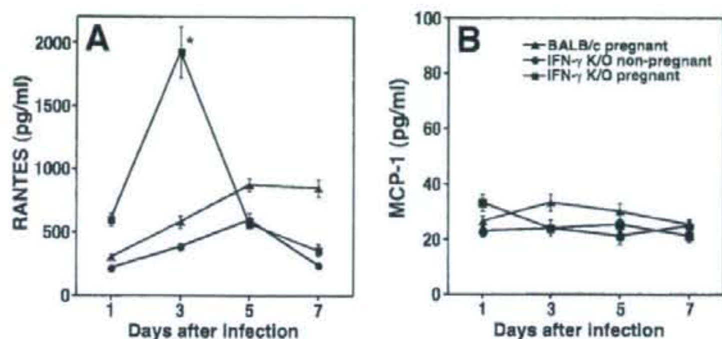

Fig. 2. Induction of RANTES in pregnant IFN-γ knockout mice by *B. abortus* infection. RANTES (A), and MCP-1 (B) serum levels were measured in each mouse by ELISA at the indicated numbers of days after infection. The means and SE for groups of 5 mice are shown. Statistically significant differences between pregnant and non-pregnant mice are indicated by asterisks (*, $P<0.01$).

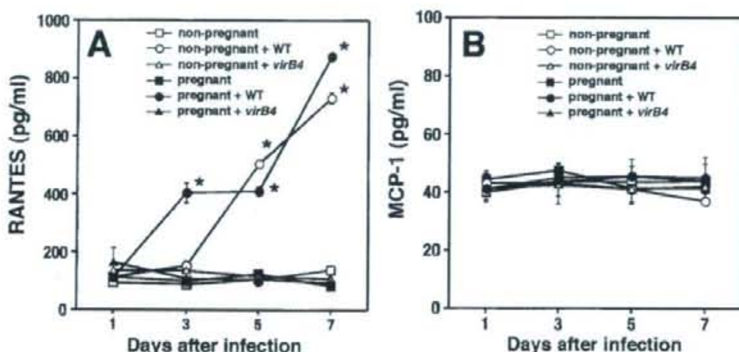

Fig. 3. Transient increase in RANTES in normal mice induced by *B. abortus* infection. Pregnant and non-pregnant normal mice were infected with wild type (WT) or *virB4* mutant (*virB4*). RANTES (A), and MCP-1 (B) serum levels were measured in each mouse by ELISA at the indicated numbers of days after infection. The means and SE for groups of five mice are shown. Statistically significant differences between mice infected with and without wild type *B. abortus* are indicated by asterisks (*, $P<0.01$).

out and normal mice induced by B. abortus infection: To examine cytokine patterns in *B. abortus* infected pregnant IFN-γ knockout mice, we conducted rough screening using a cytokine antibody array. As this showed that RANTES was greatly induced, we decided to determine if RANTES contributes to induction of abortion by *B. abortus* infection by using ELISA to measure the production of RANTES in pregnant mice. As a result, we observed a large amount of induced RANTES production in pregnant IFN-γ knockout mice at 3 days after infection with *B. abortus*, but afterwards RANTES production decreased rapidly (Fig. 2A). No significant MCP-1 production was observed in infected pregnant IFN-γ knockout mice or pregnant normal mice (Fig. 2B).

As these results imply that RANTES contributes to abortion due to *B. abortus* infection, we next examined RANTES production in normal mice with *B. abortus* infection in detail. For wild type *B. abortus* infection, RANTES was induced within 3 days of infection in pregnant mice, but the start of induction was 2 days later in non-pregnant mice. There was no induction of RANTES in pregnant and non-pregnant mice infected with *virB4* mutant, which does not have the ability of intracellular replication (Fig. 3A). No significant MCP-1 production was observed in pregnant or non-pregnant mice infected with the wild type or *virB4* mutant (Fig. 3B). These results suggest that the type IV secretion system contributes to RANTES production.

Preventing abortion by neutralizing RANTES: To determine if abortion is prevented by neutralizing the RANTES produced as a result of bacterial infection, pregnant mice

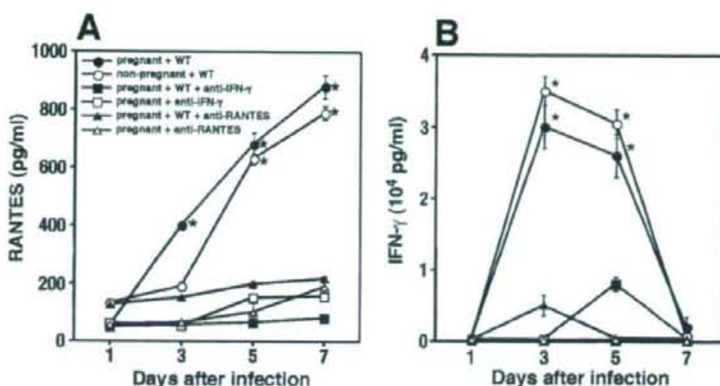

Fig. 4. Neutralization of RANTES and IFN-γ using monoclonal antibodies. RANTES and IFN-γ were neutralized in the mice by administering anti-mouse RANTES or IFN-γ monoclonal antibodies, respectively, and then the mice were infected with wild type *B. abortus* (WT). RANTES (A) and IFN-γ (B) were measured by ELISA. The means and SE for groups of 5 mice are shown. Statistically significant differences between the untreated control and antibody treated mice are indicated by asterisks (*, $P<0.01$).

were inoculated with monoclonal anti-RANTES antibodies or anti-IFN-γ antibodies 1 day before infection with *B. abortus*. Thereafter, serum levels of RANTES and IFN-γ, and abortion rates were recorded. Induction of RANTES and IFN-γ production was observed within 3 days of infection in pregnant mice, and this was inhibited by inoculation with RANTES or IFN-γ antibodies, respectively (Fig. 4A and B). Prior inoculation with anti-RANTES antibodies was also observed to inhibit abortion as compared to non-inoculated mice (Fig. 5A). However, there was no significant difference between pregnant mice inoculated with anti-RANTES antibodies and non-inoculated pregnant mice as regards bacterial growth in the spleen (Fig. 5B).

DISCUSSION

In the present study, we found that RANTES production contributes to abortion due to *B. abortus* infection in pregnant mice. We also noted that the production of RANTES and IFN-γ are influenced by each other in pregnant mice. Our previous study showed that a transient increase in IFN-γ due to *Brucella* infection causes abortion in pregnant mice [15]. 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 activate macrophages to enhance microbial killing. The role of IFN-γ in the control of *B. abortus* infections has been demonstrated by administering recombinant IFN-γ to BALB/c mice. This treatment resulted in a 10-fold decrease in the number of bacteria at 1 week after infection [24]. Similarly, the neutralization of endogenous IFN-γ through the administration of anti-IFN-γ monoclonal antibodies resulted in a decrease in control of *B. abortus* infection [35]. IFN-γ is important for control of *B. abortus* infection in BALB/c mice during the first week of infection, since an increase in bacteria has been measured in IFN-γ knockout mice at 1 week post infection [12, 13]. However, by 3 weeks post infection, the number of bacteria recovered was equivalent regardless of whether IFN-γ was present or not, and BALB/c mice survived for 10 weeks when IFN-γ was knocked out [18].

In the present study, pregnant IFN-γ knockout mice died within 15 days of *B. abortus* infection. Pregnancy leads to a generalized suppression of the adaptive immune system, typified by significantly decreased cell-mediated immunity and reduced Th1 responsiveness [21, 33, 34], and this immunosuppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents [16, 23]. This is considered to be the reason that pregnant IFN-γ knockout mice died more rapidly due to *B. abortus* infection than non-pregnant IFN-γ knockout mice.

Immune cells and their secretory products have been recognized as important pathophysiological mediators of recurrent spontaneous abortions and endometriosis in humans [7, 25, 32]. On the other hand, chemokines are involved in T-cell trafficking during normal processes and also in pathological events such as inflammation and endothelium damage [29]. RANTES is a chemokine that has recently been implicated in trophoblast and spermatozoa migration in view of its well-established chemoattractant properties [17, 26]. The specific ability of RANTES to downregulate T-cell responses suggests it might be relevant for fetal tolerance induction, but other than this no role of RANTES in pregnancy has been established [22]. A recent study reported that the secretion of RANTES, macrophage inflam-

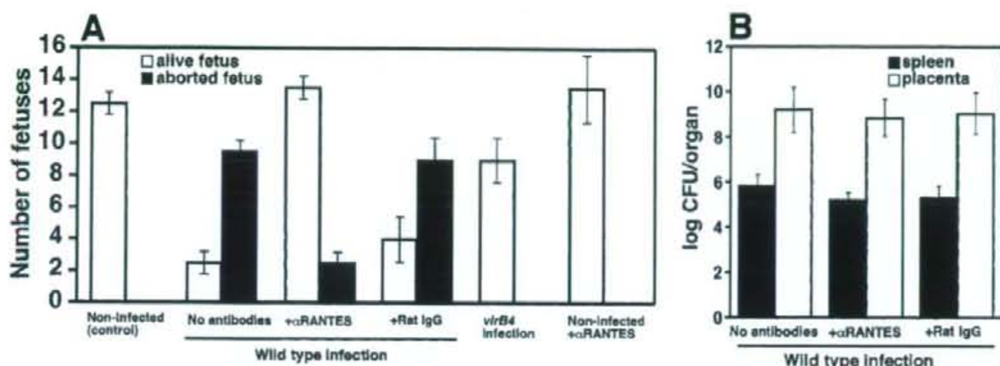

Fig. 5. Preventing abortion by neutralizing RANTES. RANTES was neutralized in the mice by administering anti-mouse RANTES monoclonal antibodies, and then they were infected with wild type (WT) or *virB4* mutant (*virB4*). The figure shows number of fetuses (A), and bacterial growth in the spleen and placenta (B).

matory protein (MIP)-1α, MIP-1β, and activated-induced, T cell-derived, and chemokine-related cytokine (ATAC) in polyclonally activated NK and T cells was closely associated with the secretion of IFN-γ [10]. RANTES is not only co-secreted to a great extent with IFN-γ at the single-cell level but also synergize functionally with IFN-γ on a common target population [10]. In this sense, the production and function of RANTES correlate with IFN-γ in *B. abortus* infected pregnant mice. Further, RANTES may act as a component of Th1 immune responses with IFN-γ in pregnant mice.

