The Role of Heme Oxygenase-1 in Infectious Abortion

（感染性流産におけるヘムオキシゲナーゼ-1 の役割）

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PREFACE

It is well known that various pathogens, such as *Brucella* spp., *Listeria monocytogenes*, *Campylobacter fetus*, *Leptospira* spp., *Bunyavirus*, and *Toxoplasma gondii*, cause infectious abortion. They infect to various livestock including cattle, pig, sheep, and goat, and inflict severe economical damage in developing countries. However, the molecular mechanisms of abortion induced by infection remain unknown. To clarify the mechanisms, I focused on *Brucella abortus* and *L. monoytogenes*. *B. abortus* is gram negative, intracellular, zoonotic bacteria. *B. abortus* survives in host cells by means of ability to inhibit phagosome and lysosome fusion. *L. monocyogenes* is gram positive, intracellular, zoonotic bacteria. *L. monocytogenes* escapes from phagosome to survive in host cell.

Heme oxygenase (HO) is the enzyme involved in heme catabolism. HO-1 is an inducible isoform produced in response to various types of stress. It was proposed that the cytoprotective enzyme HO plays a critical role in graft acceptance [1]. It was also reported that HO-1 levels at the materno–fetal interface of mice undergoing abortion are down regulated [2, 3], and that human miscarriage and preeclampsia are associated with

diminished placental HO levels [4, 5]. Moreover Sollwedel *et al.* reported that induction of HO-1 by HO-1 inducer, cobalt-protoporphyrin (Co-PP), prevented fetal rejection, and the down-regulation of HO-1 by zinc-protoporphyrin increased abortion [6].

Infection of *B. abortus* [7] or *L. monocytogenes* [8], induces the production of Th1 cytokines, such as IFN-γ, TNF-α, and IL-6. The up-regulation of Th1 cytokines leads to fetal rejection [9], while high levels of Th2 cytokines are associated with a successful pregnancy [10]. Furthermore, high levels of Th1 cytokines accompanied by decreased low HO levels are observed in rejected allografts [11].

Taken together, these findings indicate the strong relationship between HO-1 and infectious abortion. Based on these backgrounds, this study covers the following subjects:

1. Expression of heme oxygenase-1 is associated with abortion caused by *Brucella abortus* infection in pregnant mice
2. Protective role of heme oxygenase-1 in *Listeria monocytogenes*-induced abortion

CHAPTER 1

Expression of heme oxygenase-1 is associated with abortion caused by *Brucella abortus* infection in pregnant mice

INTRODUCTION

Brucellosis is a serious debilitating disease in humans and results in abortion and sterility in domestic animals. The etiologic agents of brucellosis are *Brucella* spp., small gram-negative and facultative intracellular pathogens that can multiply within professional and non-professional phagocytes [12, 13]. In contrast to other intracellular pathogens, Brucella species do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistant forms or fimbriae, and do not show antigenic variation [14]. A key aspect of the virulence of Brucella is its ability to proliferate within professional and non-professional phagocytic host cells, thereby successfully bypassing the bactericidal effects of phagocytes, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within host cells [15, 16]. Infection in humans is almost exclusively due to zoonosis, either through direct contact with infected animals or from contaminated dairy products [17]. The mouse model, particularly that using the non-pregnant mouse, has been used extensively to study some aspects of the pathogenesis of brucellosis [18]. While brucellosis is known to primarily affect the reproductive tract in the natural host and has been much studied, little is known regarding the cellular and molecular mechanisms of Brucella infection in the pregnant mouse [19]. The infectious abortion model using the pregnant mouse is a

powerful tool for investigating the mechanisms of Brucella pathogenesis and in our previous study we demonstrated that *Brucella abortus* causes abortion in pregnant mice by inoculating bacteria on day 4.5 of gestation [7]. We found that there was a higher degree of bacterial colonization in the placenta than in other organs, that there were many bacteria in trophoblast giant (TG) cells in the placenta and that abortion was not induced in an intracellular replication-defective mutant. Transient interferon-γ (IFN-γ) production induced by infection with *B. abortus* also contributes to infectious abortion and its neutralization served to prevent abortion. Pregnancy leads to a generalized suppression of the adaptive immune system, typified by significantly decreased cell-mediated immunity and reduced T helper cell (Th) 1 responsiveness [9, 20, 21].This immunosuppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents [22, 23]. Immunity against *B. abortus* is principally mediated by cellular immune responses since it is an intracellular pathogen, and involves antigen-specific T cell activation of CD4 and CD8 T cells and humoral responses. Protection of the host against *B. abortus* infection is thought to be mediated primarily by a Th1 type of immune response than a Th2 response [24]. For many other intracellular bacterial and protozoan pathogens, it has been shown that IFN-γ is an important component of Th1 immune responses and

contributes to control through its ability to stimulate macrophages to kill more microbes. The role of IFN-γ in the control of *B. abortus* infections has been demonstrated by supplementing BALB/c mice with recombinant IFN-γ, when such treatment resulted in a 10-fold decrease in the number of bacteria at 1 week after infection [25]. It has also been shown that neutralizing endogenous IFN-γ by administering anti-IFN-γ monoclonal antibodies results in a decrease in control [24]. Despite these results, however, the role of other factors in abortion induced by *B. abortus* infection in the pregnant mouse is still unknown. In the present study, I investigated the role of heme oxygenase (HO)-1 in abortion induced by *B. abortus* infection in the pregnant mouse. These results suggested that the expression of HO-1 in TG cells was down-regulated by IFN-γ treatment and *B. abortus* infection, and this led to infectious abortion.

RESULTS

Abortion induced by *B. abortus* infection is dependent on expression of HO-1 in the placenta

Previous studies have reported the presence of HO-1 in the mammalian placenta and postulated a protective role for HO during pregnancy [3, 6, 26]. To investigate the role of HO-1 in abortion induced by *B. abortus* infection, the effect of cobalt-protoporphyrin (Co-PP) on infectious abortion was tested. Treatment with Co-PP, which is known to up-regulate HO-1 expression, had a positive effect on long-term graft acceptance [11, 27] and helped prevent fetal rejection in pregnant mice [6]. In a previous study [7], we observed that *B. abortus* infection induced abortion. In the present study, treatment with Co-PP was seen to inhibit abortion due to *B. abortus* infection in pregnant mice as compared with non-treated pregnant mice (Fig. 1A). However, there was no significant difference between mice treated with Co-PP and non-treated mice as regards bacterial growth in the spleen (Fig. 1B).To find out if prevention of infectious abortion is dependent on HO-1 expression in the placenta, its amounts in the placenta were determined by immunoblotting. I observed an increase in HO-1in the placentas of uninfected pregnant mice due to Co-PP treatment (Fig. 1C).

Though there was a marked decrease in the amount of HO-1 in the placentas of *B. abortus* infected pregnant mice, the placentas of mice in which abortion had been prevented by Co-PP treatment had greater amounts of HO-1 in the placenta than mice in which abortion had not been prevented by Co-PP treatment (Fig. 1C). These results suggest that abortion induced by *B. abortus* infection is dependent on the expression of HO-1 in the placenta.

Decrease in HO-1 expression due to *B. abortus* infection in trophoblast giant cells

B. abortus has been observed to specifically infect trophoblast giant (TG) cells in the placentas of pregnant mice [7, 19]. To investigate the protective role of HO-1 further, I used an *in vitro* cell culture system for trophoblast stem (TS) cells and TG cells differentiated from TS cells. Replication of *B. abortus* in TG cells was confirmed by fluorescence microscopy (Fig. 2C). Expression of HO-1 was observed in TG cells and this was decreased by *B. abortus* infection (Fig. 2A). Also, up-regulation of HO-1 expression was observed in both infected and uninfected TG cells treated with Co-PP in a concentration dependent manner (Fig. 2A). However, there was no significant difference between TG cells treated with Co-PP and non-treated TG cells as regards intracellular growth of bacteria (Fig. 2B). Since a transient increase in IFN-γ brought

about by *B. abortus* infection was observed to promote abortion in pregnant mice [7], I investigated the effect of IFN-γ treatment on HO-1 expression in TG cells. HO-1 expression in TG cells was significantly decreased by IFN-γ treatment in a concentration dependent manner, and this down-regulation of HO-1 was further enhanced by *B. abortus* infection (Fig. 3).

