Table 2. Participants' residential bathtub type and ventilation method of bathrooms

	Recovery of *M. avium* and *M. intracellulare*		
	Culture positive	Culture negative	Total
Type of bathtub			
Bathtub supplied with hot-water	3	10	13*
Bathtub attached to a bath-boiler			
Natural circulation type[1)]	8	0	8
Forced circulation type[2)]	4	4	8
The time of draining off water from bathtub after bathing			
Shortly after bathing	2	6	8**
Next day	12	8	20
Unknown	1	0	1
Ventilation of a bathroom			
Bathroom dryer	0	3	3**
Ventilating fan			
Regular use	0	1	1
1-8 h after bathing	2	2	4
0.5-1 h after bathing	3	2	5
No bathroom dryer or no ventilating fan	10	6	16

Data are represented as the number of residences.
[1)]: Natural circulation type of bath-boiler which has two holes in a bathtub.
[2)]: Forced ciculation type of bath-boiler which has one hole in a bathtub.
*, $P < 0.01$. **, $P < 0.1$.

the use of a bathroom dryer or a ventilating fan to decrease the recovery rate of MAC ($P = 0.08$, Mann-Whitney's U test). Our results showed that the reservation of bathtub water and/or the continuance of high humidity in the bathroom appear to be conducive to MAC colonization.

DISCUSSION

The characteristics of pulmonary MAC disease, frequent recurrence and multiple infections, suggest that polyclonal MAC colonization is likely to occur in the home or hospital environment surrounding patients. Here, we demonstrated that polyclonal MAC organisms colonize predominantly inside the showerhead and the bathtub inlet of patients' bathrooms. In our previous study, MAC isolates were recovered from residential bathrooms but not from other sites within the residence (17). This uneven distribution and polyclonal colonization of MAC in the residential bathrooms suggested that MAC concentrates and colonizes in the bathroom preferentially. Furthermore, we found that MAC organisms colonized in the bathrooms for a long period of at least 3 months. These findings were in accord with a previous report that nontuberculous mycobacteria (including MAC) formed persistent colonies in a drinking water system (20). Thus MAC may also persistently colonize in bathtub inlets and the inside of the showerhead, where biofilm can be formed.

For 7 cases (47%, 7/15), the genotypes of the environmental isolates showed PFGE patterns related to their respective clinical isolates. Such a high rate of related polymorphism suggests that the bathroom is one of source of infection, although there is still a possibility that MAC bacilli might be transmitted from patients to their bathrooms. Therefore, the source of primary infection with MAC is a controversial issue. However, the fact that patients' bathrooms colonize MAC supports the idea that patients were reinfected by inhaling the MAC organisms each time they bathed. When patients repeatedly inhale pathogens, the efficacy of chemotherapy is reduced, and reinfection may be caused by the same pathogen or a genetically different strain. In fact, the cure rate with macrolide-based regimens is still low, and MAC infection frequently recurs after the sputum culture is converted to negative upon successful completion of therapy (2,3,9). Most recurrences of MAC disease after discontinuation of therapy are interpreted as reinfections with new MAC strains rather than relapse of the initial MAC strains (3,7,8). In the present study, we also demonstrated that the genotypes of reinfected strains were related to those of isolates from their respective bathrooms. This fact supports a risk of reinfection in patients. Furthermore, polyclonal infection may be involved in transferring organisms from a polyclonal environmental colonization in the bathrooms to patients. A recent report also provided evidence that showers may serve as a source of pulmonary infection caused by waterborne *M. avium* (21). The results of our questionnaire survey suggest that, in order to prevent the transference of MAC from bathroom to patient, it is important to keep the bathroom free from MAC colonization by desiccating. Indeed, Archuleta et al. (22) reported that desiccated *M. avium* loses its viability at a constant rate. It is difficult to prove that the bathroom is the source of primary infection for pulmonary MAC disease. In part, this is because pulmonary MAC disease is often asymptomatic during the early stage of infection and progresses slowly, so that many patients are uncertain of the number of months or years that have passed since the initial infection. If MAC bacilli in the bathroom are transferred to a susceptible host, they might be capable of moving between the bathroom and host during the asymptomatic time periods. Therefore, a prospective cohort study would be required to clarify that the bathroom is a source of infection.

In conclusion, we found that polyclonal MAC organisms were distributed throughout the bathrooms of our patients, but predominantly colonized in the bathtub inlets. Nearly half of the 15 bathrooms that harbored MAC strains (47%, 7/15) had strains with a genetically close relationship to their respective clinical isolates. Thus, it is considered that there is a risk of infection in bathrooms colonized by MAC.

ACKNOWLEDGMENTS

We are grateful to all participants who donated samples and the hospital nurses who took part in our study.

This research was supported by grants-in-aid from the Institute for Fermentation, Osaka (IFO), the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants), the Japan Health Sciences Foundation, and the United States-Japan Cooperative Medical Science Program against Tuberculosis and Leprosy.

REFERENCES

1. Falkinham, J.O., 3rd (1996): Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev., 9, 177-215.
2. Field, S.K., Fisher, D. and Cowie, R.L. (2004): *Mycobacterium avium* complex pulmonary disease in patients without HIV infection. Chest, 126, 566-581.
3. Griffith, D.E., Aksamit, T., Brown-Elliott, B.A., et al. (2007): An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am. J. Respir. Crit. Care Med., 175, 367-416.
4. Sakatani, M. (2005): The non-tuberculous mycobacteriosis. Kekkaku, 80, 25-30 (in Japanese).
5. Marras, T.K. and Daley, C.L. (2002): Epidemiology of human pulmonary infection with nontuberculous mycobacteria. Clin. Chest Med., 23, 553-567.
6. Songer, J.G., Bicknell, E.J. and Thoen, C.O. (1980): Epidemiological investigation of swine tuberculosis in Arizona. Can. J. Comp. Med., 44, 115-120.
7. Wallace, R.J., Jr., Zhang, Y., Brown, B.A., et al. (1998): Polyclonal *Mycobacterium avium* complex infections in patients with nodular bronchiectasis. Am. J. Respir. Crit. Care Med., 158, 1235-1244.
8. Wallace, R.J., Jr., Zhang, Y., Brown-Elliott, B.A., et al. (2002): Repeat positive cultures in *Mycobacterium intracellulare* lung disease after macrolide therapy represent new infections in patients with nodular bronchiectasis. J. Infect. Dis., 186, 266-273.
9. Kobashi, Y. and Matsushima, T. (2003): The effect of combined therapy according to the guidelines for the treatment of *Mycobacterium avium* complex pulmonary disease. Intern. Med., 42, 670-675.
10. Subcommittee of the Joint Tuberculosis Committee of the British Thoracic Society (2000): Management of opportunist mycobacterial infections: Joint Tuberculosis Committee Guidelines 1999. Thorax, 55, 210-218.
11. Aronson, T., Holtzman, A., Glover, N., et al. (1999): Comparison of large restriction fragments of *Mycobacterium avium* isolates recovered from AIDS and non-AIDS patients with those of isolates from potable water. J. Clin. Microbiol., 37, 1008-1012.
12. Covert, T.C., Rodgers, M.R., Reyes, A.L., et al. (1999): Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol., 65, 2492-2496.
13. Falkinham, J.O., 3rd, Norton, C.D. and LeChevallier, M.W. (2001): Factors influencing numbers of *Mycobacterium avium, Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. Appl. Environ. Microbiol., 67, 1225-1231.
14. Peters, M., Muller, C., Rusch-Gerdes, S., et al. (1995): Isolation of atypical mycobacteria from tap water in hospitals and homes: is this a possible source of disseminated MAC infection in AIDS patients? J. Infect., 31, 39-44.
15. Arbeit, R.D., Slutsky, A., Barber, T.W., et al. (1993): Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. J. Infect. Dis., 167, 1384-1390.
16. von Reyn. C.F., Maslow, J.N., Barber, T.W., et al. (1994): Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. Lancet, 343, 1137-1141.
17. Nishiuchi, Y., Maekura, R., Kitada, S., et al. (2007): The recovery of *Mycobacterium avium-intracellulare* complex (MAC) from the residential bathrooms of patients with pulmonary MAC. Clin. Infect. Dis., 45, 347-351.
18. Ad Hoc Committee of the Scientific Assembly on Microbiology, Tuberculosis, and Pulmonary Infections (1997): Diagnosis and treatment of disease caused by nontuberculous mycobacteria. This official statement of the American Thoracic Society was approved by the Board of Directors, March 1997. Medical Section of the American Lung Association. Am. J. Respir. Crit. Care Med., 156, S1-25.
19. Chen, Z.H., Butler, W.R., Baumstark, B.R., et al. (1996): Identification and differentiation of *Mycobacterium avium* and *M. intracellulare* by PCR. J. Clin. Microbiol., 34, 1267-1269.
20. Hilborn, E.D., Covert, T.C., Yakrus, M.A., et al. (2006): Persistence of nontuberculous mycobacteria in a drinking water system after addition of filtration treatment. Appl. Environ. Microbiol., 72, 5864-5869.
21. Falkinham, J.O., III, Iseman, M.D., Haas, P., et al. (2008): *Mycobacterium avium* in a shower linked to pulmonary disease. J. Water Health, 6, 209-213.
22. Archuleta, R.J., Mullens, P. and Primm, T.P. (2002): The relationship of temperature to desiccation and starvation tolerance of the *Mycobacterium avium* complex. Arch. Microbiol., 178, 311-314.