NK cells are large granular lymphocytes found in peripheral blood and also in the maternal decidua during pregnancy. The actions of NK cells on trophoblast lineage cells are likely mediated by NK cell secretory products, including cytokines, and may be direct or indirect. Uterine NK cells produce several cytokines and are the primary source of IFN-γ in the metrial grand [1, 2]. IFN-γ has been implicated as a major mediator of uterine NK cell function during pregnancy [3, 4]. Trophoblast cells are among a variety of different IFN-γ targets, and *in vitro* trophoblast cell differentiation, survival, and outgrowth are affected by IFN-γ [1, 5]. However, the function of IFN-γ is more diverse than the induction of bactericidal function and includes the stimulation of antigen presentation through class I and class II MHC molecules, the orchestration of leukocyte-endothelium interactions, the effects on cell proliferation and apoptosis, as well as stimulation and repression of a variety of genes whose functional significance remains obscure [6]. NK cells may play a role in controlling brucellosis in pregnant mice and shift from Th2- to Th1-polarized immune response may induce the abortion by *B. abortus* infection.

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REFERENCES

1. Ain, R., Canham, L.N. and Soares, M.J. 2003. Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Dev. Biol.* **260**: 176–190.
2. Ashkar, A.A. and Croy, B.A. 1999. Interferon-γ contributes to the normalcy of murine pregnancy. *Biol. Reprod.* **61**: 493–502.
3. Ashkar, A.A. and Croy, B.A. 2001. Functions of uterine natural killer cells are mediated by interferon gamma production during murine pregnancy. *Semin. Immunol.* **13**: 235–241.
4. Ashkar, A.A., Di Santo, J.P. and Croy, B.A. 2000. Interferon γ contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J. Exp. Med.* **192**: 259–269.
5. Athanassakis, I., Papadimitriou, L., Bouris, G. and Vassiliadis, S. 2000. Interferon-γ induces differentiation of ectoplacental cone cells to phenotypically distinct trophoblasts. *Dev. Comp. Immunol.* **24**: 663–672.
6. Boehm, U., Klamp, T., Groot, M. and Howard, J.C. 1997. Cellular responses to interferon-γ. *Annu. Rev. Immunol.* **15**: 749–795.
7. Chaouat, G., Zourbas, S., Ostoij, S., Lappree-Delage, G., Dubanchet, S., Ledee, N. and Matral, J. 2002. A brief review of recent data on some cytokine expressions at the materno-fetal interface which might challenge the classical Th1/Th2 dichotomy. *J. Reprod. Immunol.* **53**: 241–253.
8. Delrue, R.M., Martinez-Lorenzo, M., Lestrate, P., Danese, I., Bielarz, V., Mertens, P., De Bolle, X., Tibor, A., Gorvel, J.P. and Letesson, J.J. 2001. Identification of Brucella spp. genes involved in intracellular trafficking. *Cell. Microbiol.* **3**: 487–497.
9. Detileux, P.G., Deyoe, B.L. and Cheville, N.F. 1990. Entry and intracellular localization of *Brucella* spp. in Vero cells: fluores-

cence and electron microscopy. *Vet. Pathol.* **27**: 317–328.
10. Dorner, B.G., Scheffold, A., Rolph, M.S., Huser, M.B., Kaufmann, S.H.E., Radbruch, A., Flesch, I.E.A. and Kroczek, R.A. 2002. MIP-1α, MIP-1β, RANTES, and ATAC/lymphotactin function together with IFN-γ as type 1 cytokines. *Proc. Natl. Acad. Sci. U.S.A.* 99: 6181–6186.
11. Enright, F.M. 1990. The pathogenesis and pathibiology of *Brucella* infection in domestic animals. pp. 301–320. *In*: Animal Brucellosis (Nielsen, K. and Duncan, J.R. eds.), CRC Press, Boca Raton, FL.
12. Fernandes, D.M. and Baldwin, C.L. 1995. IL-10 downregulates protective immunity to *Brucella abortus*. *Infect. Immun.* **63**: 1130–1133.
13. Fernandes, D.M., Jiang, X., Jung, J.H. and Baldwin, C.L. 1996. Comparison of T cell cytokines in resistant and susceptible mice infected with virulent *Brucella* abortus strain 2308. *FEMS Immunol. Med. Microbiol.* **16**: 193–203.
14. Finlay, B. and Falkow, S. 1997. Common themes in microbial pathogenicity. *Microbiol. Mol. Biol. Rev.* **61**: 136–169.
15. Kim, S., Lee, D.S., Watanabe, K., Furuoka, H., Suzuki, H. and Watarai, M. 2005. Interferon-γ promotes abortion due to *Brucella* infection in pregnant mice. *BMC Microbiol.* **5**: 22.
16. Krishnan, L., Guilbert, L.J., Russell, A.S., Wegmann, T.G., Mosmann, T.R. and Belosevic, M. 1996. Pregnancy impairs resistance of C57BL/6 mice to *Leishmania major* infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines. *J. Immunol.* **156**: 644–652.
17. Muciaccia, B., Padula, F., Vicini, E., Gandini, L., Lenzi, A. and Stefanini, M. 2005. Beta-chemokine receptors 5 and 3 are expressed on the head region of human spermatozoon. *FASEB J.* **19**: 2048–2050.
18. Murphy, E.A., Sathiyaseelan, J., Parent, M., Zou, B. and Baldwin, C.L. 2001. Interferon-γ is critical for surviving a *Brucella abortus* infection in both resistant C57BL/6 mice and susceptible BALB/c mice. *Immunology* **103**: 511–518.
19. Pappas, G., Papadimitriou, P., Akritidis, N., Christou, L. and Tsianos, E.V. 2006. The new global map of human brucellosis. *Lancet Infect. Dis.* **6**: 91–99.
20. Pizarro-Cerda, J., Moreno, E., Sanguedolce, V., Mege, J.L. and Gorvel, J.P. 1998. Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. *Infect. Immun.* **66**: 2387–2392.
21. Raghupathy, R. 1997. Th1 type immunity is incompatible with successful pregnancy. *Immunol. Today* **18**: 478–482.
22. Ramhorst, R., Gutierrez, G., Corigliano, A., Junovich, G. and Fainboim, L. 2007. Implication of RANTES in the modulation of alloimmune response by progesterone during pregnancy. *Am. J. Reprod. Immunol.* **57**: 147–152.
23. Sano, M., Mitsuyama, M., Watanabe, Y. and Nomoto, K. 1986. Impairment of T cell-mediated immunity to *Listeria monocytogenes* in pregnant mice. *Microbiol. Immunol.* **30**: 165–176.
24. Stevens, M.G., Pugh Jr., G.W. and Tabatabai, L.B. 1992. Effects of γ-interferon and indomethacin in preventing *Brucella abortus* infections in mice. *Infect. Immun.* **60**: 4407–4409.
25. Thellin, O., Coumans, B., Zorzi, W., Igout, A. and Herinen, E. 2000. Tolerance to the foeto-placental "graft": ten ways to support a child for nine months. *Curr. Opin. Immunol.* **12**: 731–737.
26. Thirkill, T.L., Lowe, K., Vedagiri, H., Blankenship, T.N., Barakat, A. and Douglas, G. 2005. Macaque trophoblast migration is regulated by RANTES. *Exp. Cell Res.* **305**: 355–364.
27. Tobias, L., Cordes, D.O. and Schurig, G.G. 1993. Placental pathology of the pregnant mouse inoculated with *Brucella abortus* strain 2308. *Vet. Pathol.* **30**: 119–129.
28. Ugalde, R.A. 1999. Intracellular lifestyle of Brucella spp. common genes with other animal pathogens, plant pathogens, and endosymbionts. *Microbes. Infect.* **1**: 1211–1219.
29. Von Hundelshausen, P., Koenen, R.R., Sack, M., Mause, S.F., Andriaens, W., Proudfoot, A.E., Hackeng, T.M. and Weber, C. 2005. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood* **105**: 924–930.
30. Watarai, M., Makino, S-I. and Shirahata, T. 2002. An essential virulence protein of *Brucella abortus*, VirB4, requires an intact nucleoside-triphosphate-binding domain. *Microbiology* **184**: 1439–1446.
31. Watarai, M., Makino, S-I., Michikawa, M., Yanagisawa, K., Murakami, S. and Shirahata, T. 2002. Macrophage plasma membrane cholesterol contributes to *Brucella abortus* infection of mice. *Infect. Immun.* **70**: 4818–4825.
32. Wallach, E. 1999. Chemokines and human reproduction. *Fertil. Steril.* **71**: 983–993.
33. Weinberg, E.D. 1987. Pregnancy-associated immune suppression: risks and mechanisms. *Microb. Pathog.* **3**: 393–397.
34. Wegmann, T.G., Lin, H., Guilbert, L. and Mosmann, T.R. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol. Today* **14**: 353–356.
35. Zhan, Y. and Cheers, C. 1993. Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. *Infect. Immun.* **61**: 4899–4901.

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Heat shock cognate protein 70 contributes to *Brucella* invasion into trophoblast giant cells that cause infectious abortion

Kenta Watanabe[1,2], Masato Tachibana[1,2], Satoshi Tanaka[3], Hidefumi Furuoka[4], Motohiro Horiuchi[5], Hiroshi Suzuki[2,6] and Masahisa Watarai*[1]

Address: [1]Department of Veterinary Public Health, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan, [2]Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, [3]Department of Animal Resource Science and Veterinary Medical Science, the University of Tokyo, Tokyo, Japan, [4]Department of Veterinary Medical Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, [5]Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan and [6]Department of Development and Medical Technology, the University of Tokyo, Tokyo, Japan

Email: Kenta Watanabe - s11042@st.obihiro.ac.jp; Masato Tachibana - s13028@st.obihiro.ac.jp; Satoshi Tanaka - asatoshi@mail.ecc.u-tokyo.ac.jp; Hidefumi Furuoka - furuoka@obihiro.ac.jp; Motohiro Horiuchi - horiuchi@vetmed.hokudai.ac.jp; Hiroshi Suzuki - hisuzuki@obihiro.ac.jp; Masahisa Watarai* - watarai@yamaguchi-u.ac.jp

* Corresponding author

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Abstract

Background: The cell tropism of *Brucella abortus*, a causative agent of brucellosis and facultative intracellular pathogen, in the placenta is thought to be a key event of infectious abortion, although the molecular mechanism for this is largely unknown. There is a higher degree of bacterial colonization in the placenta than in other organs and many bacteria are detected in trophoblast giant (TG) cells in the placenta. In the present study, we investigated mechanism of *B. abortus* invasion into TG cells.

Results: We observed internalization and intracellular growth of *B. abortus* in cultured TG cells. A monoclonal antibody that inhibits bacterial internalization was isolated and this reacted with heat shock cognate protein 70 (Hsc70). Depletion and over expression of Hsc70 in TG cells inhibited and promoted bacterial internalization, respectively. IFN-γ receptor was expressed in TG cells and IFN-γ treatment enhanced the uptake of bacteria by TG cells. Administering the anti-Hsc70 antibody to pregnant mice served to prevent infectious abortion.

Conclusion: *B. abortus* infection of TG cells in placenta is mediated by Hsc70, and that such infection leads to infectious abortion.