HO-1 protects against cell death due to *B. abortus* infection

Since HO-1 has been reported to have antiapoptotic properties [28, 29], I investigated the rate of cell death due to infection with *B. abortus*. To examine the effect of HO-1 on TG cell death, I reduced the amount of endogenous HO-1 by transfecting HO-1-specific small interfering RNA (siRNA) duplexes into the TG cells. After 48 h of transfection with HO-1-specific siRNA, the expression level of HO-1 was no longer detectable, but was not affected by transfection with β-actin or the control siRNA (Fig. 4C). Cell death was not observed in HO-1 knockdown cells created using siRNA, IFN-γ treated cells, or no treatment cells (Fig. 4A and B). Cell death was also not observed in *B. abortus* infected TG cells significantly (Fig. 4A and B). However, infection of *B. abortus* induced cell death in HO-1 knockdown and IFN-γ treated TG cells (Fig. 4A and B).

DISCUSSION

It has been proposed that the cytoprotective enzyme HO plays a critical role in graft acceptance [1]. HO is the rate-limiting enzyme in heme catabolism, which generates free iron, biliverdin, and carbon dioxide, the main products of its action. Biliverdin is converted into bilirubin by biliverdin reductase. Accumulation of free heme, which is toxic, leads to tissue inflammation and injuries [1, 30, 31]. HO is therefore responsible, at least in part, for preventing such injuries [1, 30, 31]. Among the three identified mammalian HO isoforms, HO-1 is a stress-responsive protein, which is implicated in antioxidant defense mechanisms and modulation of vascular tone [31, 32]. Much evidence points to an up-regulation of HO-1 allowing the acceptance of mouse and rat allografts [33, 34], while its down-regulation or absence is directly related to acute graft rejection [29]. It has been reported that HO-1 levels at the materno–fetal interface of mice undergoing abortion were down regulated [2, 3], and that human miscarriage and preeclampsia were associated with diminished placental HO levels [4, 5]. In this study, I observed that expression of HO-1 was associated with abortion induced by *B. abortus* infection. *B. abortus* infection caused down-regulation of HO-1 in the placenta and I consider this to be one reason for abortion induced by bacterial

infection. Sollwedel *et al.* reported that up-regulation of HO-1 by Co-PP during the implantation window could prevent abortion in mice [6]. They also found that induction of HO-1 by Co-PP prevented fetal rejection, and the down-regulation of HO-1 by zinc-protoporphyrin increased abortion [6]. In the present study, I also observed that induction of HO-1 by Co-PP prevented abortion induced by *B. abortus* infection. Previous studies have reported that during pregnancy, different types of trophoblast cells are important sources of HOs [3-5, 35] and participate in the catabolism of the heme protein, avoiding accumulation or recirculation of free heme which could be extremely toxic for the mother and fetus. Down regulation of HO-1 is potentially very harmful at the materno–fetal interface since large amounts of free heme readily incorporate into endothelial cells, leading to oxidative injury and enhanced adhesion molecule expression [30, 36], and allowing migration of inflammatory lymphocytes into the materno–fetal interface from mice undergoing abortion [3]. Therefore, up-regulation of HO-1 is important in protection against infectious abortion. HOs are expressed in cultured human trophoblast cells [37] and my study showed that HO-1 is also detected in cultured murine TG cells. *B. abortus* infects TG cells in the placenta of pregnant mice specifically [7, 19], and this may be a reason for the down-regulation of HO-1 expression in placenta. However, the mechanism of down regulation of HO-1 in TG

cells by *B. abortus* infection remains unknown. Though the Th1/Th2 cytokines paradigm proposes that the up-regulation of proinflammatory cytokines, such as IFN-γ, TNF-α, and IL-6, would lead to fetal rejection [9], high levels of Th2 cytokines would be associated with a successful pregnancy [10]. Further, increased Th1 levels associated with low HO levels could be observed in rejected allografts [11]. The results in this study showed that HO-1 expressed in TG cells was down-regulated by IFN-γ, and that the effect of this cytokine was enhanced by *B. abortus* infection. Th1 cytokines may therefore play an important role in the regulation of HO-1 expression in TG cells. HO-1 is also believed to have antiapoptotic and tissue-protective properties. Induction of HO-1 expression by heme protects endothelial cells from TNF-α-mediated apoptosis [29, 38], but the mechanisms by which HO-1 prevents cells from undergoing apoptosis are still unclear. Since *B. abortus* infection was seen to induce cell death in HO-1 knockdown or IFN-γ treated TG cells, HO-1expression in TG cells would be associated with the induction of TG cell death by *B. abortus* infection. TG cell death in the placenta would be induced by bacterial infection, and then abortion might occur. Although the immunological mechanisms that govern the success of pregnancy in mammals are highly complex and many factors should participate in infectious abortion, HO-1 may be a putative therapeutic target in abortion by *B. abortus* infection.

MATERIALS AND METHODS

Bacterial strain and mice

B. abortus 544 (ATCC23448), a smooth virulent *B. abortus* biovar1 strain, was used. The *B. abortus* strain was maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson) or Brucella broth containing 1.5% agar [39]. Six- to ten-week-old ICR female mice were individually mated to 6- to 10-week-old ICR male mice. All of these mice were obtained from CLEA Japan. The normal gestational time for these mice is 19 days and the vaginal plug was observed on day 0.5 of gestation.

Virulence in pregnant mice

Groups of five pregnant mice were infected intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 ml saline with or without Co-PP (5 mg/kg, SIGMA) at 4.5 days of gestation [7]. On day18.5 of gestation, their fetuses, placentas, and spleens were removed and homogenized in saline. Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of CFU in each organ. Fetuses were determined to be alive if there was a heartbeat and dead if there was no heartbeat [7]. The animal experiments were permitted by Animal Research Committee of Obihiro

University of Agriculture and Veterinary Medicine.

Cell culture

Trophoblast stem (TS) cells were cultured in TS medium in the presence of FGF4, heparin and mouse embryonic fibroblast (MEF)-conditioned medium as described previously [40]. The TS medium was prepared by adding 20% fetal bovine serum (FBS), 1 mM sodiumpyruvate, 100 mM β-mercaptoethanol, and 2 mM L-glutamineto RPMI 1640. To induce differentiation to trophoblast giant (TG) cells, the cells were cultured in TS medium alone. The TG cells were seeded (1–2 × 10^5 per well) in 48 well tissue culture plates for all assays.

Efficiency of bacterial replication within cultured cells

Bacterial infection and intracellular survival assays were performed according to a modified version of the method of Kim et al [41]. *B. abortus* strains were deposited onto TG cells at a multiplicity of infection (MOI) of 10 by centrifugation at 150 × g for 10 min at room temperature. To measure intracellular replication efficiency, the infected cells were incubated at 37 °C for 30 min, washed once with TS medium and then incubated with TS medium containing gentamicin (30 mg/ml) for 2, 24, 48 and 72 h.

Next, the cells were washed three times with PBS and lysed with cold distilled water. CFU values were determined by serial dilution on Brucella plates. Percentage protection was determined by dividing the number of bacteria surviving by the number in the infectious inoculum. Co-PP or recombinant IFN-γ (Cedarlane Laboratories) was added to the TS medium at the indicated concentrations 12 h before infection.

Immunoblotting

Placenta or cell lysates were separated on 12% polyacrylamide gels and transferred to a PVDF membrane, which was incubated for1 h at room temperature with primary antibody (anti-HO-1, Stressgen) in 5% skim milk. They were then washed three times in Tris buffered saline (TBS) with 0.02% Tween 20, incubated for 30 min with a horseradish peroxidase (HRP)-conjugated secondary antibody and then washed again. Immunoreactions were visualized by ECL (GE Healthcare Life Sciences). Antibody for β-actin was purchased from SIGMA.

siRNA experiment

The siRNA duplexes used for silencing mouse HO-1 (target sequence: AACAAGTAACATGGAATAATA), and β-actin (target sequence:

CACTGACTTGAGACCAATAAA) and AllStars Negative Control siRNA were purchased from QIAGEN. TG cells were transiently transfected using oligofectamine (Invitrogen) with or without a final concentration of 10 nM for siRNAs.