Cancer Cell International

CANCER CELL INTERNATIONAL

BioMed Central

Primary research

Open Access

Growth inhibition of HeLa cell by internalization of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) Tokyo

Akira Kitamura*[1], Sohkichi Mastumoto[2] and Izumi Asahina[1]

Address: [1]Division of Oral and Maxillofacial Surgical Reconstruction and Functional Restoration, Department of Developmental and Reconstructive Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto, Nagasaki 852-8588, Japan and [2]Department of Host Defense, Osaka City University. Graduate School of Medicine, Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

Email: Akira Kitamura* - a-kit@nagasaki-u.ac.jp; Sohkichi Mastumoto - sohkichi@med.osaka-cu.ac.jp; Izumi Asahina - asahina@nagasaki-u.ac.jp

* Corresponding author

Published: 2 December 2009

Cancer Cell International 2009, **9**:30 doi:10.1186/1475-2867-9-30

Received: 19 August 2009
Accepted: 2 December 2009

This article is available from: http://www.cancerci.com/content/9/1/30

Abstract

Background: Intravesical BCG immunotherapy is effective for preventing recurrence and progression in none muscle-invasive bladder cancer but the dosing schedule and duration of treatment remain empirical. The mechanisms by which intravesical BCG treatment mediates antitumor activity are currently poorly understood.

Results: HeLa cell infected with *Mycobacterium bovis* Bacillus Calmette-Guérin(BCG) Tokyo which were different multiplicity of infection(MOI). Proliferation of HeLa cell reduced in a dose-dependent manner by live BCG. The cytoplasm of the HeLa cell showed variety lysosomal stages by internalized and interacted BCG.

Conclusion: Proliferated Live BCG secreted the protein and depressed the growth of tumor. The possibility for clinical introduction of BCG therapy for carcinoma reported with review of literature.

Background

Intravesical BCG treatment has been demonstrated to be an effective therapy for superficial transitional cell carcinoma of the bladder though the mechanism of antitumor effect still remained unclear. We studied to whether the BCG depressed the growth of malignant tumor cell or not. The proliferation of HeLa cells were inhibited by dose dependent manner infected by live BCG. HeLa cell infected by live and dead *Mycobacterium bovis* Bacillus Calmette-Guérin(BCG) Tokyo revealed variety stage of lysosome and BCG in the cytoplasm of HeLa cell. The internalization of live BCG into the HeLa cells did not blocked by heparin and cytochalasin B. The internalized live BCG secreted the secreted-protein and depressed the growth of tumor cell. Live BCG inhibited the growth of tumor cell by internalized and then the secreted protein in cytoplasm of HeLa cells suggested the possibility of new cancer therapy made of BCG combined with the drug delivery system(DDS).

Results

The growth of HeLa cell was inhibited by dose dependent manner cultured by live *Mycobacterium bovis* Bacillus Calmette-Guérin(BCG) Tokyo (Figure 1). The proliferation of HeLa cell was not inhibited when the MOI was 1. Live BCG indicated more depressed the growth of HeLa cell

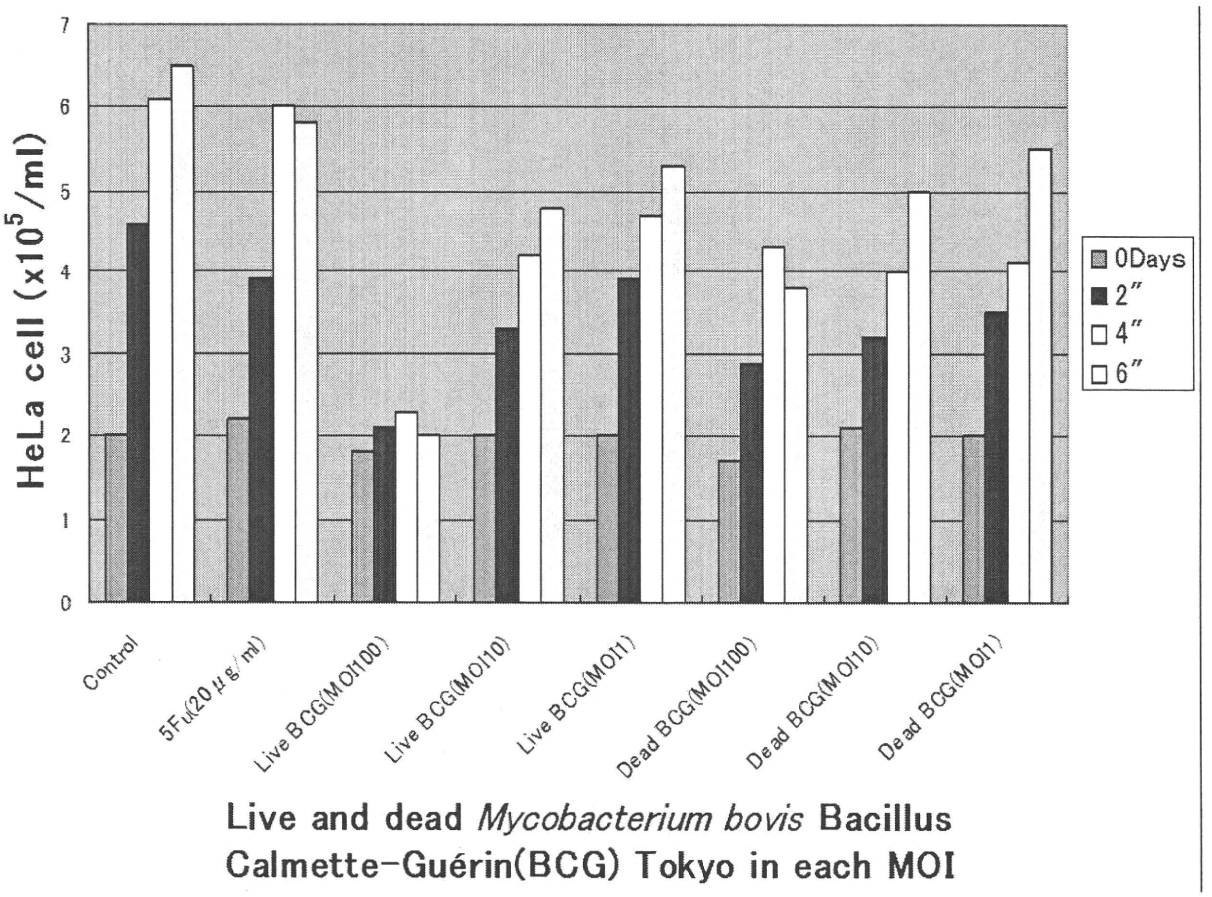

Figure 1
Growth of HeLa cells were depressed by live and dead *Mycobacterium bovis* Bacillus Calmette-Guérin(BCG) Tokyo in each multiplicity of infection(MOI). The growth of HeLa cells were inhibited in dose dependent manner cultured by live BCG. The proliferation of HeLa cell was not inhibited when the MOI was 1.

than the each membrane(0.04 μg/ml, dry weight) or cytoplasm(0.02 μg/ml, dry weight) fraction of the BCG [1] whose dosage were equivalent of MOI 100 (Figure 2).