Background

Brucellosis is a widespread and economically important infectious disease of animals and humans caused by members of the genus *Brucella*. *Brucella* spp. are small gram-negative, facultative intracellular pathogens that cause abortion, retained placenta and infertility in numerous domestic and wild mammals, and a disease known as undulant fever in humans [1-3]. Transmission of *Brucella* spp. from infected animals to humans may be either direct or indirect. Direct transmission involves the respiratory, conjunctival and cutaneous routes, and is more important in people in close contact with infected animals. Indirect

transmission is through the consumption of contaminated dairy products [3]. *Brucella* spp. occasionally causes spontaneous abortion in pregnant women [4].

There have been several histological studies on the placentas of *Brucella* infected animals [5]. Further, it has been found that *Brucella* internalizes into the caprine erythrophagocytic trophoblastic epithelial cells from the maternal circulation [6] and that the internalized bacteria replicate within the rough endoplasmic reticulum, resulting in secondary infection of adjacent trophoblastic epithelial cells [6,7]. Researches have also shown that after necrosis of infected trophoblasts, large numbers of brucellae are released, and proximity of the fetal capillaries in the ulcerated placenta to the lumenal bacteria has been proposed as the source of the fetal bacteremia and further placental infection [6,8]. However, the molecular mechanism of abortion induced by *Brucella* spp. remains unknown.

The mouse model, particularly that using the unpregnant mouse, has been used extensively to study some aspects of the pathogenesis of brucellosis [2]. While brucellosis is known to primarily affect the reproductive tract in the natural host, little is known regarding the cellular and molecular mechanisms of *Brucella* infection in the pregnant mouse [9]. Although the structure of bovine placenta is completely different from mouse placenta, the infectious abortion model using the pregnant mouse is a powerful tool for investigating the mechanisms of *Brucella* pathogenesis. In our previous study, we demonstrated that *B. abortus* causes abortion in pregnant mice by inoculating bacteria on day 4.5 of gestation [10]. 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 an intracellular replication-defective mutant did not induce abortion. These findings suggest that bacterial infection of TG cells plays a key role in abortion induced by *B. abortus* infection.

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 [11-13]. This immunosuppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents [14,15]. Our previous study showed that a transient increase in interferon (IFN)-γ due to *Brucella* infection contributes to abortion in pregnant mice [10]. In addition to examining the balance of inflammatory and regulatory cytokines in bacteria infected pregnant mice, analysis of bacterial internalization into the TG cells, a specific host cells in placenta, will help to advance our knowledge regarding the control of *Brucella*-induced abortion.

In the present study, we investigated the internalization of *B. abortus* into TG cells and identified heat shock cognate protein 70 (Hsc70) as a candidate receptor against *Brucella* or bacterial uptake-associated molecule. We noted that IFN-γ enhances bacterial internalization into TG cells.

Methods

Bacterial strains

All *B. abortus* derivatives were from 544 (ATCC23448) smooth virulent *B. abortus* biovar 1 strains. GFP expressed 544 strain was used in this study [16,17]. *B. abortus* strains were maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson) or Brucella broth containing 1.5% agar.

Mice

Six to ten-week-old ICR female mice were individually mated to 6- to 10-week-old ICR male mice. The parent mice were obtained from CLEA Japan. Day 0.5 of gestation was the day the vaginal plug was observed. The normal gestational time for these mice is 19 days.

Virulence in pregnant mice

Groups of five pregnant mice were infected intraperitoneally with approximately 10^4 colony forming unit (CFU) of brucellae in 0.1 ml saline on day 4.5 of gestation [10]. On day 18.5 of gestation, placenta and spleen were removed and homogenized in phosphate buffered saline (PBS). 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. 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 (MEFs)-conditioned medium as described previously [18]. The TS medium was prepared by adding 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine to RPMI 1640. To induce differentiation to trophoblast giant (TG) cells, the cells were cultured in the only TS medium alone for 3 days at 37°C in CO_2 incubator. The TG cells were seeded ($1-2 \times 10^5$ per well) in 48 well tissue culture plates for all assays.

Efficiency of bacterial internalization and replication within cultured cells

Bacterial infection and intracellular survival assays were performed according to a modified version of the method of Kim *et al* [19]. *B. abortus* strains were deposited onto TS or TG cells at a multiplicity of infection (MOI) of 100 which had been grown on 48-well microtiter plates containing TS medium but no antibiotics by centrifugation at 150 × g for 10 min at room temperature. To measure bacterial internalization efficiency after 30 min of incubation at 37°C, the cells were washed once with TS medium and then incubated with TS medium containing gentamicin (30 μg/ml) for 30 min. Next, cells were washed three times with PBS and lysed with cold distilled water. CFU values were determined by serial dilution on Brucella plates. To measure intracellular replication efficiency, infected cells were incubated at 37°C for 30 min, washed once with TS medium and then incubated with TS medium containing gentamicin (30 μg/ml) for 2, 24, 48 and 72 h. The cell washing, lysis and plating procedures were the same as for the bacterial internalization efficiency assay. Percentage protection was determined by dividing the number of bacteria surviving by the number in the infectious inoculum. The purified the R2–25 antibody or recombinant IFN-γ (Cedarlane Laboratories) was added to the TS medium at the indicated concentrations 2 or 12 h before infection.

F-actin staining

GFP-expressing bacteria were deposited onto the cultured cells by centrifugation and the incubation was conducted at 37°C for 30 min. The infected cells were incubated with TS medium containing gentamicin (30 μg/ml) at 37°C for 30 min to kill extracellular bacteria and were then fixed in 4% paraformaldehyde for 30 min at room temperature. Next, samples were permeabilized in 0.2% Triton X-100, washed three times with PBS and incubated with Alexa Fluor 594-phalloidin (Molecular Probes) at 20 μg/ml for 30 min at 37°C. After three washes with PBS, samples were placed in mounting medium (90% glycerol containing 1 mg/ml phenylenediamene in PBS, pH 9.0) and visualized by fluorescence microscopy.

Isolation of monoclonal antibodies

Hybridomas producing monoclonal antibodies that inhibit bacterial internalization into TG cells were obtained from fusions of BALB/c P3-X63-Ag8.653 (8-azaguanine-resistant and non-producer cell line) myeloma cells with spleen cells from Wister rats that had been immunized with TG cells. The screening of hybridoma supernatants for inhibiting antibodies was performed by adding antibodies to the TS medium in a bacterial internalization assay. Monoclonal antibodies obtained from hybridoma supernatants were purified using a protein G column (GE Healthcare Life Science) and the class and subclass of the purified monoclonal antibodies were determined using an Immunogloblin Typing Kit (WAKO Pure Chemical). The R2–25 monoclonal antibody used in this study was typed as IgG1.

Subcellular fractionation of TS and TG cells

TS and TG cells (3×10^5/ml) were seeded into each well of a 6-well plate. Protein isolation for the cytoskeleton, nuclear, membrane, and cytosol fraction was performed using a ProteoExtract Subcellular Proteome Extraction Kit as described by the manufacturer (Calbiochem).

Immunoblotting

The cell lysates (500 μg/ml) and fractionated proteins (50 μg/ml) were separated on 10% polyacrylamide gels and transferred to a PVDF membrane, which was incubated for 1 h at room temperature with primary antibody (0.5 μg/ml) in 5% skim milk. It was 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 at 0.01 μg/ml and then washed again. Immunoreactions were visualized by ECL (GE Healthcare Life Science). Antibodies for β-actin, β-tublin and histone H1 were purchased from SIGMA or Abcam. Anti-IFN-γ receptor rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology.

Mass spectrometry analysis

Identification of proteins reacting with monoclonal antibodies that inhibited bacterial internalization into TG cells was conducted by means of nano LC-MS/MS analysis and a search of MASCOT database (APRO life Science Institute, Japan).

RNA isolation and RT-PCR

The total RNA of TG cells was isolated using an RNA Purification Kit (Qiagen) and purified RNA samples were stored at -30°C until use. The RNA was quantified by absorption at 260 nm using a SmartSpec3000 spectrophotometer (Bio-Rad). RT-PCR was carried out using a Sperscript II Kit (Invitrogen). The primers used for mouse Hsc70 or β-actin amplification had the following sequence 5'-GCAGCTGGGCCTACACACAAG-3' and 5'-CCCTGTGGAACAAAGCTACAC-3', or 5'-CGTGACAT-TAAGGAGAAGCTGTGC-3' and 5'-CTCAGGAGGAG-CAATGATCTTGAT.

Expression and purification of recombinant proteins

Mouse Hsc70 cDNA (GenBank Accession No. BC066191) was amplified from RNA isolated from TG cells by means of RT-PCR with the pair of primers described above. The product was cloned into the pCR2.1-TOPO vector (Invitrogen) (pCR-Hsc70). To achieve expression of recombinant Hsc70 protein, amplified DNA encoding Hsc70 from pCR-Hsc70 in PCR was cloned into pCold TF vector

(Takara Bio Inc.). The His-tagged Hsc70 was expressed in the *E. coli* strain DH5α, and its purification and cleavage of His-tagged by HRV 3C protease were performed as described by the manufacturer (Novagen). Bovine Hsc70 and the rat anti-Hsc70 monoclonal antibody (SPA-815) were obtained from Stressgen for use as control materials.

To achieve expression of Hsc70 in TG cells, amplified DNA encoding Hsc70 from pCR-Hsc70 in PCR was cloned into the pcDNA4/TO vector in the T-Rex System (Invitrogen). pcDNA4/TO-Hsc70 was transfected into TG cells using the FuGENE 6 Transfection Reagent (Roche) with a final concentration of 1.2 μg/ml.

siRNA experiment

The siRNA duplexes used for silencing mouse Hsc70 (target sequence: AACAAGTAACATGGAATAATA), and β-actin (target sequence: CACTGACTTGAGACCAATAAA) and AllStars Negative Control siRNA were purchased from QIGEN. TG cells were transiently transfected using oligofectamine (Invitrogen) with or without a final concentration of 10 nM for siRNAs.

Immunofluorescence microscopy

Samples grown on coverslips were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and permeabilized with or without 0.2% Triton X-100 in PBS for 20 min at room temperature. After blocking with 5% BSA in PBS, the cells were incubated with primary antibody (25 μg/ml) for 1 h at 37°C, and detection was conducted with TRITC-labeled goat anti-rat IgG (0.01 μg/ml) (Chemicon). Fluorescent images were taken using an Olympus BX51 microscope and a cooled CCD camera Olympus DP70.

In vivo depletion of Hsc70

Hsc70 was neutralized in the mice by administering an anti-mouse Hsc70 monoclonal antibody (clone R2-25)*in vivo* using 100 or 200 μg of antibody in a volume of 0.3 or 0.6 ml intraperitoneally 24 h before infection. Control mice were given 100 μg of normal rat IgG in a volume of 0.1 ml according to the same injection schedule as used for the anti-Hsc70 monoclonal antibody treated groups. Bacterial infection was conducted as described previously. On day 18.5 of gestation, fetuses were removed from the mice and a judgment made as to whether they were pregnant or not. Fetuses were determined to be alive if there was a heartbeat, and dead if there was no heartbeat.