Detection of cell death

Cell death was determined by means of a JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical) according to the instructions of the manufacturer. Mitochondrial membrane potential, DJm, is an important parameter of mitochondrial function used as an indicator of cell health. Healthy cells with a high mitochondrial DJm have red fluorescence while apoptotic or unhealthy cells have a low DJm and green fluorescence [42]. Cell death was confirmed by MEBSTAIN Apoptosis Kit Direct (MBL) according to the instructions of the manufacturer.

Statistical analysis

All statistical analysis was conducted using the Student's t test.

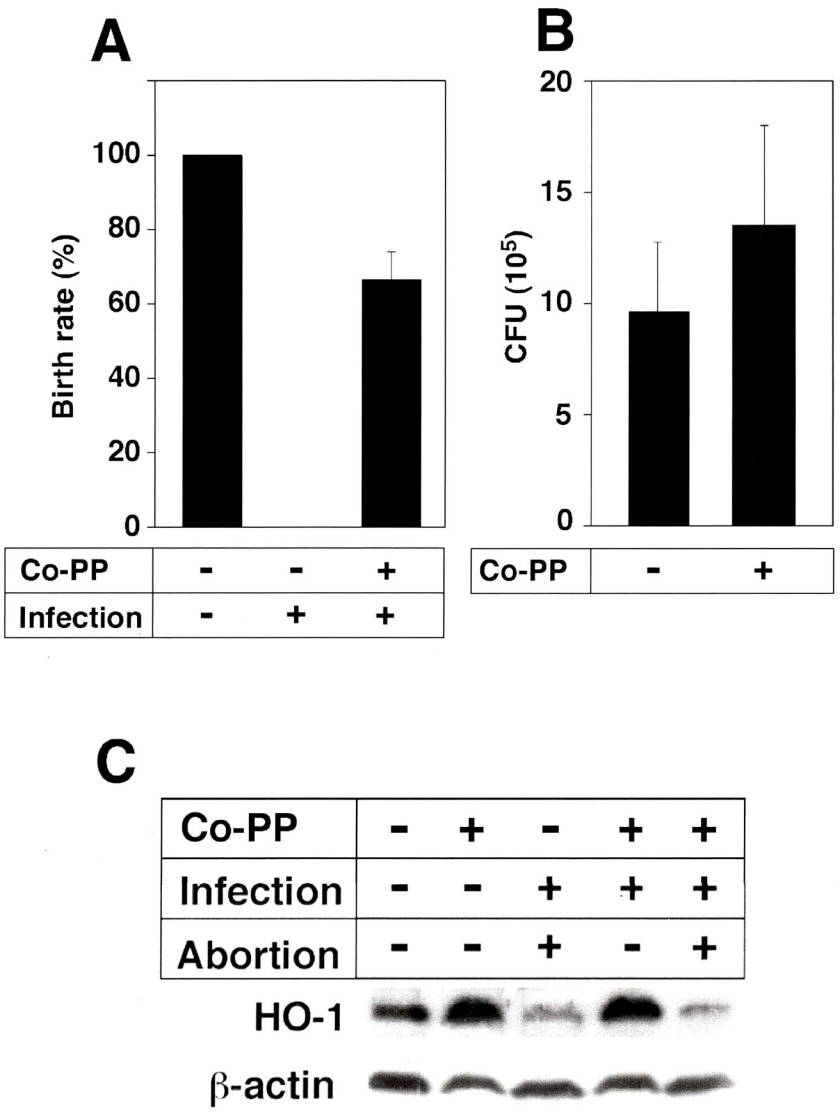


Fig. 1. Abortion prevented by treatment with Co-PP

Pregnant mice inoculated with or without Co-PP were infected with *B. abortus*. The figure shows birth rate (A), bacterial growth in spleen (B), and expression of HO-1 in immunoblotting (C).

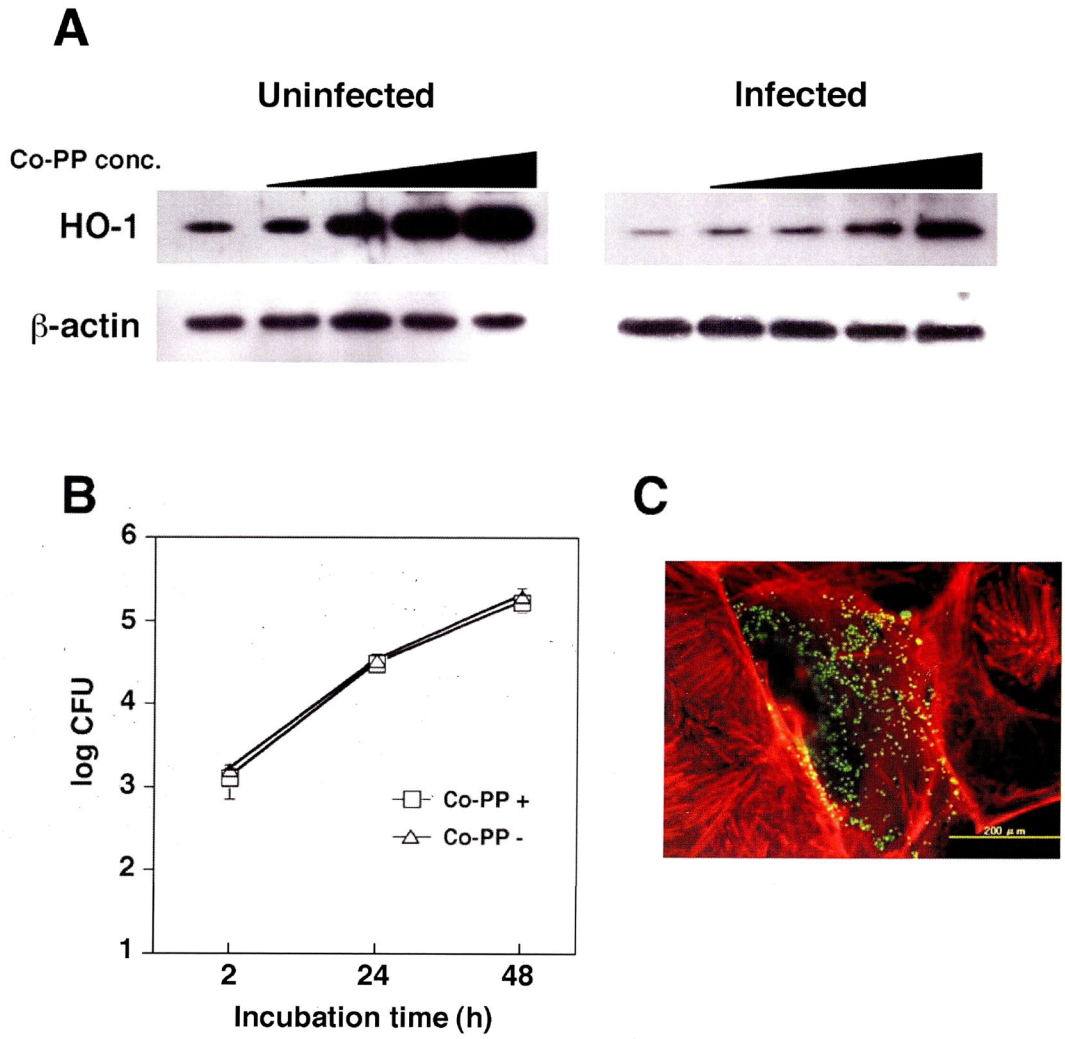


Fig. 2. Expression of HO-1 in *B. abortus* infected TG cells pretreated with Co-PP

(A)Expressions of HO-1 and β-actin (control) were examined by means of immunoblotting in TG cells treated with Co-PP (0, 0.75, 2.25, 7.5 or 22.5 μg/ml, respectively) which were infected with or without *B. abortus*. (B)Bacterial growth in TG cells treated with or without Co-PP (22.5 μg/ml) is shown. (C)Bacterial replication in TG cells. *B. abortus* was deposited onto TG cells which were then incubated for 48 h,

fixed and stained for actin filaments with Alexa Fluor 594-phalloidin. The figure shown GFP-expressed bacteria (green) and Alexa Fluor 594 channel (red) merged images.