Two days after cultured with live BCG (MOI 100), HeLa cell showed vacuole and BCG in the cytoplasm (Figure 3A). Even one hour after cultured with live BCG, the cytoplasm of HeLa cell also revealed lysosome, residual body and BCG (Figure 4A). There were several kinds of lysosome which indicated phagocytosis caused from internalized BCG (Figure 4A, C). The myelin-like multilamellar structure was also recognized in the cytoplasm of HeLa cell by dead BCG one day after incubated [2] (Figure 4C). Internalized live or dead (Figure 4A, C) BCG induced the lysosomal activity of the HeLa cell. Four days after the infected HeLa cell by live BCG showed the necrosis in which the BCG kept its shape (Figure 4E). The internalization of live BCG into the HeLa cells was found in their cytoplasm with cytochalasin B [3] (100 μg/ml, Figure 3E) or heparin [4] (0.001 U/ml.Figure 3F) was added into each well before co-culture with HeLa cell respectively. Immunoelectron microscope checked using polyclonal antibodies of the MPB70 (secreted protein, α-antigen) [5-7] and revealed the protein A gold reacted around the cell wall of live BCG. (Figure 3B, D)

Discussion

Intravesical Bacillus Calmette-Guérin(BCG) therapy has been effective in delaying or preventing recurrence and progression for transitional cell carcinoma of bladder although its outcome is still unpredictable [8]. The report suggested that BCG interacted with tumor cells or internalized into them, and yet the role of cellular attachment has been un-established. Therefore we initiate studied

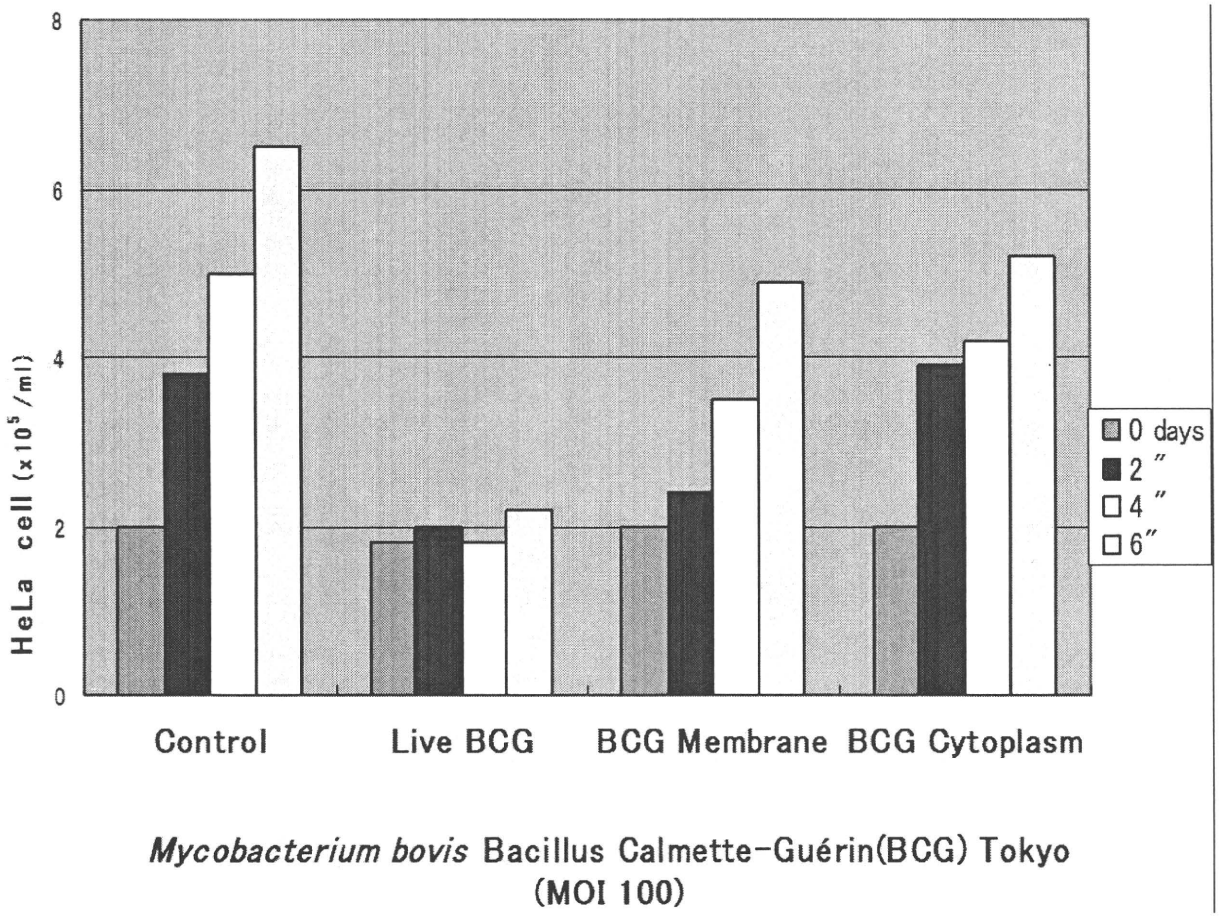

Figure 2
Growth depression of HeLa cell by different BCG component(MOI 100). Live BCG depressed the tumor growth than that of the membrane(0.04 μg/ml, DW) and cytoplasm(0.02 μg/ml, DW) fraction each dosage were equivalent of MOI 100.

using the TEM to better define for the interaction of HeLa cells with BCG and leading to the hypothesis that live BCG induced anti-tumor activity in the tumor cells. The growth inhibition of the HeLa cell was more distinct by live BCG to compare dead BCG, the cytoplasm or membrane fraction of BCG [1]. Those indicated live BCG invaded and proliferated in the tumor cells. Since BCG infection inhibits the proliferation and differentiation of HeLa cells, question arises as to the mechanism whether inducing the bacteriological function from inside or outside. Several bacterial components have already been reported the affected proliferation by infection [9]. The possibility remains that poor nutrition of the cells caused by intracellular proliferation of BCG as well as the BCG stimulus on the cell surface receptor may also be involved in the suppression of cellular proliferation and differentiation [2].

Infection and growth of live BCG in the host cell and released BCG-related cytokine were estimated as the reasons for the depression of the tumor cells. MPB70 [7] (α antigen) known to be an immunogenic mycobacterial protein secreted in large amounts from culture filtrated of *Mycobacterium bovis* Bacillus Calmette-Guérin(BCG) Tokyo. This protein is thought to be crucial for binding phagocytic cell having fibronectin receptors and this function might be a direct effect of BCG immunotherapy[10,11]. BCG is thought to bind to the bladder wall via interaction between the bacterial antigen complex and fibronectin [12]. Similar observations with fibronectin attachment protein(FAP) demonstrated a Type I response inducing IL12 and IFNγ production in normal human peripheral blood lymphocytes. These data suggest that a Type I response is required for antitumor activity by BCG [13].