Statistical analysis

All statistical analysis was conducted using the Student *t* test.

Results

B. abortus internalizes and replicates in trophoblast giant cells

We previously reported that there were many bacteria in trophoblast giant (TG) cells in the placenta by inoculation of pregnant mice with *B. abortus* [10]. To examine this bacterial infection into TG cells further, we used *in vitro* cell culture system of trophoblast stem (TS) cells and TG cells differentiated from TS cells. The *B. abortus* internalized into TG cells more efficiently than TS cells (Fig. 1A). We also investigated the intracellular replication of *B. abortus* in TS and TG cells. The bacteria replicated more efficiently in TG cells than TS cells (Fig. 1B).

Several intracellular pathogens attached to the host plasma membrane induce actin polymerization around the site of bacterial attachment and the process is essential for bacterial entry [20]. We therefore examined actin polymerization by means of fluorescence microscopy after 30 min and 48 h of incubation of TS and TG cells infected with *B. abortus*. It has been noted that differentiated TG cells dramatically rearrange their actin cytoskeleton into thick bundles of stress fibers [21]. There was no apparent actin polymerization around the site of the bacterial entry after 30 min incubation on TG cells or 48 h of incubation of infected TG cells (Fig. 1C).

Isolation of monoclonal antibodies that inhibit bacterial internalization into TG cells

To identify a receptor against *B. abortus* on TG cells, rats were immunized with TG cells, and monoclonal antibodies that inhibit bacterial internalization into TG cells were isolated. Seventy-five clones of bacterial internalization inhibiting antibodies (3.9%) were isolated from 1,920 hybridoma supernatants. From among the monoclonal antibodies, we selected R2-25, since it significantly inhibited internalization and showed clear reactions with protein in immunoblotting. The purified R2-25 antibody significantly inhibited bacterial internalization concentration dependently, but there was no inhibition with rat IgG (negative control) (Fig. 2A). The R2-25 antibody reacted with protein of around 70 kDa which was localized in membrane and cytosol, with the protein amount in the membrane fraction being especially large (Fig. 2C). On examining the distribution of protein reacting with R2-25 on the surface of TG cells by immunofluorescence microscopy, we observed an islet-like distribution on the surface of TG cells which was no permeabilized cells (Fig. 2B). It was difficult to detect the protein reacting with the R2-25 antibody on the surface of permeabilized cells.

Antibody inhibiting bacterial internalization reacts with heat shock cognate protein 70

We performed mass spectrometry analysis to identify the protein reacting with the R2-25 antibody. Proteins of the

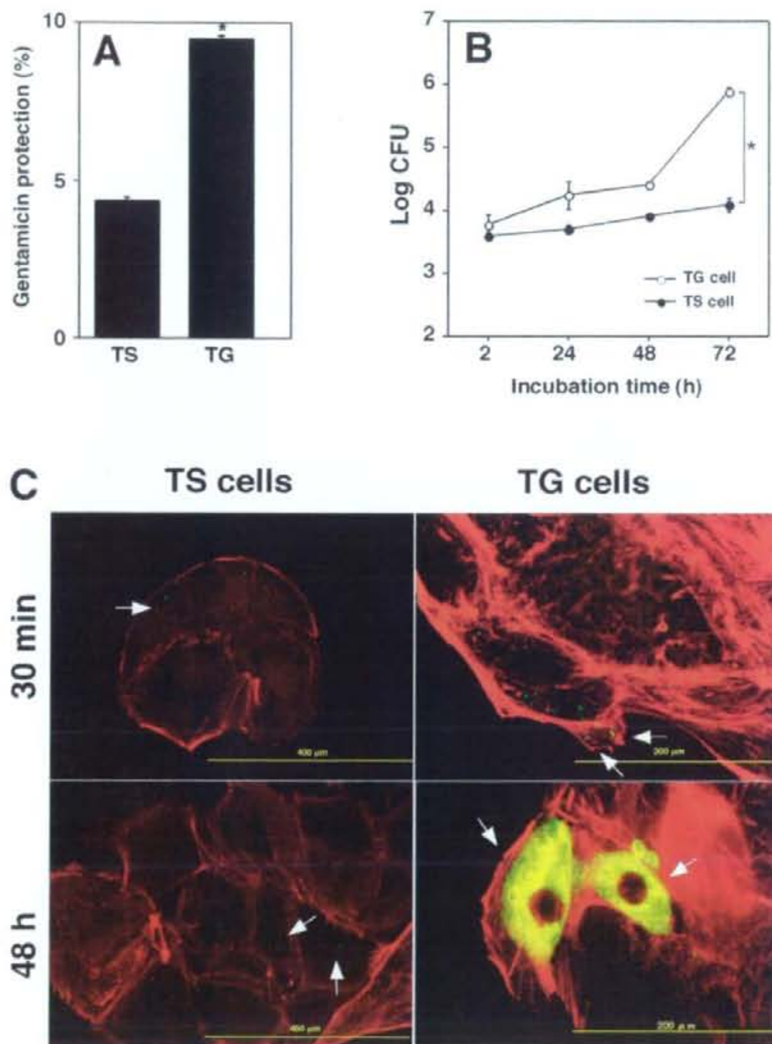

Figure 1

***B. abortus* infection in trophoblast giant cells**. (A) Bacterial internalization into trophoblast stem (TS) and giant (TG) cells. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant difference between the bacterial internalization into TS and TG cells is indicated by asterisk (*, $P < 0.01$). (B) Intracellular replication of *B. abortus* in TS and TG cells. Datum points and error bars represent the mean of CFU of triplicate samples from a typical experiment (performed at least four times) and their standard deviations. Statistically significant difference between bacterial replication of *B. abortus* in TS and TG cells after 72 h of inoculation is indicated by asterisk (*, $P < 0.01$). (C) F-actin staining of bacteria infected cells. The figure shows GFP (bacteria) and Alexa Fluor 594 (actin filaments) channel merged images. Arrows indicate TS and TG cells containing bacteria.

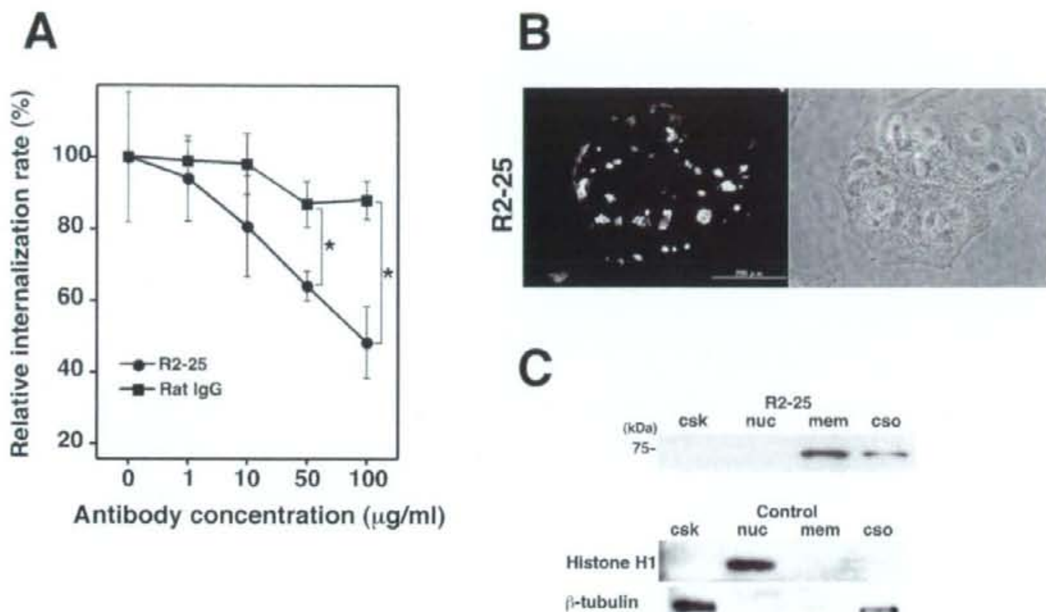

Figure 2

Characterization of the monoclonal antibody that inhibits bacterial internalization into TG cells. (A) Inhibition of bacterial internalization by the R2–25 antibody treatment. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization into TG cells treated with the R2–25 antibody and those treated with rat IgG are indicated by asterisks (*, $P < 0.01$). (B) Distribution of protein reacting with monoclonal antibodies in TG cells. The left panels show fluorescence microscopy of the antibody stained TG cells and the right panels phase contrast microscopy of the corresponding microscopic fields. (C) Immunoblot analysis was performed on TG cell subcellular fractions with the monoclonal antibodies R2–25. Cells were fractionated to cytoskeleton (csk), nucleus (nuc), membrane (mem) and cytosol (cso). The anti-histone H1 and anti-β-tubulin antibody were used for fraction control for the nucleus and cytoskeleton.

membrane fraction were separated by SDS-PAGE and transferred to a PVDF membrane (Fig. 3A). The protein reacting with R2–25 was extracted from the PVDF membrane and the subjected to LC-MS/MS analysis. Through a search of the MASCOT database, the protein reacting with R2–25 was determined to be heat shock cognate protein 70 (Hsc70) (Fig. 3D). The anti-Hsc70 antibody also reacted with this protein (Fig. 3A). To confirm that the R2–25 antibody reacted with Hsc70, we tested its reactivity with mouse and bovine recombinant Hsc70. As expected, the R2–25 and anti-Hsc70 antibodies reacted with both types of recombinant Hsc70 (Fig. 3B and 3C). The mouse recombinant Hsc70 had a slightly greater molecular weight than the bovine recombinant Hsc70 because 25 amino acid residues had been added to the former after HRV 3C protease cleavage. These results indicate that the protein reacting with R2–25 was Hsc70.