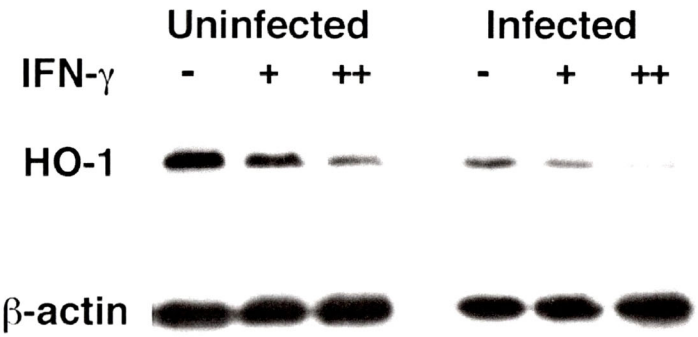


Fig. 3. Expression of HO-1 in IFN-γ treated TG cells

IFN-γ (0, 2.5 or 7.5 × 10^3 units/ml) treated TG cells were infected with or without *B. abortus*, and then the expression of HO-1 and β-actin (control) were examined by means of immunoblotting.

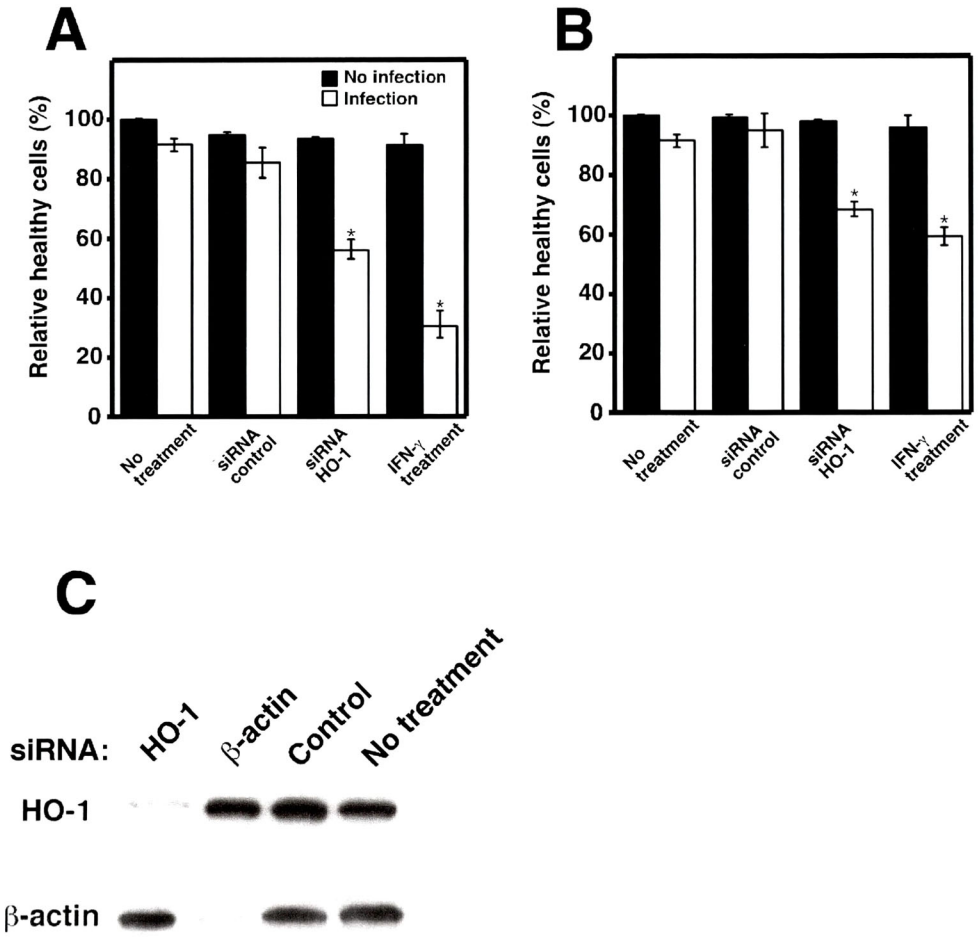


Fig. 4. Expression of HO-1 correlated with TG cell death due to *B. abortus* infection

HO-1 depleted and IFN-γ treated TG cells were infected with or without *B. abortus*, and cell death was determined using a JC-1 Mitochondrial Membrane Potential Assay Kit (A) and MEBSTAIN Apoptosis Kit Direct (B). Quantitative analysis of cell death (A and B). One hundred TG cells per coverslip were examined to determine the total number of live or dead TG cells. Data are the average of triplicate samples from

three identical experiments, and the error bars represent the standard deviation. Statistically significant differences in live cell numbers between TG cells depleted in HO-1 (siRNA HO-1) and without HO-1 (siRNA control), and IFN-γ treated and non-treated TG cells are indicated by asterisks (*, $P < 0.01$). TG cells were treated for 48 h with siRNA targeting HO-1, received no treatment (no treatment), or were treated with siRNA targeting β-actin or the control (QIAGEN AllStars Negative Control) (C). Expression of the indicated proteins was examined by means of immunoblotting. β-actin was used as an internal control.

CHPTER 2

Protective role of heme oxygenase-1 in *Listeria monocytogenes*-induced abortion

INTRODUCTION

Listeriosis is caused by gram-positive *Listeria monocytogenes*. In humans, this pathogen has the ability to cross the intestinal, placental, and blood-brain barriers, leading to gastroenteritis, maternofetal infections, and meningoencephalitis, respectively. A key feature of the virulence of *L. monocytogenes* is its ability to avoid the killing mechanisms of professional and non- professional phagocytic host cells [43-46]. *L. monocytogenes* infections in humans are caused mainly by injection of contaminated food, such as daily products, raw vegetables, fish, poultry, processed chicken, and beef [47].

L. monocytogenes induces cell death *in vitro* and *in vivo* in various cell types including hepatocytes [48], lymphocytes [49], and dendritic cells [50]. Cell death induced by *L. monocytogenes* is associated with listeriolysin O, a pore-forming toxin that allows bacteria to lyse the phagosomal membrane and escape into the cytosol.

In a previous study, I investigated abortion induced by brucella infections and demonstrated that it was associated with cell death of placental immune cells, the trophoblast giant (TG) cells. Furthermore, I found that heme oxygenase (HO)-1 expression inhibited infectious abortions *in vivo* and cell death *in vitro* [51]. HO-1 plays

a key role in cytoprotection, anti-oxidation, and anti-inflammation. Most of the physiological functions of HO-1 are associated with its enzymatic activity in heme catabolism [52, 53]. In humans, HO-1 deficiency is associated with susceptibility to oxidative stress and an increased pro-inflammatory state, leading to severe endothelial damage [54]. Mice lacking HO-1 develop progressive inflammatory disease [55] and show enhanced lipopolysaccharide-induced toxemia [56]. Although the protective properties of HO-1 have been studied using various inflammatory models [57-62], the molecular mechanisms, timing, and mode of HO-1 function during disease remains largely unknown. HO-1 expression is known to be associated with B-cell lymphoma-extra large (Bcl-XL) expression [63]. Bcl-XL is one of the several anti-apoptotic proteins that are members of the Bcl-2 family [64].

L. monocytogenes infection causes abortion in pregnant mice [65]. However, the factors involved in abortion induced by *L. monocytogenes* infection in these animals remain unknown. In the present study, I investigated the roles of the anti-apoptotic factors, HO-1 and Bcl-XL, in abortion induced by *L. monocytogenes* infection. HO-1 and Bcl-XL expression was down-regulated by *L. monocytogenes* infection or interferon (IFN)-γ treatment, leading to infectious abortion. HO-1 and Bcl-XL overexpression suppressed this infectious abortion. These results suggest that HO-1 and Bcl-XL play a

critical role in the control of infectious abortion induced by *L. monocytogenes*.

RESULTS

L. monocytogenes infection decreased HO-1 and Bcl-XL expression in TG cells

L. monocytogenes has been shown to infect the placenta and induce cell death *in vitro* and *in vivo* [66-68]. TG cells are placental immune cells existing in maternal-fetal interface and these cells are important for maintaining pregnancy [69]. In a previous study, I demonstrated that HO-1 plays a role in inhibiting cell death induced by *Brucella abortus* infection. To investigate the mechanisms through which *L. monocytogenes* induces cell death in placenta, I measured HO-1 expression in TG cells. HO-1 was expressed in TG cells, but its expression decreased on *L. monocytogenes* infection (Fig. 1A). Furthermore, HO-1 expression was enhanced by the HO-1 inducer cobalt protoporphyrin (Co-PP), in a concentration-dependent manner (Fig. 1A). No significant difference was observed in intracellular growth of bacteria between Co-PP-treated and non-treated TG cells (Fig. 1B, C). These results indicate that *L. monocytogenes* infection decreases HO-1 expression. To investigate the mechanism of HO-1, Bcl-XL expression was analyzed (Fig. 1A). Bcl-XL, an anti-apoptotic protein induced by HO-1, belongs to the Bcl-2 family [28, 29]. Bcl-XL expression was enhanced by the HO-1 inducer Co-PP and decreased by *L. monocytogenes* infection as well as HO-1.