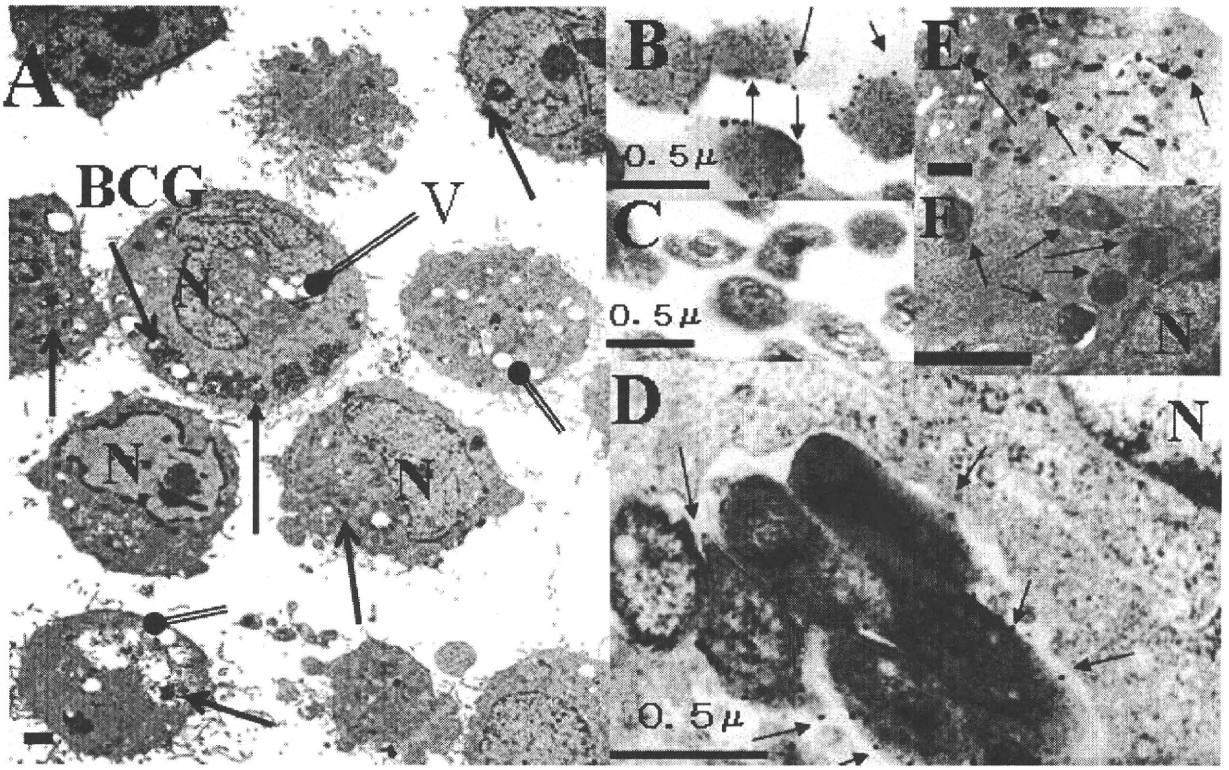

Figure 3

A; HeLa cell and live BCG were co-cultured after 2 days. There were vacuole(V) and BCG(↑) in the cytoplasm of HeLa cell. B, D; Protein A gold(↑) attached on the cell wall of BCG in the HeLa cell co-cultured with live BCG after two days by immunoelectron microscope (anti MPB70 polyclonal antibody). C; Dead BCG did not reacted at the cell wall. Cytochalasin B (E, 100 μg/ml) or Heparin (F, 0.001 U/ml) was added in each culture well of HeLa cell before infection by live BCG also showed the internalized BCG(↑) after 24 hours. N; Nucleus

Antitumor effects of BCG against superficial urinary bladder cancer were known to be strong when BCG is directly infused into the bladder, but its immunological mechanisms are poorly understood [14]. The internalization was inhibited by cytochalasin B(200 μg/ml)[3]; conditions known to inhibit phagocytosis [15]. Heparin (1.25 U/Kg) also induced the aggregation of the local expression of fibronectin and sequentially lessen FN-mediated BCG attachment to bladder wall [4]. But cell membrane-expressed fibronectin did not seem to be crucially involved in the internalization of BCG by transitional bladder cancer. A correlation between cellular fibronectin expression and the ability of transitional cell carcinoma to internalize BCG may be considered as a fortuitous coincidence [16]. Our data showed the internalization of live BCG into the HeLa cells were not blocked by heparin or cytochalasin B. These phenomena suggested another possible reaction between the membrane of the HeLa cell and BCG. Further experiments will be necessary to clarify the biological relevant related between the internalization and phagocytosis or autophagy which is associated with the role of mycobacterial infection and intracellular killing of the cell [17]. Autophagy, the process in which cellular organelles are targeted for degradation in lysosome, represents another potential tumor resistance mechanism and further adding to the complexity of cell death pathways when tumor cells are exposed to various agents [18,19]. IFN-γ induction of autophagy has not been previously reported in immune or phagocyte cells but has been observed in HeLa cells [2,20]. We reported that mycobacterial infection induces the Th1-type immune response lead on the immunological environment rich in IFN-α, which is a suppressive mediator of the Th2-type immune reaction [21]. Th1-stimulating cytokines played an important role in BCG-induced macrophage cytotoxicity and that combination of BCG with selected Th1-stimulating cytokines, either supplemented or expressed by BCG, may enhance the effect of BCG in the treatment of bladder cancer patients [22]. Early stages of BCG infection into osteoblastic-like cell (MC3T3-E1) secreted IL-6 and then

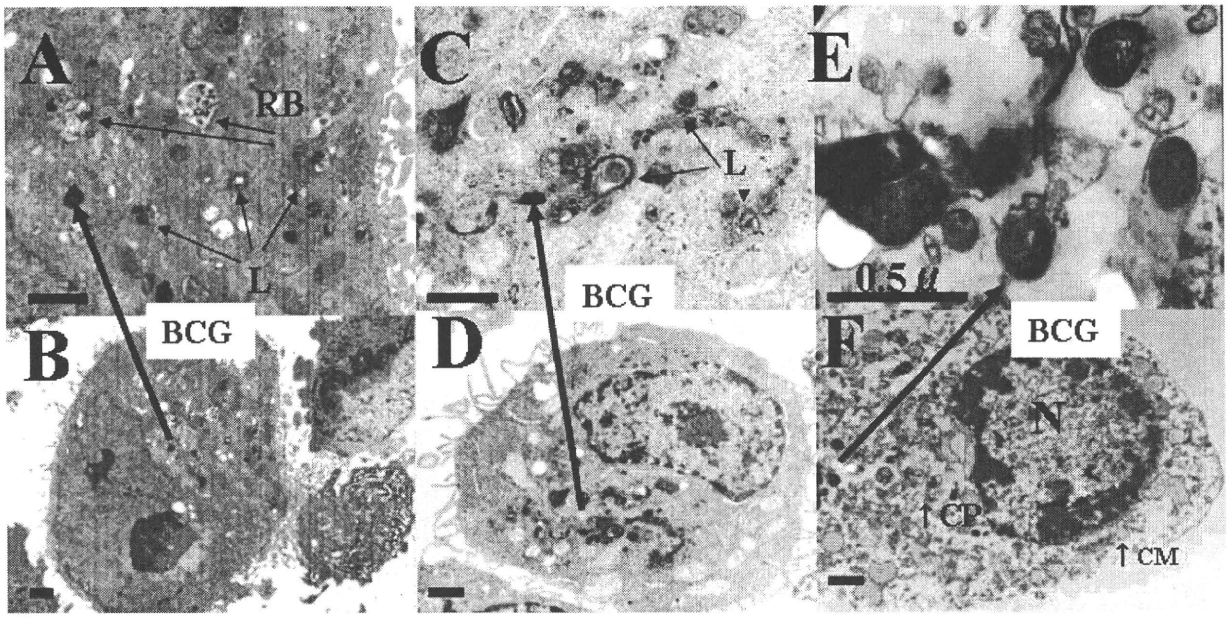

Figure 4
A, B; HeLa cells were infected with live BCG after 1 hour and showed BCG(↑), residual body(RB) and lysosome(L) in the cytoplasm. Different stages of the lysosomal activity were induced by internalized BCG. C, D; HeLa cells were co-cultured with dead BCG(↑) which were also internalized after 1 hour and showed the lysosome and vacuole. E, F; HeLa cells co-cultured with live BCG after four days. HeLa cell showed lack of cell membrane(↑CM), lost of cytoplasm(↑CP), nuclear degeneration(N) and BCG(↑). The shape of BCG was keeping in the HeLa cell.

depressed the proliferation of host [23]. The findings that live BCG infected and internalized in the HeLa cells as shown here, lysosomal activity is an important connection between immune mediator and associated intracellular depression of the host cells. These data suggested that live BCG invaded and proliferated in the cell then released the BCG-related protein have direct effect to inhibit the growth of the host.