Hsc70 contributes to bacterial internalization into TG cells

To examine the effect of Hsc70 on bacterial internalization into TG cells further, we reduced the amount of endogenous Hsc70 by transfecting Hsc70-specific small interfering RNA (siRNA) duplexes into the TG cells. After 48 h of transfection with Hsc70-specific siRNA, the expression level of Hsc70 was no longer detectable, but was not affected by transfection with β-actin or the control siRNA (Fig. 4A and 4D). Thus, the internalization efficiency of *B. abortus* into TG cells was significantly reduced by transfection with Hsc70-specific siRNA (Fig. 4C). Next, excessive production of endogenous Hsc70 was induced

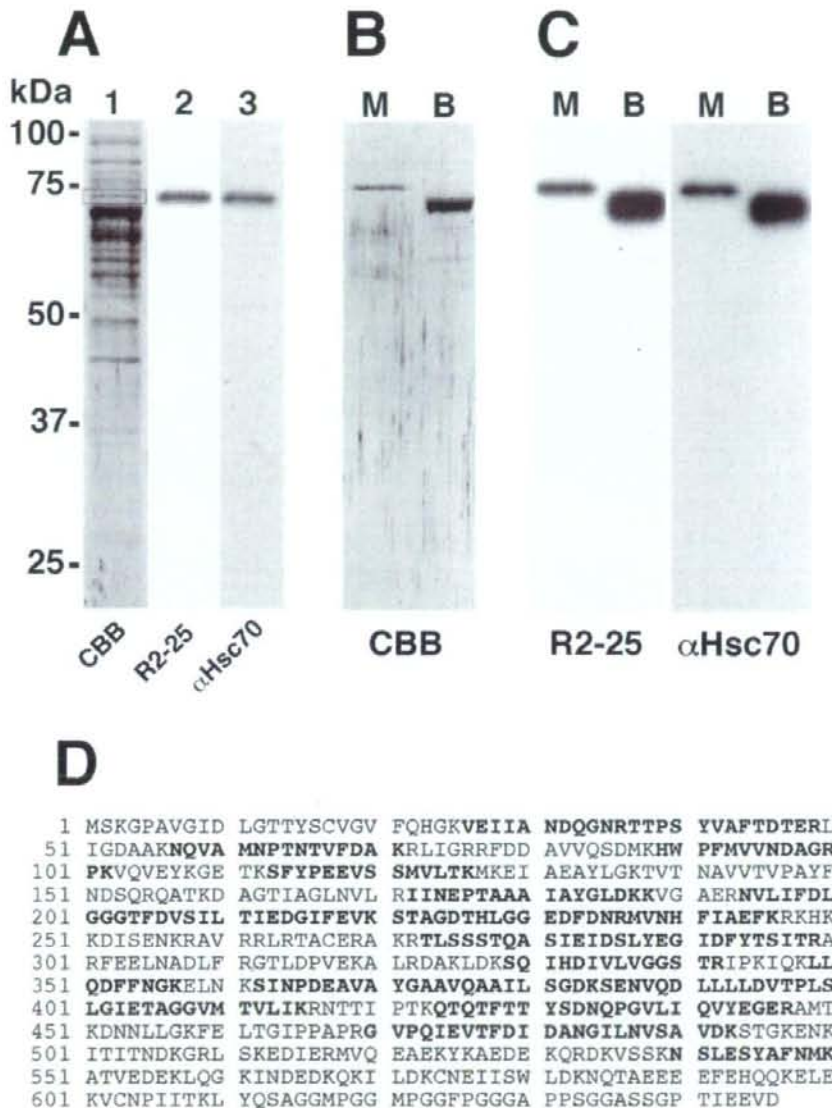

Figure 3

Bacterial internalization inhibiting antibodies react with Hsc70. (A) A membrane to which TG cell membrane fraction proteins had been transferred was stained with Coomassie brilliant blue (CBB) (lane 1), immunoblotted with the R2–25 antibody (lane 2) and the anti-Hsc70 antibody (clone SPA-815) (lane 3). (B) CBB staining of membrane to which mouse (M) or bovine (B) recombinant Hsc70 had been transferred. (C) Immunoblot analysis of mouse (M) or bovine (B) recombinant Hsc70 with R2–25 antibody and anti-Hsc70 antibody (clone SPA-815). (D) Amino acid sequences of Hsc70. The protein reacting with R2–25 was extracted from the PVDF membrane (square in lane 1 of panel A) and was used for LC-MS/MS analysis. The amino acids detected for Hsc70 in the analysis are shown in bold.

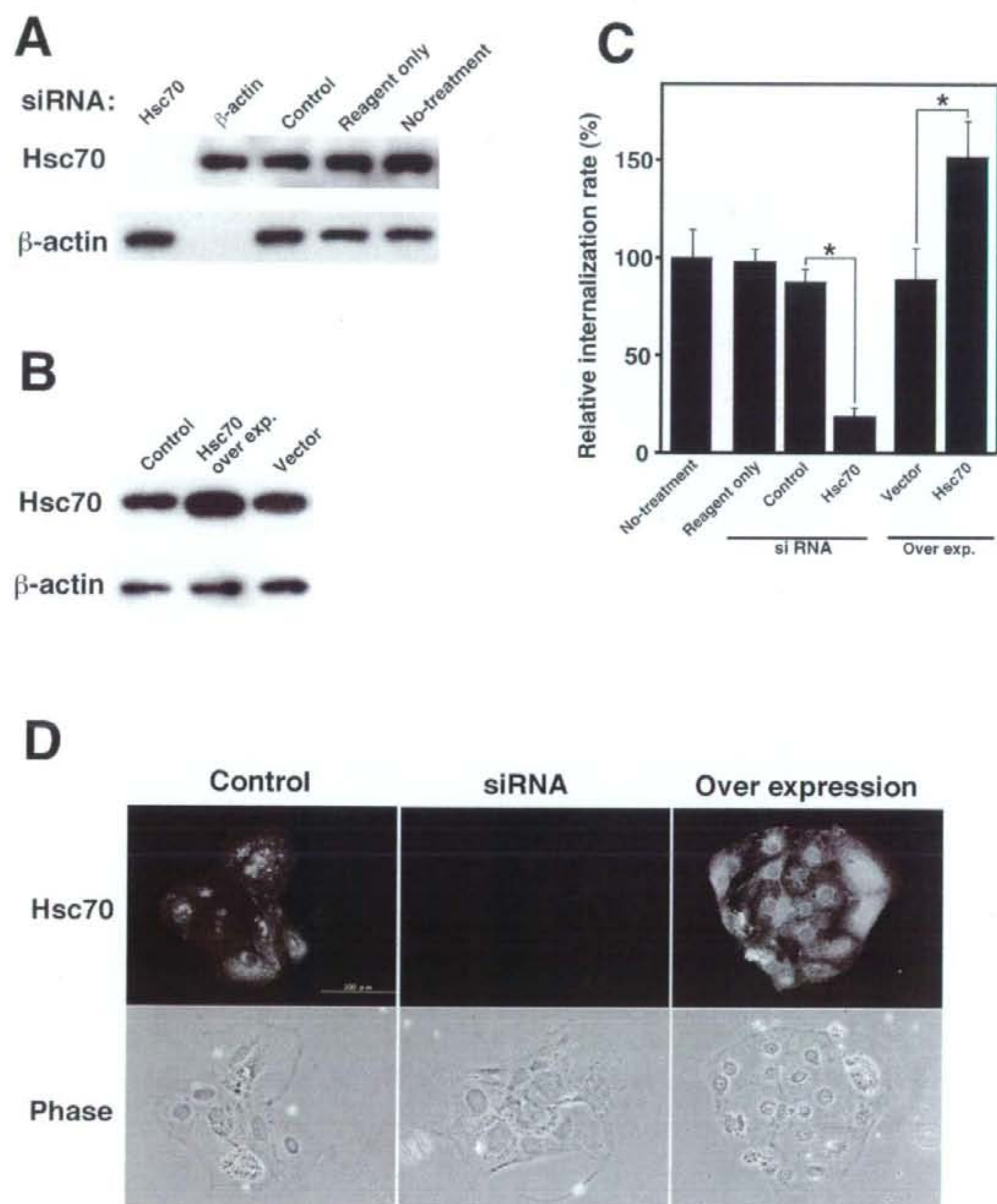

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
Effect of depletion and over expression of Hsc70 in TG cells on bacterial internalization. (A) Depletion of Hsc70. TG cells were treated for 48 h with siRNA targeting Hsc70 or without it (reagent only or no treatment), or β-actin or the control (QIAGEN AllStars Negative Control). Expression of the indicated proteins was monitored by immunoblotting. β-actin was used as an internal control. (B) Over expression of Hsc70. TG cells were transfected with or without (control) pcDNA4/TO-Hsc70 or vector only. (C) Bacterial internalization into Hsc70 depleted (siRNA) or over expressed (over exp.) TG cells was studied in a bacterial internalization assay. Lanes correspond to panels A and B. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization into TG cells with (Hsc70) and without siRNA (control), and over expression and the control (vector) are indicated by asterisks (*, $P < 0.01$). (D) Distribution of Hsc70 in non-treated (control), Hsc70 depleted (siRNA), or over expressed (over expression) TG cells. Fluorescence microscopy of stained TG cells with the R2–25 antibody (upper panels) and phase contrast microscopy of the corresponding microscopic fields (lower panels) are shown.

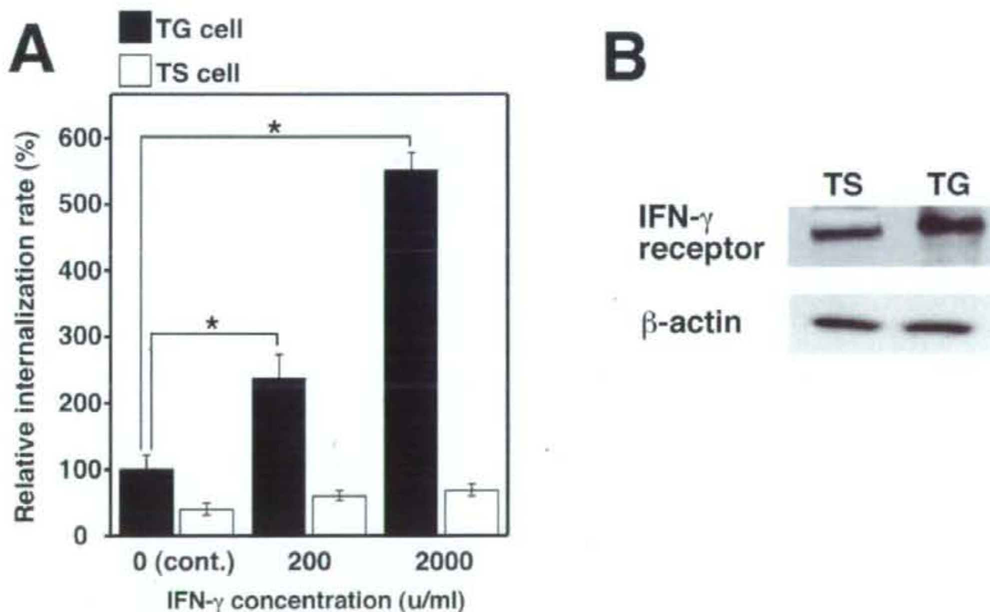

Figure 5
IFN-γ promotes bacterial internalization into TG cells. (A) Bacterial internalization into IFN-γ treated TS or TG cells. *B. abortus* was deposited onto TS and TG cells which were treated with or without (cont.) IFN-γ at the indicated concentrations. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization in TG cells with and without IFN-γ treatment are indicated by asterisks (*, $P < 0.01$). (B) Expression of IFN-γ receptor in TS and TG cells. Immunoblot analysis was performed with anti-IFN-γ receptor and anti-β-actin rabbit polyclonal antibody.

by transfecting the Hsc70 expression vector into TG cells. After 48 h, expression levels of Hsc70 were significantly higher than the control levels (Fig. 4B and 4D). The internalization efficiency of *B. abortus* into TG cells in which Hsc70 was over-expressed was significantly higher than the control levels (Fig. 4C).