Furthermore, I showed that this reduction in expression was recovered by Co-PP.

Since an increase in IFN- γ due to *L. monocytogenes* infection was observed to promote abortion in pregnant mice [8], I investigated the effect of IFN-γ treatment on HO-1 and Bcl-XL expression in TG cells. HO-1 and Bcl-XL expression in TG cells decreased significantly in a concentration-dependent manner on treatment with IFN-γ, with the down-regulation being enhanced further by *L. monocytogenes* infection (Fig. 2A).

HO-1 and Bcl-XL protect against cell death induced by *L. monocytogenes* infection

To examine whether HO-1 and Bcl-XL inhibited cell death, TG cells were infected with *L. monocytogenes* with or without Co-PP treatment and the rate of cell death was determined measuring mitochondrial membrane potential. Mitochondrial membrane potential has been used as an indicator of cell death. In this experimental system, cell death induced cells with low mitochondrial membrane potential were detected as unhealthy cells (Fig. 2B). Treatment with Co-PP inhibited cell death induced by *L. monocytogenes* infection in TG cells as compared with untreated TG cell. In contrast, cell death induced by *L. monocytogenes* infection in IFN-γ-treated TG cells was enhanced compared to that in untreated TG cells (Fig. 2B). Treatment with

cytochalasin D, which is known to inhibit *L. monocytogenes* internalization, was found to inhibit the death of TG cells by *L. monocytogenes* infection compared with non-treated TG cells (Fig. 2B). These results indicate that internalization of *L. monocytogenes* decreases HO-1 and Bcl-XL expression leading to enhancement of cell death.

To confirm the effect of HO-1 and Bcl-XL on TG cell death following infection with *L. monocytogenes*, I reduced the amount of endogenous HO-1 and Bcl-XL by transfecting HO-1-specific small interfering RNA (siRNA) duplexes into TG cells. After 48 h of transfection with HO-1-specific siRNA, HO-1 and Bcl-XL expression levels were no longer detectable, but were not affected by transfection with β-actin or control siRNA (Fig. 3A). HO-1 or Bcl-XL knockdown did not induce cell death in TG cells (Fig. 3C). While *L. monocytogenes* infection resulted in a slight induction of cell death in TG cells, HO-1 or Bcl-XL knockdown enhanced cell death in infected TG cells (Fig. 3C). Bcl-XL overexpression in the T-Rex system inhibited cell death compared to cells not expressing the protein (Fig. 3C). There was no significant difference in bacterial growth between transfected and non-transfected TG cells (Fig. 3B). These results suggest that HO-1 and Bcl-XL play critical roles in the inhibition of cell death induced by *L. monocytogenes* infections.

Abortion induced by *L. monocytogenes* infection is dependent on HO-1 and Bcl-XL expression in the placenta

Previous studies have reported the presence of HO-1 in the mammalian placenta and postulated that it has a protective role during pregnancy [3, 6, 26]. I assume that the inhibitory action of HO-1 and Bcl-XL on cell death leads to a successful pregnancy. To examine whether HO-1 and Bcl-XL actually block abortion induced by *L. monocytogenes* infection, I measured HO-1 and Bcl-XL expression levels in the placenta of *L. monocytogenes*-infected mice. Both HO-1 and Bcl-XL were expressed in the placenta of mice, with levels being decreased by *L. monocytogenes* infection (Fig. 4C). Moreover, injection of *L. monocytogenes* with Co-PP restored HO-1 and Bcl-XL expression levels (Fig. 4C). I next investigated the role of HO-1 and Bcl-XL expression on abortion induced by *L. monocytogenes*. Infection of *L. monocytogenes* induced abortion in pregnant mice (Fig. 4A). HO-1 and Bcl-XL expression induced by Co-PP injection blocked abortion in *L. monocytogenes*-infected mice (Fig. 4C). There was no significant difference in the growth of bacteria in livers (Fig. 4B) and placenta (data not shown) between Co-PP-treated and untreated mice. These results suggest that abortion induced by *L. monocytogenes* infection is dependent on HO-1 and Bcl-XL expression in

the placenta.

DISCUSSION

Heme oxygenases (HOs) are heme catabolic enzymes. Heme is degraded to carbon monoxide, biliverdin, and ferrous ion. Biliverdin is converted to bilirubin, which is believed to be a potent anti-oxidant. Three isoforms of HOs have been identified. HO-1 is an inducible isoform produced in response to various types of stress, such as oxidative stress, heat stress, endotoxin stress, hypoxia, heavy metal stress, and cytokine stress [70]. Furthermore, HO-1 plays a role in cytoprotection, anti-oxidation, anti-inflammation, and graft acceptance [1, 11, 27]. HO-1 is also down-regulated at the fetal maternal interface during spontaneous abortion in both humans and mice [2, 4, 5, 26]. Up-regulation of HO-1 by Co-PP prevents abortion, while down-regulation by zinc protoporphyrin increases the chances of abortion [6]. It has been reported that during pregnancy, all placental cell types are positive for HOs and that different types of trophoblast cells are important sources of these enzymes [3-5, 35]. As I anticipated, HO-1 was associated with infectious abortion. It is well known that various pathogens, such as *Brucella* spp., *L monocytogenes*, *Leptospira* spp., *Buniyavirus*, and *Toxoplasma gondii*, cause infectious abortion. However, the mechanisms responsible for infectious abortion remain unclear. Previously, I reported that HO-1 was associated with abortion

induced by *B. abortus* infection. *B. abortus* are gram-negative, intracellular, and zoonotic bacteria that cause down-regulation of HO-1 in the placenta leading to abortion. However, it remains unclear whether HO-1 is a common regulator for abortion induced by various pathogens. In this study, I used gram-positive, intracellular, and zoonotic *L. monocytogenes* to examine this possibility.

In order to investigate the detailed mechanisms of infectious abortion induced by *L. monocytogenes*, I studied TG cells *in vitro*. TG cells are immunocompetent cells present in the placenta [71-73] and play a critical role in implantation and pregnancy [69, 71]. HO-1 expression in TG cells was decreased by *L. monocytogenes* infection (Fig. 1A) and treatment with IFN-γ (Fig. 2A). Furthermore, it is well known that IFN-γ is induced by *L. monocytogenes* infection in mice [8] and there is evidence that Th1 cytokines, such as IFN-γ inhibit HO-1 expression resulting in allograft rejection [11]. These results indicate that Th1 cytokines induced by *L. monocytogenes* infection control HO-1 expression.

Although HO-1 appears to play a critical role in the control of infectious abortion, the mechanisms of this control remain unclear. I focused on Bcl-XL since HO-1 enhances the expression of this anti-apoptotic protein [63]. I found that Bcl-XL expression was enhanced by a HO-1 inducer, Co-PP (Figs. 1A, 4A) and furthermore that

Bcl-XL overexpression prevented cell death induced by *L. monocytogenes* infection (Fig. 3C). These results suggest that Bcl-XL is a key factor that protects placenta cells from injury induced by *L. monocytogenes* infection, thereby resulting in successful pregnancy.

I also observed that HO-1 and Bcl-XL expression was down-regulated in the placenta of pregnant mice by *L. monocytogenes* infection (Fig. 4A), while it was up-regulated by Co-PP and inhibited infectious abortion (Fig. 4B). These results suggest that HO-1 and Bcl-XL have critical roles against infectious abortion induced by *L. monocytogenes*.

Although HO-1 and Bcl-XL play an important role to protect cells from cell death, it is still unknown how HO-1 and Bcl-XL inhibit cell death induced by *L. monocytogenes* infection. Caspase-9 is an apoptotic protein and its activation is inhibited by Bcl-XL [74]. In TG cells, however, *L. monocytogenes* infection failed to induce caspase-9 activation (data not shown). These results may indicate that *L. monocytogenes* induces cell death trough alternative pathways involved with Bcl-XL.