The novel method such as using target would allow further improve the bacteria to satisfy a variety of requirements for clinical use. These therapies elicit active immune response against the tumor so that they kill off primary as well as metastases [24]. Successful cancer therapy required close contact between BCG and tumor cells, a host capable of developing and expressing delayed hypersensitivity type reactions to mycobacterial antigens, limited tumor size and an adequate number of viable BCG [25]. It is also necessary to establish the effective drug delivery system(DDS) to be internalized into malignant cell in vivo treatment [26]. The difference between the live and dead BCG which internalized in the cytoplasm of HeLa cell is the existence of secreted protein or not. BCG interacted with tumor cells and were internalized into them suggested future development of anti-tumor agents made from bacterial cell wall [27]or secreted protein [28].

Conclusion

Live BCG depressed the growth of the HeLa cell by dose dependent manner.

Live BCG internalized and secreted protein in the host cell suggested the depression of tumor cell.

Methods

HeLa cell

HeLa cell (1×10^5 cells/ml) were maintained in minimal essential medium (MEM, Gibco BRL, Tokyo Japan) supplemented with 10% fetal bovine serum and 100,000 U/1 penicillin at 37°C in humidified atmosphere with 5% CO_2. The cultured medium was replaced every 3 days. Cells were rinsed 2 times with phosphates-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4) before addition of fresh medium.

Bacillus Calmette-Guérin(BCG) [7,1,29]

Mycobacterium bovis Bacillus Calmette-Guérin(BCG) Tokyo was cultured in Middle brock 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% Albumin-dextrose-catalase(ADC; Difco laboratories) enrichment and 0.1% Tween 80. The cells(approximate 8×10^{10} cells/ml) were harvested with shaking at 37°C until 0.8 of an optical density(OD) at 590 nm. It was centri-

fuged and the pelletized cells re-suspended with MEM were divided and used for experiments. Dead BCG dosage (1×10^7 cells/ml) as MOI 100 was prepared and treated by an autoclave (121 °C, 10 min).

Fraction of BCG membrane and cytoplasm [1]

BCG(Tokyo) was sedimented (3,000 × g, 10 min, 4 °C), suspended in TMNSH buffer, and lysed by sonication in a Bioruptor UCD-200T sonicator (Toso, Tokyo, Japan). The cell lysate was centrifuged at 10,000 × g for 10 min at 4 °C twice. The supernatant was then centrifuged at 30,000 × g for 30 min at 4 °C. The pellet was used as the membrane fraction.

The supernatant solution was centrifuged under the same conditions, and the supernatant thus obtained was then centrifuged at 105,000 × g for two hours at 4 °C. The pellet was used as the cytoplasm fraction.

The pellets obtained in each step were suspended in TMNSH buffer. Freeze drying membrane and cytoplasm fraction were prepared 0.04 μg/ml and 0.02 μg/ml respectively for the experiment as MOI 100 and keeping at the department of oral bacteriology Nagasaki University. BCG also had been cultured in the same department.

Polyclonal antibody [7]of MPB70 (secreted protein, antigen) [5,6,10]

Purified MPB70 was provided by Dr. Nagai. BALB/c mice at 7-10 weeks of age were immunized intravenously with MPB 70 (10 μg diluted 200 ul of PBS) which was served as the most abundant protein in the culture filtrate from BCG(Tokyo). After 30 days, same amount of MPB 70 was injected intraperitoneally to boost the immune response. After 1 week later, sera were collected from the eye vein of immunized mice and pooled at -80 °C until use. Animal had been keeping at animal center of Nagasaki Univ.

HeLa and BCG cells co-culture

Two millimeter of HeLa cells (1×10^5/ml) were inoculated in 24-well plates and cultured for 1 days. Then BCG at various doses and type were added to the wells. After 72 hours fresh MEM exchanged one ml.

Different BCG dosage even, ten and hundred times of multiplicity of infection (MOI) BCG were prepared and cultured with HeLa cell.

Growth inhibition of HeLa cell checked by its fraction of membrane (0.04 μg/ml, Dry weight) and cytoplasm (0.02 μg/ml, Dry weight) were also prepared as equivalent dosage of MOI 100 and added in the medium of HeLa cells.

Internalization of the BCG checked using each cytochalasin B (100 μg/ml, Wako pure C.I. Japan) or heparin sodium (0.001 U/ml, OSTUKA Pharm. Japan) was added into the well before co-cultured with HeLa cell respectively.

Cell count

Every other day during the incubation period with the HeLa and BCG, MEM changed to the usual saline solution and treated 2% tripsin treated for 5 minutes. HeLa cells were mounted on the erythrocytometer after 0.3% trypan-blue stain. The number of cells expressed as the mean for three times.

Transmission electron microscope (TEM) [28]

The HeLa cell washed in the normal saline solution and centrifuged. The cells were fixed in solution of 2% glutaraldehyde in 0.1 M phosphate buffer solution, pH 7.3 for 2 hours and 1% osmium tetraoxide for 2 hours. After two times washed PBS and water the cells were dehydrated with increasing concentrations of ethanol; and gradually infiltrated with Epon 812. Before inspection by TEM the trimmed bloc of epon was orientated and stained with toluidine blue for light microscopy orientation. The ultrathin section with silver to gold interference color were picked up in a nickel grid and stained with uranyl acetate and lead nitrate in the usual manner.

For Immuno-TEM HeLa cell fixed 1% paraformaldehyde at 4 °C for one hour. Then dehydrated in ethanol and embedded by LR white(Okenshoji, Japan) for 2 days at -20 °C. The same interference color picked up on the collodion coated mesh (Nissinn EM, Japan) specimens were reacted with 2% hydrogen peroxide for 30 minutes twice and blocked bovine serum for 30 minutes. They were reacted with hundred times diluted anti-sera (polyclonal Aanti-MPB70) for 12 hours at 8 °C and protein A gold (15 nm, FUNAKOSI, Japan) for three hours at room temperature in moisture chamber and then double stained for 5 minutes each. The specimens were examined in a H800 electron microscope (Hitachi, Japan) operating at 75 kV.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AK counted the HeLa cells, carried out the morphologic study of TEM and immuno-TEM, drafted the manuscript and designed this experiment. SM cultured the BCG, prepared MPB70 and BCG fraction of membrane and cytoplasm and made the polyclonal antibody of MPB70 (secreted protein, α-antigen). IA carried out the co-culture of BCG and HeLa cell. All authors read and approved the final manuscript.

References

1. Ohara N, Naito M, Miyazaki C: **HrpA, a new ribosome-associated protein which appears in heat-stressed *Mycobacterium bovis* Bacillus Calmette-Guérin.** *J Bacteriol* 1997, **179**:6495-6498.