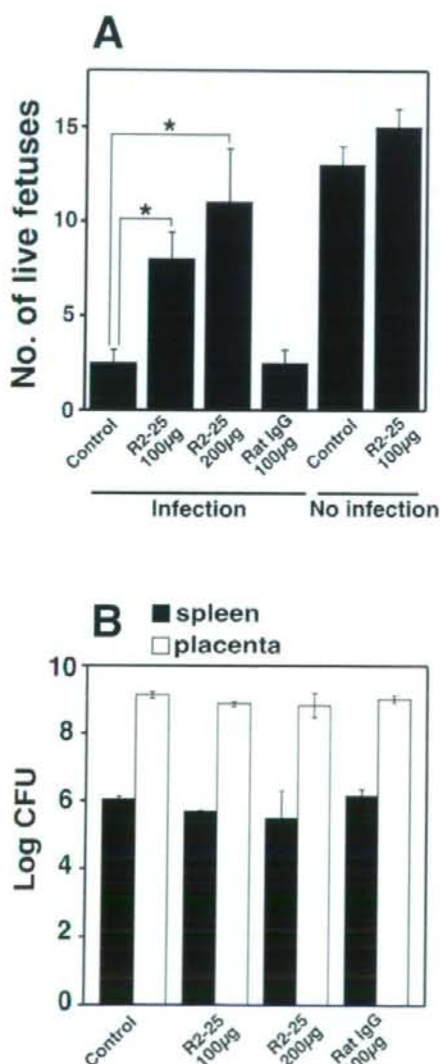

Figure 6

Figure 6

Preventing abortion by inoculating with anti-Hsc70 antibody. (A) Number of live fetuses. Hsc70 was neutralized in the mice by administering with or without (control) an anti-mouse Hsc70 monoclonal antibody (clone R2–25)*in vivo* using 100 or 200 μg of the antibody. The control mice were given 100 μg of normal rat IgG. Statistically significant differences between the untreated control and antibody treated mice are indicated by asterisks (*, $P < 0.01$). (B) Bacterial numbers in spleen and placenta. On day 18.5 of gestation, the placenta and spleen were removed and homogenized in PBS. Tissue homogenates were serially diluted with PBS and plated on Brucella agar in order to count the number of CFU in each organ.

IFN-γ enhances bacterial uptake by TG cells

Since a transient increase in IFN-γ brought about by *Brucella* infection promotes abortion in pregnant mice [10], we investigated the effect of IFN-γ treatment on bacterial internalization and Hsc70 expression in TG cells. IFN-γ treatment significantly increased the internalization efficiency of *B. abortus* into TG cells as their concentration, but had no effect in TS cells (Fig. 5A). To determine whether the enhancement of bacterial internalization by IFN-γ treatment was due to up-regulate Hsc70 expression or not, RNA was isolated from IFN-γ treated TG cells and subjected to RT-PCR. This showed that IFN-γ treatment did not affect Hsc70 expression (data not shown). IFN-γ receptor was expressed in TS and TG cells (Fig. 5B).

Preventing abortion by inoculating pregnant mice with anti-Hsc70 antibody

To determine if abortion is prevented by neutralizing the Hsc70 expressed on TG cells in the mouse placenta, pregnant mice were inoculated with the R2–25 antibody 24 h before infection with *B. abortus*, which was done on day 4.5 of gestation. While there was no change in the number of abortions observed in the non-inoculated mice, there was a significant increase in number of live fetuses in the inoculated mice (Fig. 6A). Inoculation of uninfected pregnant mice with the R2–25 antibody did not affect on pregnancy (Fig. 6A). Upon examining bacterial numbers in the spleen and placenta of infected pregnant mice, it was found that bacterial numbers were similar in both mice inoculated with the R2–25 antibody and those not inoculated with it (Fig. 6B).

Discussion

Previous mouse model studies have shown that *Brucella abortus* specifically replicates in trophoblast giant (TG) cells in the placenta [9,10]. TG cells are polyploid cells that play a crucial role in implantation, in remodeling of the embryonic cavity, and preventing maternal blood

flow to the implantation site [22]. Since *B. abortus* internalizes into TG cells and replicates in them, cell functions are not exhibited completely, which leads to abortion since implantation and placental development are inhibited. Therefore, it is thought that bacterial infection of TG cells is a key event in inducing abortion. To analyze the molecular mechanisms of *B. abortus* infection of TG cells *in vitro*, we used trophoblast stem (TS) cells and TG cells differentiated from TS cells for the infection assay in this study. Although TG cell differentiation is fairly well understood at the morphological and molecular level [23], the role of immune responses in fighting against pathogens of TG cells is poorly understood and in this regard a model of host-pathogen interaction using TG cells would be useful for obtaining new information of the effect of TG cell functions on pregnancy.

Hsc70 has been reported to be present on the surface of several types of cells [24]. In this regard, though Hsc70 congregates on the surface of TG cells, it is present to a much lesser extent on the surface of TS cells (data not shown). This may be a reason that the internalization of *B. abortus* into TG cells was greater than that into TS cells. As Hsc70 and many other factors will be present on TG cells differentiated from TS cells, there is a possibility that other receptors or bacterial uptake-associated molecules may contribute to *B. abortus* infection of TG cells. Little is known about how Hsc70, a protein with no signal sequence for secretion, exits cells by mechanisms other than escape from cells undergoing necrotic lysis. In previous studies, Hsc70 has seen to be released from a late endsomal lysosomal location where it participates in protein degradation [25,26]. Further, the secretion of the Hsp70 family and its association with lipid rafts have also been observed in epithelial cells under normal conditions, and a lipid raft-based mechanism has been suggested for the membrane delivery and release of Hsp70 family [27]. Although receptors for the extracellular Hsp70 family have still not been fully defined, several cell surface receptors have been suggested, such as CD14, CD40, CD91 and scavenger receptor Lox-1 [28-31]. Since it has also been noted that class A scavenger receptor (SR-A) contributes to *B. abortus* infection in macrophages [32], SR-A may be receptors for Hsc70, and the mechanism for *B. abortus* internalization into TG cells may be the same pathway as that for Hsc70 uptake by TG cells. Hsc70 may have a function that is catching antigens and anti-Hsc70 would inhibit binding between Hsc70 and antigens. IFN-γ treatment enhanced bacterial internalization into TG cells and these observations agreed with results obtained in pregnant mice model [10], and thus expression of unidentified receptors against Hsc70 may be upregulated by IFN-γ treatment. IFN-γ should therefore promote internalization of *B. abortus* into TG cells *in vivo* and this would be one of ways in which infectious abortion is induced.

Conclusion

The finding of this study that the anti-Hsc70 antibody prevents abortion caused by *B. abortus* infection is expected to be applied in the development of methods of preventing abortion. Since intracellular bacteria such as *Brucella* replicate in host cells, it is difficult to completely eliminate them from the host through treatment with antibiotics and develop effective vaccines against them. An alternative strategy in treating infection due to *Brucella* would be inhibition of bacterial internalization into TG cells and this could be an effective means of protecting against abortion due to brucellosis. Recently, Carvalho Neta *et al.* reported that *B. abortus* modulates innate immune response by bovine trophoblastic cells [33]. Although the structure of bovine placenta is completely different from mouse placenta, bovine and mouse trophoblastic cells may have similar function in the immune system. However, it is not known whether the mechanism of host-pathogen interaction observed in this study could be used to develop protective methods against other abortion-inducing pathogen infections, and thus further analysis of TG cell function in the immune system will be needed to clarify host defense mechanisms in the placenta and those contributing to the success of pregnancy.

Authors' contributions

MW conceived the study. MW, HS and KW designed the experiments, interpreted the results and worked on the manuscript. KW and MT carried out most of the experimental work. ST, HF and MH participated in cell culture and pathological experiments. HS and MW participated in animal experiments. All authors read and approved the final manuscript.

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References

1. Givens MD: **A clinical, evidence-based approach to infectious causes of infertility in beef cattle.** *Theriogenology* 2006, **66**:648-54.
2. Ko J, Splitter GA: **Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans.** *Clin Microbiol Rev* 2003, **16**:65-78.
3. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV: **The new global map of human brucellosis.** *Lancet Infect Dis* 2006, **6**:91-99.
4. Khan MY, Mah MW, Memish ZA: **Brucellosis in pregnant women.** *Clin Infect Dis* 2001, **32**:1172-1177.
5. Samartino LE, Enright FM: **Pathogenesis of abortion of bovine brucellosis.** *Comp Immunol Microbiol Infect Dis* 1993, **16**:95-101.
6. Anderson TD, Cheville NF, Meador VP: **Pathogenesis of placentitis in the goat inoculated with *Brucella abortus*. II. Ultrastructural studies.** *Vet Pathol* 1986, **23**:227-239.
7. Meador VP, Deyoe BL: **Intracellular localization of *Brucella abortus* in bovine placenta.** *Vet Pathol* 1989, **26**:513-515.