In humans, it was reported that *L. monocytogenes* infects extravillous trophoblasts (EVTs), and spreads across maternal-fetal barrier [75]. However, there is less information about molecular mechanisms by which *L. monocytogenes* passes

maternal-fetus barrier. Since trophoblast cells such as EVTs in human or TG cells in mouse exists in maternal-fetal interface, down regulation of HO-1 and Bcl-XL leading to enhancement of cell death may be a key event for *L. monocytogenes* to spread across the barrier.

In conclusion, these results indicate that down-regulation of HO-1 induced by various pathogens may be a key event in infectious abortion. Antimicrobial drugs are usually used in the treatment of listeriosis. However, an increasing number of multidrug-resistant *L. monocytogenes* have been reported [76, 77]. It is noteworthy that the HO-1 inducer Co-PP suppressed abortion induced by *L. monocytogenes*. Therefore, HO-1 has potential as a putative therapeutic target in infectious abortion.

MATERIALS AND METHODS

Bacterial strains

L. monocytogenes EGD was maintained as a frozen glycerol stock and cultured in brain heart infusion (BHI) broth (Becton Dickinson) or on BHI broth containing 1.5 % agar.

Cell culture

Mouse trophoblast stem (TS) cell line was gifted from Dr. Tanaka [44, 49]. TS cells were cultured in TS medium in the presence of fibroblast growth factor-4, heparin, and mouse embryonic fibroblast-conditioned medium as described previously [40]. The TS medium was prepared by adding 20% fetal bovine serum, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine to RPMI 1640. To induce differentiation to TG cells, the cells were cultured in TS medium alone for 3 days at 37 °C in a CO_2 incubator. The cells were then seeded in a 48-well (1-2 × 10^5 per well) or a 12-well (4-8 × 10^5 per well) tissue culture plate.

Immunoblotting

The protein samples were separated on a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, which was incubated for 16 h at 4 °C with anti-HO-1 rabbit polyclonal antibody (Stressgen) or anti-Bcl-XL rabbit polyclonal antibody (Cell Signaling Technology) at a dilution of 1:5000 or 1:1000 in 5% skim milk. The membrane was washed three times in Tris-buffered saline with 0.02% Tween 20, incubated for 30 min with 0.01 μg/ml horseradish peroxidase-conjugated secondary antibody, and washed again. The immunoreactions were visualized using the enhanced chemiluminescence detection system (GE Healthcare Life Science). The β-actin antibody was purchased from Sigma.

Efficiency of bacterial replication within cultured cells

L. monocytogenes strains were deposited onto TG cells at a multiplicity of infection (MOI) of 10 by centrifugation at 150 × g for 10 min at room temperature. To measure the intracellular replication efficiency, the infected cells were incubated at 37 °C for 30 min, washed once with TS medium, and then incubated in TS medium containing gentamicin (50 μg/ml) for 0.5, 2, 6, and 12 h. The cells were washed three times with phosphate-buffered saline (PBS) and lysed with cold distilled water. Colony

forming unit (CFU) was determined by serial dilution on BHI agar plates. Cytochalasin D (Wako), recombinant IFN-γ (Cedarlane Laboratories) or Co-PP was added to the TS medium at the indicated concentrations 2, 16, and 24 h before infection.

Immunofluorescence microscopy

Bacteria were deposited onto TG cells grown on coverslips by centrifugation at 150 × g for 5 min at room temperature and were then incubated at 37 °C for 30 min. The samples were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed three times with PBS, and incubated successively three times for 5 min in blocking buffer (5% bovine serum albumin in PBS) at room temperature. The samples were permeabilized in 0.2% Triton X-100 and washed three times with PBS, followed by treatment with 5 μg/ml anti-*L. monocytogenes* polyclonal rabbit antibody (Viro Stat) diluted in blocking buffer to identify intracellular bacteria. After incubation for 1 h at 37 °C, the samples were washed three times for 5 min with blocking buffer, stained with FITC-labeled goat anti-rabbit IgG (0.01 μg/ml, Chemicon) in blocking buffer, and incubated for 1 h at 37 °C. Fluorescent images were obtained using a FluoView FV100 confocal laser scanning microscope (Olympus).

Expression of recombinant protein

Total RNA was isolated from TG cells using the RNA Purification Kit (Qiagen), and the purified RNA samples were stored at −30 °C until use. RNA was quantified by absorption at 260 nm using the SmartSpec 3000 spectrophotometer (Bio-Rad). RT-PCR was performed using Superscript II Kit (Invitrogen). The primers used for mouse Bcl-XL amplification were 5′- ATGTCTCAGAGCAACCGGG AG -3′ and 5′-TCACTTCCGACTGAAGAGTGA -3′. To express Bcl-XL in TG cells, amplified DNA encoding Bcl-XL from TG cells in RT-PCR was cloned into the pcDNA4/TO vector of the T-Rex System (Invitrogen). pcDNA4/TO-Bcl-XL was transfected into TG cells using the FuGENE 6 Transfection Reagent (Roche) at a final concentration of 1.2 μg/ml.

siRNA experiment

siRNA duplexes used for silencing mouse HO-1 (target sequence: CAGCCACACAGCACTATGTAA) and Bcl-XL (target sequence: AAAGTGCAGTTCAGTAATAAA) and AllStars Negative Control siRNA were purchased from QIAGEN. TG cells were transfected transiently using the X-tremeGENE siRNA Transfection Reagent (Roche) with or without a final

concentration of 10 nM for siRNAs.

Determination of cell death

Cell death was determined using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical) according to the manufacturer's instructions. Mitochondrial membrane potential, DJm, an important parameter of mitochondrial function, is used as an indicator of cell health. Healthy cells have a high mitochondrial DJm and red fluorescence, while apoptotic or unhealthy cells have a low DJm and green fluorescence [42].

Mice

Six to 10-week-old BALB/c female mice were mated individually to 6- to 10-week-old BALB/c male mice. The parent mice were obtained from Kyudo Co., Ltd.. Vaginal plug was observed at day 0.5 of gestation. The normal gestational time for these mice is 19 days.

Virulence in pregnant mice

Groups of five pregnant mice were infected intravenously at 13.5 days of

gestation with approximately 10^5 cells of *L. monocytogenes* in 0.1 ml saline with or without Co-PP (5 mg/kg, Sigma). On day 16.5 of gestation, their livers were removed and homogenized in saline. The tissue homogenates were serially diluted with PBS and plated on BHI agar plates to estimate the number of CFU. Fetuses were classified as alive if there was a heartbeat and as dead if there was no heartbeat. The animal experiments were approved by the Animal Research Committee of Yamaguchi University (permit number: 141).

Statistical analyses

Statistical analyses were performed using Student's t test. Statistically significant differences compared with the controls are indicated by asterisks (*, $P < 0.05$). Data are expressed as the mean of triplicate samples from three identical experiments, and the error bars represent the standard deviations.

