (control) ticks both after blood feeding and FAC injection. Ferric iron accumulation demonstrated by increased staining on native HlFER was observed from 72 h after iron injection in both the whole tick and the midgut. Furthermore, weak iron staining was observed after *Hlfer* knockdown. These results show that tick ferritins are crucial antioxidant molecules that protect the hard tick from iron-mediated oxidative stress during blood feeding.

In Chapter 3, I determined whether HlFERs have a role in tick innate immunity. Iron sequestration is a known component of innate immunity against many pathogens. After *Hlfer* silencing, ticks were injected with live or heat-killed EGFP-*E. coli*, and then monitored for survival rate. Hemolymph was also collected for hemocyte examination and *E. coli* culture to demonstrate bacterial viability. Low survival rates were observed in *Hlfer*-silenced ticks after live or heat-killed GFP-*E. coli* injection. The number of *E. coli* inside and outside the hemocytes of *Hlfer*-silenced ticks was also very high compared to that of the control ticks. Cultures of hemolymph from *Hlfer*-silenced ticks also yielded more colonies than the control ticks. These results indicate that HlFERs may limit the multiplication of some pathogens through their iron-binding function.

The good results of RNAi experiments suggested that HlFERs might be good candidate vaccine antigens for tick control. In Chapter 4, rabbits were immunized with recombinant HlFERs (rHlFERs) before tick infestation to investigate the effects of antibodies against HlFERs to ticks. Tick feeding and reproduction parameters were evaluated to determine vaccine efficacy. To demonstrate the effects of host antibodies, oxidative stress was detected in the eggs and larvae. rHlFERs stimulated host antibody production as demonstrated by ELISA. After infestation, significantly lower bodyweight was observed in the ticks infested from the rHlFER2-immunized rabbit compared to those from the control rabbit. Reduced oviposition and hatching rate were observed in both rHlFER-immunized groups. rHlFER2 showed a higher vaccine efficacy. The antibodies against rHlFERs were detected in the eggs, and higher levels of oxidative stress biomarkers in the eggs and larvae, of ticks from rHlFER vaccinated rabbits. These results showed that HlFER2 has a good potential as an anti-tick vaccine antigen that may affect multiple tick species.

Taken altogether, the results of my studies show that both types of HlFERs prevent oxidative stress in *H. longicornis* during blood feeding, which is essential for reproduction. Through their iron storage function, the intracellular HlFER1 also serves as an “in-house” antioxidant of cells, whereas the secretory HlFER2, which can be transported within the tick, may also serve as a systemic antioxidant molecule. The results of vaccination trial suggest that HlFER2 can be a good candidate molecule as an anti-tick vaccine antigen that may possibly affect multiple tick species.

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学位論文審査の結果の要旨

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審査結果の要旨：

マダニは病原微生物を媒介する吸血性の外部寄生虫であり、マダニ体内は吸血する際に血液に含まれる大量の鉄分子に暴露されることが予想される。鉄分子はマダニの生命恒常維持において不可欠であるが、時には鉄分子の過剰摂取はマダニにとって有毒になることも考えられる。しかし、マダニ体内における鉄代謝のメカニズムについては完全に明らかになっていない。そこで、本論文ではフタトゲチマダニを用いて鉄代謝を制御することが予想される分泌型フェリチン 2 を新規に同定し、すでに同定されている細胞内型フェリチン 1 とそれらの特性について比較し、フェリチンの鉄代謝における役割について検討を行った。

第 1 章では、RNA 干渉法によるフェリチン遺伝子のノックダウンマダニは、飽血後体重の減少と高い死亡率を引き起こし、産卵と中腸の形態に変化を及ぼすことを示した。すなわち、フタトゲチマダニの吸血や産卵において、フェリチンは鉄分子の供給や鉄分子の毒性に対して、重要な制御的役割を果たす必須な分子であることが考えられた。

第 2 章では、鉄代謝による酸化ストレスからマダニを保護するフェリチンの役割を検証するために、フェリチン遺伝子ノックダウン後のマダニを吸血させるか、あるいは鉄分子接種による酸化ストレスバイオマーカーである過酸化脂質の指標となるマロンジアルデヒドとタンパク質酸化によるカルボニル化タンパク質の検出を行った。その結果、フェリチン遺伝子ノックダウンマダニ全体あるいは中腸内のマロンジアルデヒドとカルボニル化タンパク質が高濃度で検出されたことから、第 1 章で示されたマダニの飽血後体重の減少、高い死亡率、中腸の形態変化は鉄分子による酸化ストレスの可能性が考えられた。

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第３章では鉄分子の捕捉機能がマダニ初期免疫に重要であると考え、フェリチン遺伝子ノックダウンマダニに大腸菌を接種したところ、生存率が低下した。そこで、フェリチン遺伝子ノックダウンマダニの血体腔内の大腸菌数を調べたところ、大腸菌数の有意な増加が認められた。その理由として、フェリチン遺伝子ノックダウンマダニ体内ではフェリチンによる鉄分子の捕捉機能が低下したため、高濃度の鉄分子が生じ、その環境が大腸菌数の増加につながったことが考えられた。

第１、２、３章から、マダニの鉄代謝や細菌感染防御にとって、フェリチンがマダニの生命恒常維持において不可欠な分子であることが示されたため、フェリチンを抗マダニワクチンの標的抗原の候補とした。第４章の抗マダニワクチン実験では、大腸菌で発現した組換えフェリチンが高い免疫原性を示した。そこでフェリチン免疫群にマダニを吸血させたところ、マダニの飽血体重が顕著に減少し、産卵数と幼ダニの孵化率が低下した。特に分泌型フェリチン２免疫群が顕著であった。また、組換えフェリチン２免疫群を吸血させたマダニの卵では形態の異常が観察された。これらの現象は、組換えフェリチン免疫群を吸血させたマダニの卵と幼ダニでは過酸化脂質とタンパク質酸化の酸化ストレスが起こり、これは抗体がフェリチン２の機能を阻害したことによって生じた現象と思われる。以上の結果から、分泌型フェリチン 2 がフタトゲチマダニの抗マダニワクチンの候補抗原として有用であることが示された。

以上をまとめると、細胞内型フェリチン１と分泌型フェリチン２がフタトゲチマダニの吸血中の酸化ストレスを防ぎ、鉄運搬において必須の分子であることが分かった。すなわち、細胞内型フェリチン１は抗酸化分子として鉄分子を保持する機構で細胞を保護し、一方で分泌型フェリチン 2 はマダニ体内で鉄分子輸送と抗酸化分子として作用することが示唆された。以上により、本論文は博士（獣医学）の学位論文として十分な価値を有するものと判定した。