8. Anderson TD, Meador VP, Cheville NF: **Pathogenesis of placentitis in the goat inoculated with *Brucella abortus*. I. Gross and histologic lesions.** *Vet Pathol* 1986, **23**:219-226.
9. Tobias L, Cordes DO, Schurig GG: **Placental pathology of the pregnant mouse inoculated with *Brucella abortus* strain 2308.** *Vet Pathol* 1993, **30**:119-129.
10. Kim S, Lee DS, Watanabe K, Furuoka H, Suzuki H, Watarai M: **Interferon-γ promotes abortion due to *Brucella* infection in pregnant mice.** *BMC Microbiol* 2005, **5**:22.
11. Raghupathy R: **Th1-type immunity is incompatible with successful pregnancy.** *Immunol Today* 1997, **18**:478-482.
12. Wegmann TG, Lin H, Guilbert L, Mosmann TR: **Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon?** *Immunol Today* 1993, **14**:353-356.
13. Weinberg ED: **Pregnancy-associated immune suppression: risks and mechanisms.** *Microb Pathog* 1987, **3**:393-397.
14. Krishnan L, Guilbert LJ, Russell AS, Wegmann TG, Mosmann TR, Belosevic M: **Pregnancy impairs resistance of C57BL/6 mice to *Leishmania major* infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines.** *J Immunol* 1996, **156**:644-652.
15. Sano M, Mitsuyama M, Watanabe Y, Nomoto K: **Impairment of T cell-mediated immunity to *Listeria monocytogenes* in pregnant mice.** *Microbiol Immunol* 1986, **30**:165-176.
16. Watarai M, Makino S, Shirahata T: **An essential virulence protein of *Brucella abortus*, VirB4, requires an intact nucleoside-triphosphate-binding domain.** *Microbiology* 2002, **148**:1439-1446.
17. Watarai M, Makino S, Fujii Y, Okamoto K, Shirahata T: **Modulation of *Brucella*-induced macropinocytosis by lipid rafts mediates intracellular replication.** *Cell Microbiol* 2002, **4**:341-355.
18. Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J: **Promotion of trophoblast stem cell proliferation by FGF4.** *Science* 1998, **282**:2072-2075.
19. Kim S, Watarai M, Kondo Y, Erdenebaatar J, Makino S, Shirahata T: **Isolation and characterization of mini-Tn5Km2 insertion mutants of *Brucella abortus* deficient in internalization and intracellular growth in HeLa cells.** *Infect Immun* 2003, **71**:3020-3027.
20. Pizarro-Cerda J, Cossart P: **Bacterial adhesion and entry into host cells.** *Cell* 2006, **124**:715-727.
21. Parast MM, Aeder S, Sutherland AE: **Trophoblast giant-cell differentiation involves changes in cytoskeleton and cell motility.** *Dev Biol* 2001, **230**:43-60.
22. Cross JC: **Genetic insights into trophoblast differentiation and placental morphogenesis.** *Semin Cell Dev Biol* 2000, **11**:105-113.
23. Cross JC: **How to make a placenta: mechanisms of trophoblast cell differentiation in mice-a review.** *Placenta* 2005, **26(Suppl A)**:S3-9.
24. Multhoff G, Hightower LE: **Cell surface expression of heat shock proteins and the immune response.** *Cell Stress Chaperones* 1996, **1**:167-176.
25. Isenman LD, Dice JF: **Secretion of intact proteins and peptide fragments by lysosomal pathways of protein degradation.** *J Biol Chem* 1989, **264**:21591-621596.
26. Terlecky SR, Olson TS, Dice JF: **A pathway of lysosomal proteolysis mediated by the 73-kilodalton heat shock cognate protein.** *Acta Biol Hung* 1991, **42**:39-47.
27. Broquet AH, Thomas G, Masliah J, Trugnan G, Bachelet M: **Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release.** *J Biol Chem* 2003, **278**:21601-21606.
28. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK: **HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine.** *Nat Med* 2000, **6**:435-442.
29. Becker T, Hartl FU, Wieland F: **CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes.** *J Cell Biol* 2002, **158**:1277-1285.
30. Delneste Y, Magistrelli G, Gauchat J, Haeuw J, Aubry J, Nakamura K, Kawakami-Honda N, Goetsch L, Sawamura T, Bonnefoy J, Jeannin P: **Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation.** *Immunity* 2002, **17**:353-362.
31. Srivastava P: **Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses.** *Annu Rev Immunol* 2002, **20**:395-425.
32. Kim S, Watarai M, Suzuki H, Makino S, Kodama T, Shirahata T: **Lipid raft microdomains mediate class A scavenger receptor-dependent infection of *Brucella abortus*.** *Microb Pathog* 2004, **37**:11-19.
33. Carvalho Neta AV, Stynen AP, Paixão TA, Miranda KL, Silva FL, Roux CM, Tsolis RM, Everts RE, Lewin HA, Adams LG, Carvalho AF, Lage AP, Santos RL: **Modulation of the bovine trophoblastic innate immune response by *Brucella abortus*.** *Infect Immun* 2008, **76**:1897-907.

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ANIMAL EXPERIMENTATION

Beneficial effect of desialylated erythropoietin administration on the frozen-thawed canine ovarian xenotransplantation

Hiroshi Suzuki · Takako Ishijima · Saori Maruyama · Yoshiko Yanagimoto Ueta · Yasuyuki Abe · Hideki Saitoh

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Abstract

Purpose The main drawback of ovarian cryopreservation followed by transplantation is that a large proportion of follicles are lost after transplantation. Thus, effects of erythropoietin (EPO) and desialylated EPO administration on the frozen-thawed canine ovarian xenotransplantation were examined.

Methods The protective and survival-promoting effects of EPO and desialylated EPO on the follicles of frozen-thawed canine ovaries after transplantation were examined using NOD-SCID mice. Frozen-thawed dog ovarian tissue with 400 U/kg of EPO or asialo EPO was placed into the ovarian bursa.

Results At 4 weeks after the transplantation, the ovaries were removed and subjected to histological examination. The survival rate of early primary follicles was 15.2% in the EPO group and 157.6% in the asialo EPO group, in contrast to 10.1% in the untreated group.

Conclusions These results demonstrate that administration of asialo EPO could be effectively used to enhance the survival of the follicles of transplanted cryopreserved ovaries.

Capsule Administration of asialo EPO enhanced the survival of the follicles of transplanted cryopreserved ovaries.

H. Suzuki (✉) · T. Ishijima · S. Maruyama · Y. Yanagimoto Ueta · Y. Abe
Research Unit for Functional Genomics,
National Research Center of Protozoan Diseases,
Obihiro University of Agriculture and Veterinary Medicine,
Inada, Obihiro 080-8555, Japan
e-mail: hisuzuki@obihiro.ac.jp

H. Saitoh
Chugai Pharmaceutical Co., Ltd.,
Nihonbashimuromachi, Tokyo 103-8324, Japan

Keywords Cryopreservation · Dog · Erythropoietin · Fertility · Follicle · Ovary · Transplantation

Introduction

Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly increased the life expectancy of premenopausal women with cancer, but have also resulted in a growing population of adolescent and adult long-term survivors of malignancies [1] with infertility problems due to induced premature ovarian failure [2]. Several options are currently available to preserve fertility in cancer patients and provide the opportunity for mothering when they have overcome their disease: embryo cryopreservation, oocyte cryopreservation or ovarian tissue cryopreservation [2]. Among these, cryopreservation of ovarian tissue is the only option available for prepubertal girls and woman in need of immediate chemotherapy [3–8]. The main drawback of ovarian cryopreservation followed by transplantation is that a large proportion of follicles are lost during the initial ischemia which occurs after transplantation [9–16]. Therefore, reducing the damage due to the ischemic interval between transplantation and revascularization is essential to maintaining both follicular reserve and the function of the graft.

Erythropoietin (EPO) is an acidic glycoprotein hormone which promotes the differentiation and proliferation of erythroid progenitor cells, and is mainly produced by the kidney. EPO plays a central role in maintaining erythrocyte homeostasis *in vivo*, and it is clinically used for the treatment of anemia as well as pre- and post-operative management. Furthermore, it is well established that EPO functions not only as a hematopoietic factor but also to inhibit apoptosis and/or protect tissues in nerve cells,

myocardial cells, renal proximal tubular epithelial cells, etc. [17, 18]. These two functions of EPO are attributed to two different signal transduction pathways. When EPO acts on the EPO receptor homodimer, it induces hematopoiesis through the intracellular JAK2 signal transduction pathway. When EPO acts on the heterodimer of the EPO receptor and a common β receptor, however, it induces an anti-apoptotic effect through the intracellular ERK1/2 signal transduction pathway [19].

Since xenotransplantation of cryopreserved ovarian tissue can be used to evaluate the tissue developmental potential before an elective retransplantation [20, 21], the protective and survival-promoting effects of EPO and desialylated EPO (asialo EPO), digested by neuraminidase [22], on the follicles of frozen-thawed canine ovary after transplantation were examined by using non obese diabetic-severe combined immunodeficient (NOD-SCID) mice. Since asialo EPO has a higher specific activity compared with intact EPO [22], effect of asialo EPO was examined in addition to EPO.

Materials and methods

Female and male NOD-SCID mice were purchased from a commercial supplier (CLEA Japan, Tokyo, Japan), and were bred in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animals were housed in polycarbonate cages, and maintained in a specific pathogen-free environment in light-controlled (lights-on from 07:00 to 19:00) and air-conditioned rooms (temperature: 24±1°C, humidity: 50±10%). They had access to standard laboratory chow (CE-2; CLEA Japan) and water *ad libitum*. The ovaries from a 4-month-old dog were frozen-thawed and transplanted into the ovarian bursa of 8-wk-old NOD-SCID mice. The freezing and thawing procedures and ovarian transplantation were performed according to the method of Ishijima *et al.* [21]. Ovarian tissue was minced into 1.0-1.5 mm cubes, which were immersed in 1M dimethyl sulfoxide (DMSO) at room temperature for 60 s and then placed in a 1-ml cryotube (Nalge Nunc International KK, Tokyo, Japan) containing 5 μl of DMSO, and the tube was cooled on ice for 5 min After addition of DAP 213 (2M DMSO, 1M acetamide, 3M propylene glycol) solution [23] precooled on ice, the tube was cooled on ice for 5 min and immersed into liquid nitrogen. For thawing, the tube was removed from the liquid nitrogen, the liquid nitrogen in the tube was discarded and then the tube was allowed to stand at room temperature for 60 s. After the addition of 900 μl of 0.25M sucrose prewarmed to 37°C into the tube, the suspension was quickly stirred by mild pipetting and washed with PBI [24] five times. A portion of the extirpated ovaries was fixed with 10% formalin to prepare pre-transplant ovarian tissue samples.

Adult NOD-SCID mice (n=9) were anesthetized by intraperitoneal administration of sodium pentobarbital (5 mg/ml. Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and then the dorsal skin was incised to draw out the ovaries. An incision was made in the lateral side of each ovary to remove the mouse ovary in the ovarian bursa, leaving a part to ensure blood flow to a dog ovarian xenograft after transplantation, and a piece of frozen-thawed dog ovarian tissue was placed there (i.e. into the ovarian bursa). A hemostatic gelatin sponge (Spongel, S022Y01, Astellas, Japan) soaked with 400 U/kg of EPO (r-hu-EPO; EPOGIN S1500, Chugai Pharmaceuticals, Tokyo, Japan) or asialo EPO [22] was also placed into the ovarian bursa. As a control, Spongel soaked with an equivalent amount of physiological saline was placed into the ovarian bursa. The skin incision was closed with a clip (9-mm auto clip, 427631, Becton Dickinson). The operated mice were placed on a warm plate until sufficient recovery had occurred to allow movement. At 4 weeks after the operation, the transplanted ovaries were removed and fixed with 10% formalin and subjected to hematoxylin and eosin staining together with the pre-transplant ovarian tissue samples. To evaluate the effects of EPO and asialo EPO, follicles that visibly contained an ovum (oocyte) with a nucleus were counted according to the classification of Oktay *et al.* [25] as follows. Primordial follicles comprise follicles containing an oocyte partially or completely encapsulated by squamous pregranulosa cells; early primary follicles are follicles in which at least one of the pregranulosa cells had become columnar (enlarged); primary follicles are follicles in which all of the granulosa cells exhibit enlargement and a single layer of granulosa cells; transitional follicles comprise follicles containing an oocyte encapsulated by a 1-2 layer of columnar granulosa cells; preantral follicles are made up of follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with no antrum formation; antral follicles are follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with antrum formation. For pre-transplant ovarian tissues, ten tissue samples were randomly selected and the number of follicles in the ten tissue samples was counted. The number of follicles in a circle of 900 μm in diameter, i.e., a view field of 0.64 mm^2, containing the highest number of follicles in each selected tissue sample was counted (for a total of 10 view fields). This number was recorded as the number of follicles before transplantation. For transplanted ovarian tissue, five sections (7 μm in thickness) were sequentially prepared for a tissue specimen (a block). A total of six graft samples were examined in each experimental group. The distance between sections was 40-50 μm. The number of follicles in a circle of 900 μm in

diameter, i.e., a view field of 0.64 mm^2, containing the highest number of follicles in each section, was counted (in a total of 5 fields of view). The survival rates of follicles were calculated as number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples x 100. Statistical analysis was performed by using Wilcoxon's signed rank test. P values less than 0.05 were considered to be significant.