2. Gutierrez G, Master SS, Singh SB: **Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages.** *Cell* 2004, **119**:753-766.
3. Luo YI, Szilvasi A, Chen X: **A novel method for monitoring *Mycobacterium bovis* BCG trafficking with recombinant BCG expressing green fluorescent protein.** *Clin Diagn Lab Immunol* 1996, **3**:761-768.
4. Shen Z, Wang Y, Ding GQ: **Study on enhancement of fibronectin-mediated bacillus Calmette-Guérin attachment to urinary bladder wall in rabbits.** *World J Urol* 2007, **25**:525-529.
5. Harboe M, Nagai N: **MPB70, a unique antigen of *Mycobacterium bovis* BCG.** *Am Rev Respir Dis* 1984, **129**:444-452.
6. Nagai S, Wiker HG, Harboe M: **Isolation and partial characterization of Major protein antigens in the culture fluid of *Mycobacterium tuberculosis*.** *Infect Immun* 1991, **59**:372-382.
7. Mastumoto S, Matsuo T, Ohara N: **Cloning and sequencing of a unique antigen MPT70 from *Mycobacterium tuberculosis* H37Rv and expression in BCG using *E. coli*-Mycobacteria Shuttle Vector.** *Scand J Immunol* 1995, **41**:281-287.
8. Decobert M, LaRue H, Harel F: **Maintenance bacillus Calmette-Guérin in high-risk nonmuscle-invasive bladder cancer. How much is enough?** *Cancer* 2008, **113**:710-716.
9. Kuroda K, Brown EJ, Telle WB: **Characterization of the internalization of bacillus Calmette-Guerin by human bladder tumor cells.** *J Clin Invest* 1993, **91**:69-76.
10. Ohara N, Kitaura H, Hotokezaka H: **Characterization of the gene encoding the MPB51, one of the major secreted protein antigens of *Mycobacterium bovis* BCG, and identification of the secreted protein closely related to the fibronectin binding 85 complex.** *Scand J Immunol* 1995, **41**:433-442.
11. Kuromatsu I, Matsuo K, Takamura S: **Induction of effective antitumor immune responses in a mouse bladder tumor model by using DNA of an α antigen from mycobacteria.** *Cancer Gene Ther* 2001, **8**:483-490.
12. Mitropoulos DN: **Novel insights into the mechanism of action of intravesical immunomodulators.** *In vivo* 2005, **19**:611-612.
13. Sinn HW, Elzey BD, Jensen RJ: **The fibronectin attachment protein of bacillus Calmette-Guerin (BCG) mediates antitumor activity.** *Cancer Immunol Immunother* 2008, **57**:573-579.
14. Oh BR, Jeong DW: **Antitumor effects of bcg and cytokines against MBT-2 mouse bladder tumor cell.** *Bri J Urol* 1997, **80(SUPPL 45)**:.
15. Becich MJ, Carroll S, Ratiff TL: **Internalization of bacillus calmette-guerin by bladder tumor cells.** *J Urol* 1991, **145**:1316-1324.
16. Bevers RFM, De Boer EC, Kurth H: **BCG internalization in human bladder cancer cell lines, especially with regard to cell surface-expressed fibronectin.** *Actuel Urol* 2000, **31**:31-34.
17. Biswas D, Qureshi OS, Lee W-Y: **ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/macrophages.** *BMC Immuno* 2008, **9**:35.
18. DiPaola RS, Lattime EC: **Bacillus Calmette-Guerin mechanism of action: The role of immunity, apoptosis, necrosis and autophagy.** *J Urolo* 2007, **178**:1840-1841.
19. Chen N, Karantza-Wadsworth V: **Role and regulation of autophagy in cancer.** *Biochim Biophys Acta* 2009, **1973**:1516-1523.
20. Inbal B, Bialik S, Sabanay I: **DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death.** *J cell boil* 2002, **157**:455-468.
21. Matsumoto S, Yukitake H, Kanbara H: ***Mycobacterium bovis* Bacillus Calmette-Guérin induces protective immunity against infection by *Plasmodium yoelii* at blood-stage depending on shifting immunity toward Th1 type and inducing protective IgG2a after the parasite infection.** *Vaccine* 2001, **19**:779-787.
22. Luo Y, Yamada H, Evanoff DP: **Role of Th1-stimulating cytokines in bacillus Calmette-Guérin(BCG)-induced macrophage cytotoxicity against mouse bladder cancer MBT-2 cells.** *Clin Exp Immunol* 2006, **146**:181-188.
23. Hotokezaka H, Kitamura A, Mastumoto : **Internalization of *Mycobacterium bovis* Bacillus Calmette-Guérin into Osteoblast-like MC3T3-EL cells and bone resorptive response of the cells against the infection.** *Scand J Immunol* 1998, **47**:453-458.
24. Wei MQ, Mengesha A, Good D: **Bacterial targeted tumour therapy-dawn of a new area.** *Cancer Lett* 2008, **259**:16-17.
25. Zbar B, Rapp HJ: **Immunotherapy of guinea pig cancer with BCG.** *Cancer* 1974, **34**:1532-1540.
26. Kitamura A, Tobita T, Fuzisawa A: **Evaluation on the cell-killing effects of the alpha-antigen by electroporation.** *Jpn J Cancer Res* 2001, **92(Supp 555)**:.
27. Morales A: **Evolution of intravesical immunotherapy for bladder cancer: Mycobacterial cell wall preparation as a promising agent.** *Expert Opin Investig Drugs* 2008, **17**:1067-1073.
28. Kitamura A: **Bleomycin-mediated electrochemotherapy in mouse NR-S1 carcinoma.** *Cancer Chemother Pharmacol* 2003, **51**:359-362.
29. Ikeda N, Honda I, Yano I: **Bacillus Calmette-Guerin Tokyo172 substrain for superficial bladder cancer: Characterization and antitumor effect.** *J Urolo* 2005, **173**:1507-1512.

Mycobacteria Exploit Host Hyaluronan for Efficient Extracellular Replication

Yukio Hirayama[1], Mamiko Yoshimura[1], Yuriko Ozeki[1,2], Isamu Sugawara[3], Tadashi Udagawa[3], Satoru Mizuno[3], Naoki Itano[4], Koji Kimata[5], Aki Tamaru[6], Hisashi Ogura[7], Kazuo Kobayashi[8], Sohkichi Matsumoto[1]*

1 Department of Bacteriology, Osaka City University Graduate School of Medicine, Osaka, Osaka, Japan, **2** Sonoda Women's University, Amagasaki, Hyogo, Japan, **3** Mycobacterial Reference Center, The Research Institute of Tuberculosis, Kiyose, Tokyo, Japan, **4** Department of Molecular Oncology, Division of Molecular and Cellular Biology, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, Nagano, Japan, **5** Research Complex for the Medicine Frontiers, Aichi Medical University, Yazako, Nagakute, Aichi, Japan, **6** Department of Infectious Diseases, Bacteriology Division, Osaka Prefectural Institute of Public Health, Osaka, Japan, **7** Department of Virology, Osaka City University Graduate School of Medicine, Osaka, Osaka, Japan, **8** Department of Immunology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan

Abstract

In spite of the importance of hyaluronan in host protection against infectious organisms in the alveolar spaces, its role in mycobacterial infection is unknown. In a previous study, we found that mycobacteria interact with hyaluronan on lung epithelial cells. Here, we have analyzed the role of hyaluronan after mycobacterial infection was established and found that pathogenic mycobacteria can grow by utilizing hyaluronan as a carbon source. Both mouse and human possess 3 kinds of hyaluronan synthases (HAS), designated HAS1, HAS2, and HAS3. Utilizing individual HAS-transfected cells, we show that HAS1 and HAS3 but not HAS2 support growth of mycobacteria. We found that the major hyaluronan synthase expressed in the lung is HAS1, and that its expression was increased after infection with *Mycobacterium tuberculosis*. Histochemical analysis demonstrated that hyaluronan profoundly accumulated in the granulomatous legion of the lungs in *M. tuberculosis*-infected mice and rhesus monkeys that died from tuberculosis. We detected hyaluronidase activity in the lysate of mycobacteria and showed that it was critical for hyaluronan-dependent extracellular growth. Finally, we showed that L-Ascorbic acid 6-hexadecanoate, a hyaluronidase inhibitor, suppressed growth of mycobacteria *in vivo*. Taken together, our data show that pathogenic mycobacteria exploit an intrinsic host-protective molecule, hyaluronan, to grow in the respiratory tract and demonstrate the potential usefulness of hyaluronidase inhibitors against mycobacterial diseases.