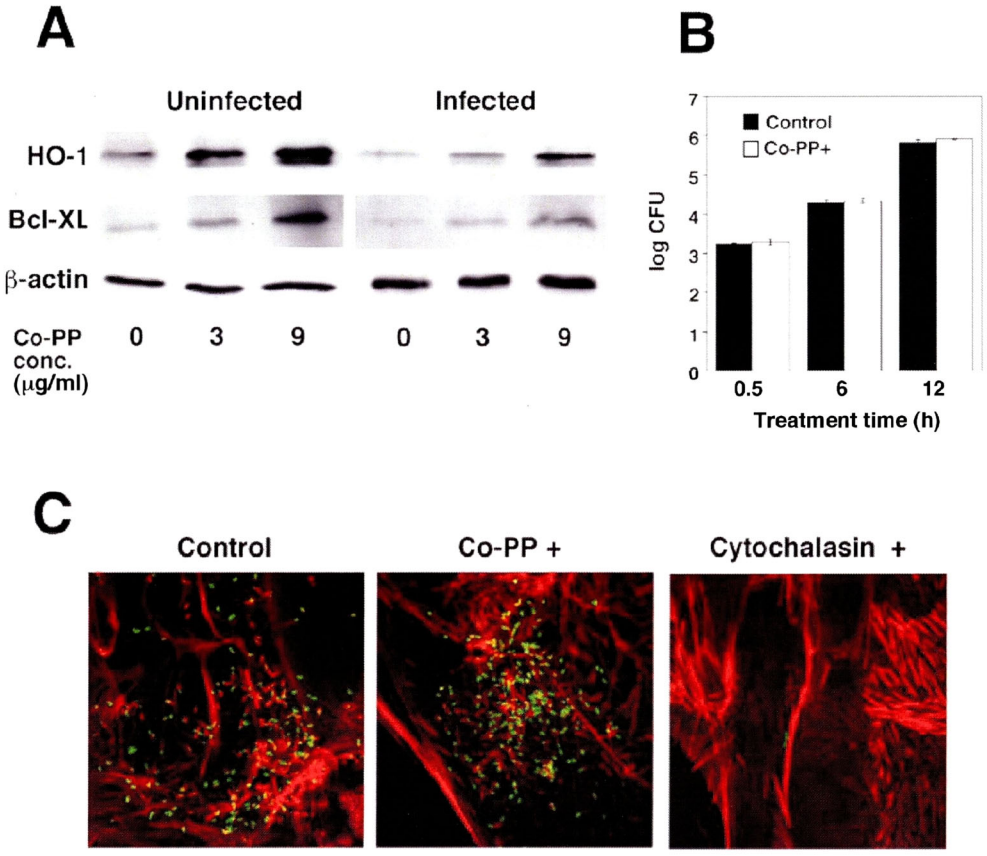


Fig. 1. Decreased HO-1 and Bcl-XL expression in TG cells infected with *L. monocytogenes*.

(A) TG cells were first treated with Co-PP and then infected with *L. monocytogenes*. The infected cells were cultured in 50 μg/ml of gentamicin. After 6 h, HO-1 and Bcl-XL expression was analyzed by immunoblotting. A representative immunoblot of three independent experiments is shown. (B) TG cells were treated with Co-PP and then infected with *L. monocytogenes*. The infected cells were cultured in 50 μg/ml of gentamicin. After 0.5, 2, and 6 h incubation, the infected cells were washed

with PBS and lysed with cold distilled water. CFU were determined by serial dilution on BHI agar plates. (C) *L. monocytogenes* was deposited on TG cells by centrifugation at 150 ×g for 10 min at room temperature, incubated for 6 h, fixed, and stained. The figure shows FITC-labeled bacteria (green) and Alexa Fluor 594-labeled actin filaments (red) merged images. The left-hand panel shows untreated cells, the center panel Co-PP (9 μg/ml) treated cells, and the right-hand panel, cytochalasin D-treated cells.

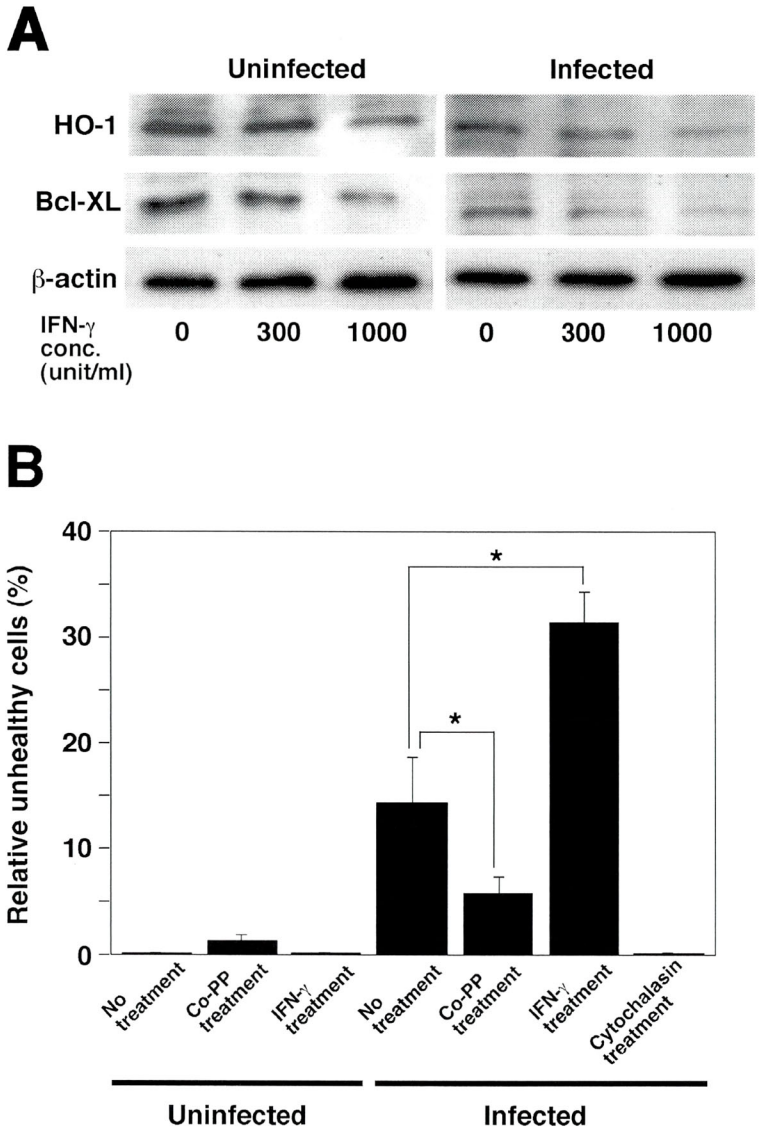


Fig. 2. Induction of cell death by *L. monocytogenes* infection

(A) TG cells were treated with IFN-γ (0, 300, and 1,000 units/ml) for 24 h and infected with *L. monocytogenes* for 6 h. HO-1 and Bcl-XL expression in TG cells was analyzed by immunoblotting. A representative immunoblot of three independent experiments is shown. (B) Cell death was determined using the JC-1 Mitochondrial Membrane Potential Assay Kit. One hundred TG cells per coverslip were examined to

determine the total number of live or dead cells. All values represent the average and the standard deviation of three identical experiments. Statistically significant differences compared with the control are indicated by asterisks (*, $P < 0.05$).