The tissues and animals used in this study were treated under the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

Results

The average number of primordial, early primary, primary, transitional, preantral and antral follicles per 0.64 mm^2 in frozen-thawed ovarian sections was 14.8±11.9, 3.3±2.1, 4.1±1.9, 3.6±1.7, 0.8±0.7 and 0, respectively. As shown in Table 1, the average number of primordial follicles per 0.64 mm^2 in ovarian sections was 0.3±0.31 in the untreated control group, 0.4±0.57 in the EPO group, and 3.9±2.47 in the asialo EPO group at 4 weeks after transplantation, in contrast to 14.8±11.9 before transplantation, which indicated the asialo EPO group in particular showed a significantly higher survival rate (26.6%) as compared with the untreated group (2.3%). The survival rate of early primary follicles was 15.2% in the EPO group and 157.6% in the asialo EPO group, in contrast to 10.1% in the untreated group. The proportion of early primary follicles to total follicles in untreated, EPO and asialo EPO group was 43, 42, and 54%, respectively. These results clearly indicate the primordial follicles have partially grown into early primary follicles in the asialo EPO group. Moreover, it was found that the asialo EPO group had a tendency to higher survival rates of primary follicles and transitional follicles as compared with the untreated group.

These results demonstrate that administration of EPO, especially asialo EPO, can be effectively used for the enhancement of survival of transplanted organ tissues.

Discussion

It is believed that the reason the primordial follicle is observably resistant to cryoinjury is because the oocyte it contains has a relatively inactive metabolism, as well as the lack of meiotic spindle, zona-pellucida and cortical granules [2]. In fact, a high percentage of oocytes as well as granulosa cells survive the cryopreservation and thawing procedure [21, 26–28]. Our previous study has shown that there was no difference in morphology and in the average number of primordial and primary follicles between the vitrified-warmed by DAP213 and fresh ovarian tissues in dog [21]. Recovery rates of the grafts in cryopreserved ovarian tissues were equivalent and much better than those in fresh ovarian tissues when the tissues were transplanted to NOD-SCID mice and recovered at 4 weeks post-operation [21]. Also, it has been shown that proliferating cell nuclear antigen was detectable in many of the granulosa cells in the primary follicles of the grafts when canine ovarian tissues were cryopreserved by DAP213 and transferred into ovarian bursa of NOD-SCID mice [21]. However, a majority of primordial follicles in frozen-thawed canine ovarian tissues reportedly disappears after transplantation (Table 1). The main reason for the follicular loss after cryopreservation and xenografting seems to be the ischemic effect which takes place after transplantation rather than cryopreservation *per se* [16, 29]. Several attempts have been

Table 1 Effect of erythropoietin administration on the average number of follicles and the survival rate of follicles relative to the number of follicles in ovarian tissues before transplantation in frozen-thawed canine ovary at 4 weeks after transplantation

Exp. Group	Classification of follicle (% of survival)					
	Primordial	Early primary	Primary	Transitional	Preantral	Antral
Untreated	$0.3±0.31^{a}$	$0.3±0.42^{a}$	$0.1±0.23^{a}$	0^{a}	0	0
	(2.3)	(10.1)	(3.3)	(0)	(0)	-
EPO	$0.4±0.57^{a}$	$0.5±0.71^{a}$	$0.3±0.42^{a}$	0^{a}	0	0
	(2.7)	(15.2)	(7.3)	(0)	(0)	-
AsialoEPO	$3.9±2.47^{b}$	$5.2±3.62^{b}$	$0.4±0.53^{a}$	$0.1±0.12^{a}$	0	0
	(26.6)	(157.6)	(9.8)	(1.9)	(0)	-

The results are shown as the mean±SD. The different superscript letters within a column indicate significantly different values ($P<0.05$). Total six graft samples were examined in each experimental group. Five sections (7 μm in thickness) were sequentially prepared for a tissue specimen (a block). The distance between sections was 40-50 μm. The number of follicles in a circle of 900 μm in diameter, i. e., a view field of 0.64 mm^2, containing the highest number of follicles in each section, was counted (in a total of 5 fields of view). The survival rates (% of survival)of follicles were calculated as number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples x 100

made to prevent or at least decrease the follicular loss of cryopreserved ovarian tissues after transplantation. However, an effective solution has not been found to date. It has been reported that functional vessels within the graft were detected by both magnetic resonance imaging and histological exam from day 7 onwards when rat ovaries were transplanted into the muscles of castrated nude mice [30]. Kim *et al.*, [31] showed that the ovarian tissue could tolerate ischaemia for at least 2 h at 0°C or at room temperature, and that a water soluble antioxidant (ascorbic acid) reduces apoptosis in ovarian cortex by up to 24 h in the case of incubation *in vitro*. It has been reported that treatment with vitamin E, a lipid soluble antioxidant, improved the survival of follicles in ovarian grafts by reducing ischemic injury [32]. Prolonged exogenous stimulation promoted primordial follicle maturation but also caused a loss of primordial follicles in xenotransplanted frozen-thawed ovaries [28, 33]. Our present study clearly shows that the administration of asialo EPO is effective for enhancing the survival of transplanted cryopreserved ovarian follicles (Table 1). In addition, the increasing number of early primary follicles in the frozen-thawed ovarian tissues treated with asialo EPO indicates a growth promoting effect of asialo EPO for primordial follicles. It is well known that EPO functions not only as a hematopoietic factor, but also inhibits apoptosis and/or protects several kinds of cells such as nerve [18] and myocardial cells [17]. However, a protective effect of EPO for ovarian or follicular cells has not been reportedly demonstrated both *in vitro* and *in vivo*. The reason why the protective effects of asialo EPO for follicular cells were much higher than those in EPO (Table 1) seems to be related with the finding that asialo EPO showed a four-times-higher specific activity *in vitro* compared with intact EPO [22]. Asialo EPO binds to its receptor faster than the intact form [22]. Although it is still unclear how asialo EPO initiates the differentiation and proliferation of the ovarian follicular cells, previous study indicates that female reproductive organs/tissues including ovarian follicles at various stages express EPO receptor, and that signal transduction of EPO contributes to the cyclic changes in the female reproductive organs [34]. In conclusion, the administration of asialo EPO appears to be effective for the prevention of follicular loss in frozen-thawed ovary after transplantation.

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References

1. Blatt J. Pregnancy outcome in long-term survivors of childhood cancer. Med Pediatr Oncol 1999;33:29–33. doi:10.1002/(SICI)1096-911X(199907)33:1<29::AID-MPO6>3.0.CO;2-2.
2. Donnez J, Matinez-Madrid B, Jadoul P, Van Langendonckt A, Demylle D, Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update 2006;12:519–35. doi:10.1093/humupd/dml032.
3. Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196C. Hum Reprod 1994;9:597–603.
4. Donnez J, Bassil S. Indications for cryopreservation of ovarian tissue. Hum Reprod Update 1998;4:248–59. doi:10.1093/humupd/4.3.248.
5. Meirow D, Ben Yehuda D, Prus D, Poliak A, Schenker JG, Rachmilewitz EA, Lewin A. Ovarian tissue banking in patients with Hodgkin's disease: is safe? Fertil Steril 1998;69:996–8. doi:10.1016/S0015-0282(98)00093-4.
6. Oktay K, Newton H, Aubard Y, Salha O, Gosden RG. Cryopreservation of immature human oocytes and ovarian tissue: an emerging technology? Fertil Steril 1998;69:1–7. doi:10.1016/S0015-0282(97)00207-0.
7. Donnez J, Godin PA, Qu J, Nisolle M. Gonadal cryopreservation in young patient with gynaecological malignancy. Curr Opin Obstet Gynecol 2000;12:1–9. doi:10.1097/00001703-200002000-00001.
8. Donnez J, Dolmans MM, Matinez-Madrid B, Demylle D, Van Langendonckt A. The role of cryopreservation for women prior to treatment of malignancy. Curr Opin Obstet Gynecol 2005;17:333–8. doi:10.1097/01.gco.0000175348.72566.47.
9. Newton H, Aubard Y, Rutherford A, Sharma V, Gosden RG. Low temperature storage and grafting of human ovarian tissue. Hum Reprod 1996;11:1487–91.
10. Candy CJ, Wood MJ, Whittingham DG. Effect of cryoprotectants on the survival of follicles in frozen mouse ovaries. J Reprod Fertil 1997;110:11–9. doi:10.1530/jrf.0.1100011.
11. Gunasena KT, Villines PM, Critser ES, Critser JK. Live births after autologous transplant of cryopreserved mouse ovaries. Hum Reprod 1997;12:101–6. doi:10.1093/humrep/12.1.101.
12. Aubard Y, Piver P, Cognie Y, Fermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. Hum Reprod 1999;14:2149–54. doi:10.1093/humrep/14.8.2149.
13. Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG. Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196C. Endocrinology 1999;140:462–71. doi:10.1210/en.140.1.462.
14. Nisolle M, Godin PA, Casanas-Roux F, Qu J, Motta P, Donnez J. Histological and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. Fertil Steril 2000;74:122–9. doi:10.1016/S0015-0282(00)00548-3.
15. Oktay K, Newton H, Gosden RG. Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. Fertil Steril 2000;73:599–603. doi:10.1016/S0015-0282(99)00548-8.
16. Liu J, Van der Elst J, Van den Broecke R, Dhont M. Early massive follicle loss and apoptosis in heterotopically grafted newborn mouse ovaries. Hum Reprod 2002;17:605–11. doi:10.1093/humrep/17.3.605.
17. Calvillo L, Latini R, Kajstura J, Leri A, Anversa P, Ghezzi P, Salio M, Cerami A, Brines M. Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. Proc Natl Acad Sci USA 2003;100:4802–6. doi:10.1073/pnas.0630444100.
18. Sakanata M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, Sasaki R. In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci USA 1998;95:4635–40. doi:10.1073/pnas.95.8.4635.
19. Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, Latini R, Xie QW, Smart J, Su-Rick C, Pobre E, Diaz D, Gomez D,