Citation: Hirayama Y, Yoshimura M, Ozeki Y, Sugawara I, Udagawa T, et al. (2009) Mycobacteria Exploit Host Hyaluronan for Efficient Extracellular Replication. PLoS Pathog 5(10): e1000643. doi:10.1371/journal.ppat.1000643

Editor: William Bishai, Johns Hopkins School of Medicine, United States of America

Received March 24, 2009; **Accepted** October 5, 2009; **Published** October 30, 2009

Funding: This work was supported by the Japan Health Sciences Foundation; Ministry of Health, Labour and Welfare (Research on Emerging and Re-Emerging Infectious Diseases, Health Sciences Research Grants); Ministry of Education, Culture, Sports, Science, and Technology; and the United States-Japan Cooperative Medical Science Program against Tuberculosis and Leprosy. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sohkichi@med.osaka-cu.ac.jp

Introduction

Infectious diseases caused by mycobacteria are serious threats to human health. Tuberculosis is caused by infection with mycobacteria, most frequently with *Mycobacterium tuberculosis* but also with *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium canetii* and kills around 2 million people annually. Leprosy is caused by *Mycobacterium leprae* and the globally registered prevalence of leprosy was around 22,000 cases at the beginning of 2006.

The major portal of entry for mycobacterial pathogens is through the respiratory tract. The primary phase of the infection begins with inhalation of bacteria, which are then phagocytosed by alveolar macrophages in the periphery of the lungs. In addition, several lines of evidence indicate that mycobacteria interact with epithelial cells in the respiratory tract [1–4]. The recent reports show the significant role of type II pneumocytes in the pathology of tuberculosis [3,5,6]. The onset of mycobacterial diseases frequently occurs after a long latent phase. Mycobacteria are an intracellular bacterium, multiplying within host cells, but also grow extracellularly [7,8].

Macrophages phagocytose mycobacteria through interaction with several cell surface receptors, including complement receptors, mannose receptors, surfactant protein A, scavenger receptors, and Fc receptors [9]. By contrast, mycobacteria attaches to or invades lung epithelial cells through interactions with glycosaminoglycans (GAG) [10]. *M. tuberculosis*, *M. bovis* bacillus Calmette-Guerin (BCG), and *M. leprae* produce two types of GAG interacting adhesins, heparin-binding hemagglutinin (HBHA) [10,11] and mycobacterial DNA-binding protein 1 (MDP1, also called histone-like protein and laminin-binding protein in *M. leprae*) [1,12]. HBHA is secreted to the extracellular milieu from mycobacteria [13], whereas MDP1 is tightly attached on the mycobacterial cell wall [14].

We previously demonstrated that hyaluronan is a major portal for infection of mycobacteria into A549 human lung epithelial cells

Author Summary

Mycobacterium tuberculosis and *Mycobacterium bovis* are major bacterial pathogens that kill approximately 2 million people annually by causing tuberculosis. The *M. tuberculosis* complex has several strategies to parasitize the host. After infection is established, these pathogens are rarely eliminated from the host, and nowadays approximately a third of the world's human population is infected with the *Mycobacterium tuberculosis* complex. The elucidation of the parasitic mechanisms of the *M. tuberculosis* complex is important for the development of novel strategies against the disease. The major portal entry of *M. tuberculosis* complex is through the respiratory tract. On the surface of the airway, hyaluronan retains bactericidal enzymes so that they are "ready-to-use", protecting tissues from invading pathogens. Furthermore, fragmented hyaluronan produced as a result of infection is used by the immune system as a sensor of infection. Thus, hyaluronan plays a pivotal role in host defenses in the respiratory tract. However, in this study, we observed that the *M. tuberculosis* complex utilizes hyaluronan as a carbon source for multiplication. We also found that the *M. tuberculosis* complex has hyaluronidase activity and showed that it is critical for hyaluronan-dependent growth of the *M. tuberculosis* complex. This study demonstrates a novel parasitic mechanism of the *M. tuberculosis* complex and suggests that mycobacterial hyaluronidase is a potential drug target.

by interacting with MDP1 [1]. Hyaluronan is a nonsulfated linear GAG composed of thousands of repeating units of GlcNAc- (beta-1, 4)-GlcUA- (beta-1, 3) and is synthesized by 3 isoforms of hyaluronan synthases (HAS), designated HAS1, HAS2, and HAS3 in both mice and humans [15–18]. In vertebrates, hyaluronan is a ubiquitous structural component of the extracellular matrix, and is abundant in the chondral and vitreous tissues. Recent findings demonstrated that hyaluronan has a pivotal role in diverse dynamic biological functions such as embryonic development [19], cell migration [20,21], tumor transformation, [22,23], wound healing [24], and inflammation [25–27].

On the mucosal surface of the airway, hyaluronan retains bactericidal enzymes so that they are "ready-to-use", protecting mucosal tissues from invading pathogens [28]. Furthermore, in the alveolar tracts, released fragmented HA stimulates innate immune responses by activating Toll-like receptor 2 and 4 dependent pathways and initiating lung inflammation [25]. By contrast, during resolution of respiratory inflammation, immuno-stimulatory hyaluronan is taken up via the hyaluronan receptor CD44 on alveolar macrophages [26]. Thus hyaluronan plays a pivotal role in host defenses in the respiratory tract, but its role in mycobacterial infection had not been elucidated so far. In this study, we analyzed the role of hyaluronan after mycobacterial infection was established.

Results

Hyaluronan enhances the extracellular growth of mycobacteria after attachment to A549 cells

A549 cells, a type II human lung epithelial cell line, were exposed to recombinant BCG expressing luciferase (rBCG-Luc) under the control of the HSP60 promoter [14] at a multiplicity of infection (MOI) of 10 for 16 hours. Cells were then washed and various doses of hyaluronan added into the culture. Growth of BCG was monitored by luciferase activity at each time point, which is indicative of viable bacteria [14,29]. We found that exogenously added hyaluronan enhances bacterial growth in a dose-dependent manner (Figure 1A). We also confirmed this effect by counting viable bacteria using a colony forming units (CFU) assay (Figure 1C).

In our experimental setting, around 60% of the bacteria adhere to the cell surface and the remaining 40% are internalized by the cells [1]. Therefore, we next examined whether hyaluronan enhances extracellular or intracellular growth by treatment with gentamicin, which kills extracellular but not intracellular bacteria. After infection, we added gentamicin (50 μg/ml) into the culture for 6 hours and then added hyaluronan after removing gentamicin. The results showed that gentamicin treatment abrogated the growth of BCG (Figure 1B), indicating that bacterial growth occurred extracellularly. The enhanced effect of hyaluronan on bacterial growth was also abolished by gentamicin treatment (Figure 1B). This suggests that hyaluronan enhances growth of BCG attached to these cells.

We next examined if the same effects of hyaluronan can be seen in *M. tuberculosis* growth after infection to A549 cells. We infected *M. tuberculosis* H37Rv to A549 cells, then added hyaluronan, and monitored growth by counting colony-forming units (CFU). Similar to the case of BCG, we found that presence of hyaluronan enhances the growth of *M. tuberculosis* in a dose dependent manner (Figure 1D). Gentamicin treatment also abrogated the growth of *M. tuberculosis* and growth-enhancing effect of hyaluronan.

BCG utilizes hyaluronan as a carbon source

To determine why hyaluronan enhances the growth of BCG, we hypothesized that BCG can utilize it as a carbon source because hyaluronan is a polymer of disaccharides. We cultured BCG-Luc in 7H9 based carbon-starved broth in the presence (0.5 mg/ml) or absence of hyaluronan. As expected, in the carbon-starved media BCG did not grow, while the addition of hyaluronan supported the growth of BCG (Figure 2A), demonstrating that BCG can utilize hyaluronan as a carbon source.

We next compared hyaluronan with other GAG in terms of their growth supporting effect. BCG-Luc was cultured in 7H9-based carbon starved media or media including 0.5 mg/ml of each GAG as the sole carbon source. The results showed that BCG did not grow in the media supplemented with heparin or heparan sulfate. Both hyaluronan and chondroitin sulfate encouraged the growth, but hyaluronan sustained higher growth rates of BCG than chondroitin sulfate (Figure 2A). We also demonstrated that the growth supporting effect of hyaluronan is comparable to an equivalent amount of glucose (0.5 mg/ml) (Figure 2B).