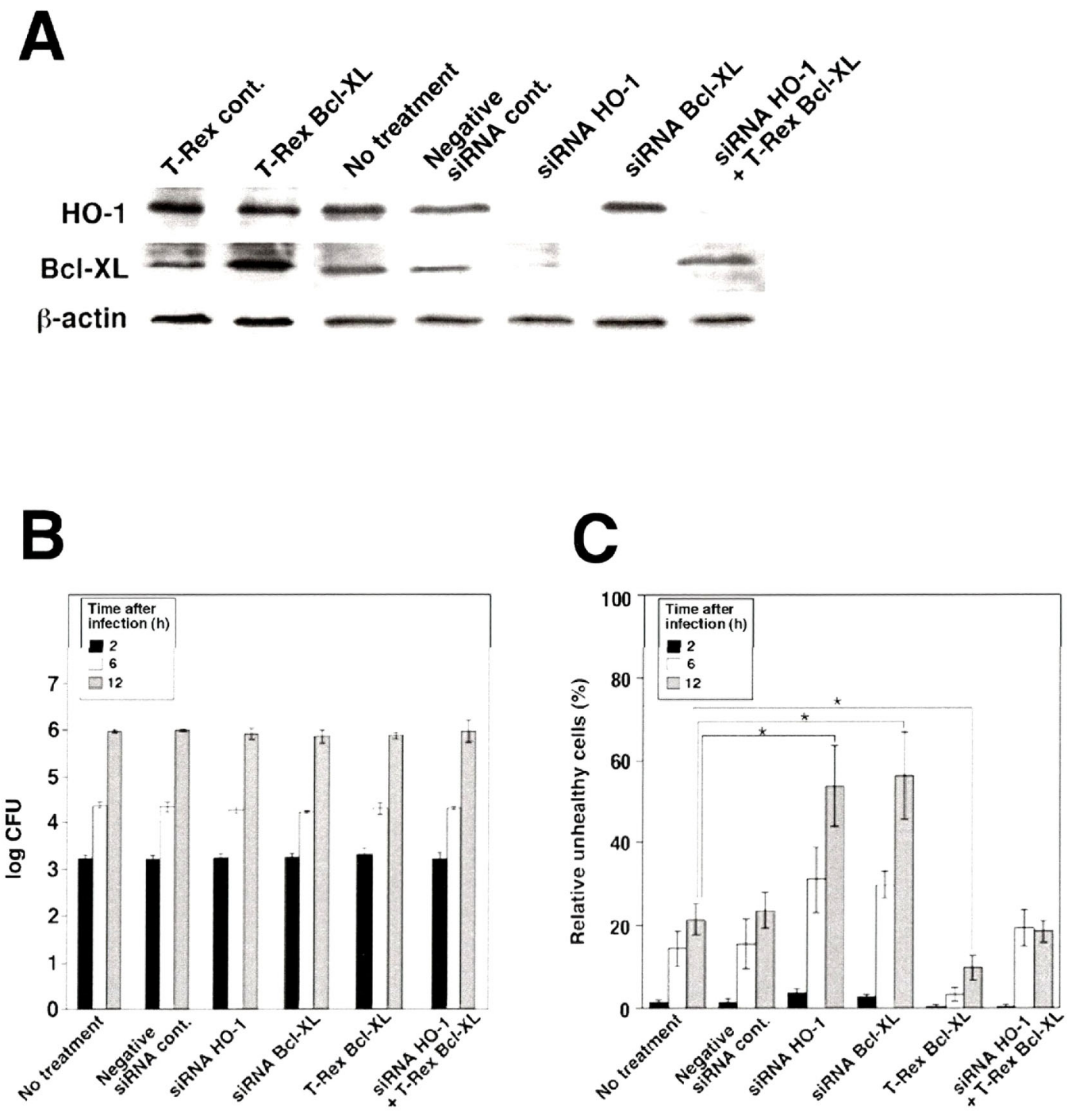
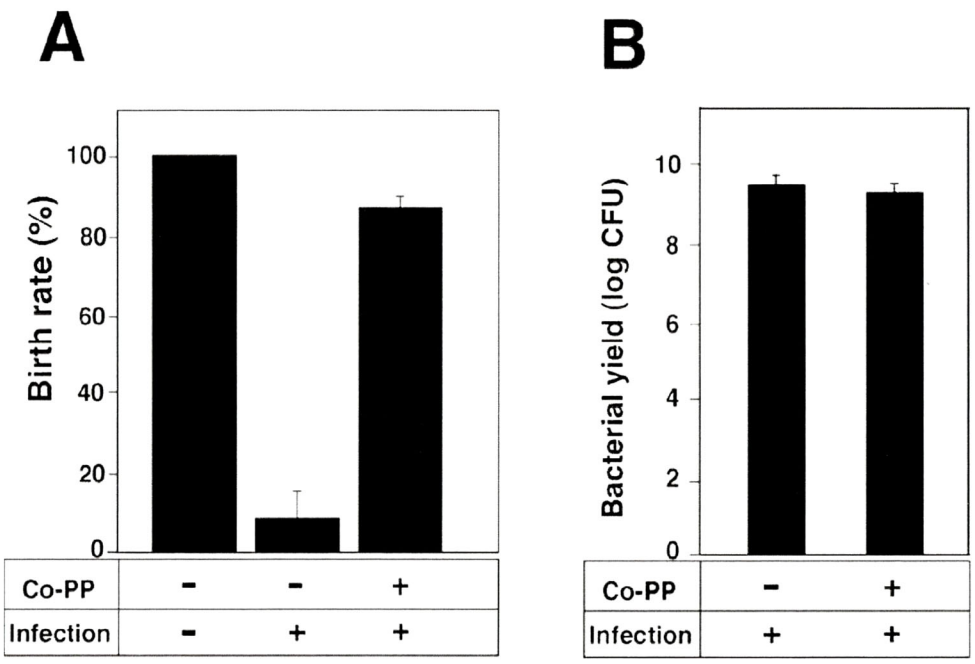


Fig. 3. Prevention of cell death by HO-1 and Bcl-XL expression

(A) TG cells were treated for 48 h with either siRNA targeting HO-1, Bcl-XL, or control siRNA (QIAGEN AllStars Negative Control). Bcl-XL overexpression was achieved by transfecting the cells with pcDNA4/TO-Bcl-XL. HO-1 and Bcl-XL expression was monitored by immunoblotting. β-actin was used as an internal control. A

representative immunoblot of three independent experiments is shown. (B) TG cells were infected with *L. monocytogenes*. The infected cells were cultured with media containing 50 μg/ml gentamicin for 2, 6, and 12 h. The cells were then washed with PBS and lysed with cold distilled water. CFU was determined by serial dilution on BHI agar plates. All values represent the average and the standard deviation of three identical experiments. (C) Cell death was determined using the JC-1 Mitochondrial Membrane Potential Assay Kit. One hundred TG cells per coverslip were examined to determine the total number of live or dead cells. All values represent the average and the standard deviation of three identical experiments. Statistically significant differences compared with the control are indicated by asterisks (*, $P < 0.05$).



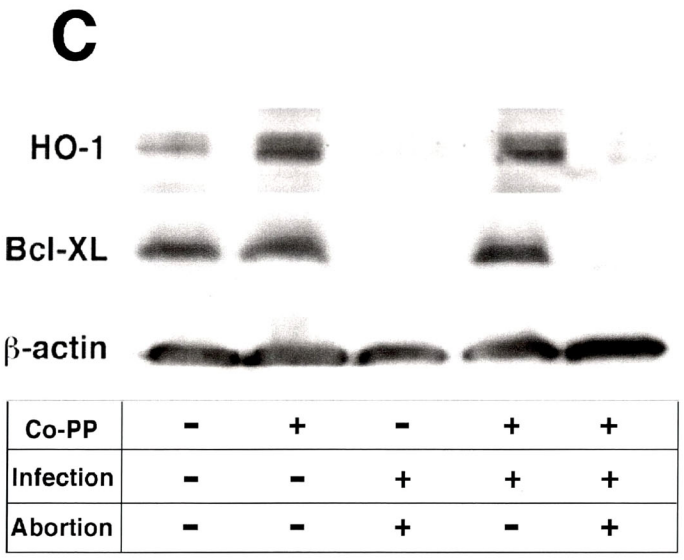


Fig. 4. Prevention of infectious abortion by HO-1 and Bcl-XL expression

(A) Pregnant mice were infected with 10^5 cells of *L. monocytogenes* in 0.1 ml of saline at day 13.5 of pregnancy with or without Co-PP treatment (5 mg/kg). At day 16.5,

the placentas, fetuses, and livers were removed. HO-1 and Bcl-XL expression in the placenta was analyzed by immunoblotting. A representative immunoblot of three independent experiments is shown. (B) Survival rates were determined by the presence or absence of a heartbeat in the fetuses. (C) Livers were homogenized in saline and diluted with PBS. CFU was determined by plating the diluted samples on BHI agar plate.

CONCLUSION

In this study, I demonstrated that HO-1 played an important role in preventing infectious abortion caused by *B. abortus* or *L. monocytogenes*.

There are a lot of pathogens that cause infectious abortion in animals including human. However, it is little known about molecular mechanisms of infectious abortion. To clarify the mechanisms of infectious abortion caused by bacteria, I focused on typical two types of bacteria, gram negative (*B. abortus*) and gram positive (*L. monocytogenes*) bacteria. *B. abortus* is a gram negative bacterium, and it survives in host cell by inhibiting fusion of phagosome and lysosome. *L. monocytogenes* is a gram positive bacterium, and it survives in host cell by escape from phagosome.

In chapter 1, I showed that HO-1 expression avoided the abortion by *B. abortus* infection. In chapter 2, I demonstrated that HO-1 expression suppressed the abortion by *L. monocytogenes* infection. These results may suggest that HO-1 plays an important role in preventing infectious abortion caused by various types of bacteria. In addition, administration of HO-1 inducer prevented infectious abortion.

Although HO-1 expression prevented infectious abortion, bacterial replications

were not inhibited by HO-1 expression. Since *B. abortus* and *L. monocytogenes* are intracellular pathogens which can replicate inside of host cells, it is difficult to eliminate and control them using antibiotics. In addition, an increasing number of multidrug-resistant bacteria have been reported. Therefore, alternative approaches other than antibiotics are necessary to control infectious diseases including abortion.

Overall, my results strongly suggest that HO-1 is a key molecule in controlling infectious abortion and a putative therapeutic target. Although more detail investigation should be done, the HO-1 inducer would be a universal therapeutic agent against abortion caused by various pathogens.

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