In order to evaluate uptake of hyaluronan during hyaluronan-dependent growth of mycobacteria, we cultured BCG in the presence of ^{3}H-labeled hyaluronan in the media containing hyaluronan as a sole carbon source. As shown in Figure 2C, live BCG incorporated hyaluronan, whereas heat-killed bacteria did not, showing actual uptake of hyaluronan into bacteria.

M. tuberculosis can utilize hyaluronan as a carbon source, whereas neither *M. avium* nor *M. smegmatis* can

We next assessed the action of hyaluronan in the growth of virulent *M. tuberculosis* (strain H37Rv), and environmental mycobacterial species such as *M. smegmatis* (strain mc^{2}155) and *M. avium* (ATCC25291). In carbon-starved media, none of the three strains grew. However, *M. tuberculosis* H37Rv, along with BCG, multiplied in the media containing hyaluronan as a sole carbon source while neither *M. smegmatis* nor *M. avium* proliferated. After 12 days culture, optimal density (OD) at 630 nm of *M.*

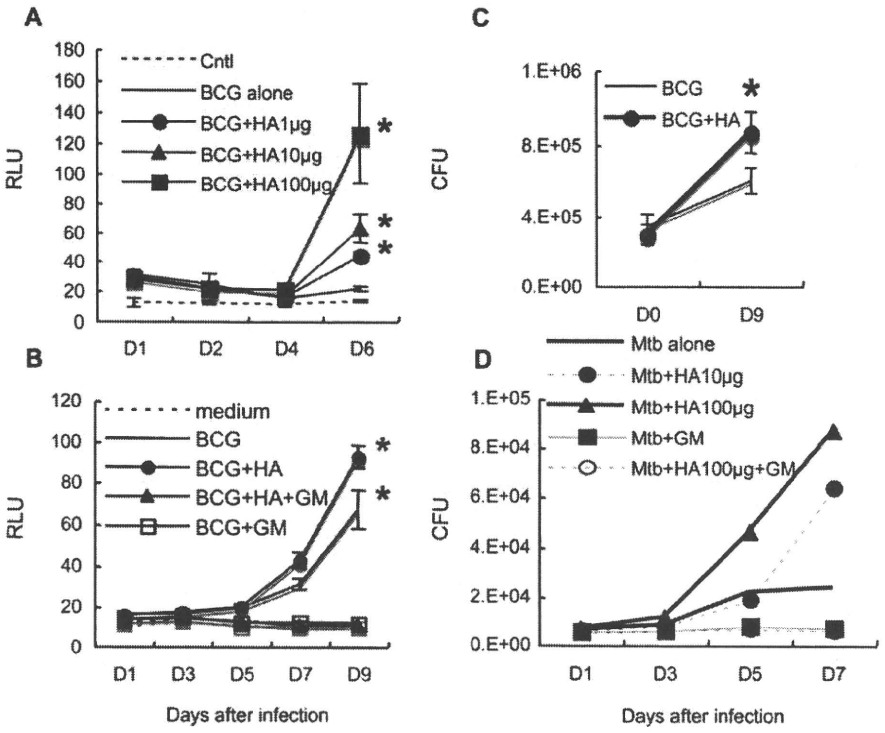

Figure 1. Effect of exogenously added hyaluronan on the growth of BCG and *M. tuberculosis* after infection of A549 cells. (A), A549 cells were infected with BCG-Luc for 16 hours at a multiplicity of infection (MOI) of 10. After removal of non-infected bacteria, different amounts of hyaluronan (HA) were added; 0 μg/200 μl (BCG alone), 1 μg/200 μl (BCG+HA1μg), 10 μg/200 μl (BCG+HA10μg), and 100 μg/200 μl (BCG+HA100μg) before culture at 37°C under 5% CO_2. Cells were lysed by adding 5% Triton X (0.5% final) at each time point (1, 2, 4, and 6 days) and bacterial growth was monitored by luciferase activity. The results are expressed as mean±the standard deviation ($n=3$). Relative luciferase unit (RLU). Cntl, control without BCG-Luc infection. For statistical analysis, a two-way ANOVA with Bonferroni Post tests were used to obtain *P*-values for each time point, comparing the various growth conditions to the control. *$P<0.01$. (B), Gentamicin (GM) treatment abrogated the growth of BCG-Luc after infection of A549 cells. A549 cells were infected with BCG-Luc for 16 hours at MOI of 10. After removal of non-infected bacteria, hyaluronan was added to be 500 μg/ml for some wells (BCG+HA, BCG+HA+GM) and cultured at 37°C under 5% CO_2 in the presence or absence of 10 μg/ml GM (BCG+HA+GM, BCG+GM). Growth of BCG was monitored by luciferase activity. The results are expressed as mean±the standard deviation ($n=3$). RLU. Cntl, control without BCG-Luc infection. (C), The enhancing effect of hyaluronan on BCG growth was confirmed by colony forming unit (CFU). A549 cells were infected with BCG-Luc for 16 hours at MOI of 10. After removal of non-infected bacteria, BCG-Luc was grown in the presence or absence of 50 μg/ml HA. Cells were lysed at each time point and serial 10-fold dilutions were plated in duplicate on Middlebrook 7H11 agar (Difco) supplemented with oleic acid, albumin, dextrose and catalase (Difco). After incubation for 3–4 weeks at 37°C, colonies were counted and the number of CFU was calculated per well (1 ml). The results are expressed as mean±the standard deviation ($n=6$). (D), A549 cells were infected with *M. tuberculosis* H37Rv and then different amounts of hyaluronan (HA) were added; 0 μg/200 μl (Mtb alone), 10 μg/200 μl (Mtb+HA10μg), and 100 μg/200 μl (Mtb+HA100μg). Gentamycin (50 μg/ml) was added to some wells with (Mtb+HA100 μg+GM) or without (Mtb+GM) 100 μg/200 μl hyaluronan. Cells were lysed by adding 5% Triton X (0.5% final) and the number of viable bacteria was determined by plating dilutions of the samples for CFU on 7H11-OADC agar.

doi:10.1371/journal.ppat.1000643.g001

tuberculosis culture increased to 0.32±0.038 from 0.01 (day 0). We then compared hyaluronan and other GAGs in terms of growth supportive effects on *M. tuberculosis*. Similar to the case of BCG, hyaluronan most effectively enhanced the growth of *M. tuberculosis* among tested GAGs (Figure 3).

Detection of hyaluronidase activity in mycobacteria

Because hyaluronan is a long chain consisting of the repeat of two monosaccharides at over 2×10^5 Da, we hypothesized that extracellular cleavage of the polymer would be required before taken up by cells. Therefore, we next assessed hyaluronidase activity in mycobacteria. Hyaluronan was incubated in the presence or absence of cell lysates derived from BCG before precipitation by phenol/chloroform extraction. Precipitates were then fractionated by polyacrylamide gel electrophoresis (PAGE) and visualized by alcian blue staining as described previously [30]. Hyaluronan was separated into discrete ladder-like bands by electrophoresis after incubation with BCG lysate (Figure 4A), demonstrating that BCG possesses hyaluronidase activity.

Hyaluronidase activity is critical for hyaluronan-dependent growth

We then addressed whether hyaluronidase activity is crucial for hyaluronan -dependent growth of mycobacteria. L-Ascorbic acid 6-hexadecanoate (Vcpal) is shown to be a potent inhibitor of hyaluronidase [31]. We investigated the effect of Vcpal on hyaluronidase activity of BCG and found that hyaluronidase activity was abolished in the presence of 25 μM Vcpal (Figure 4A, lane 4).

We next examined the effects of Vcpal on the growth of BCG. BCG-Luc was cultured in modified 7H9 media containing hyaluronan (0.5 mg/L) as the sole carbon source or 7H9-ADC

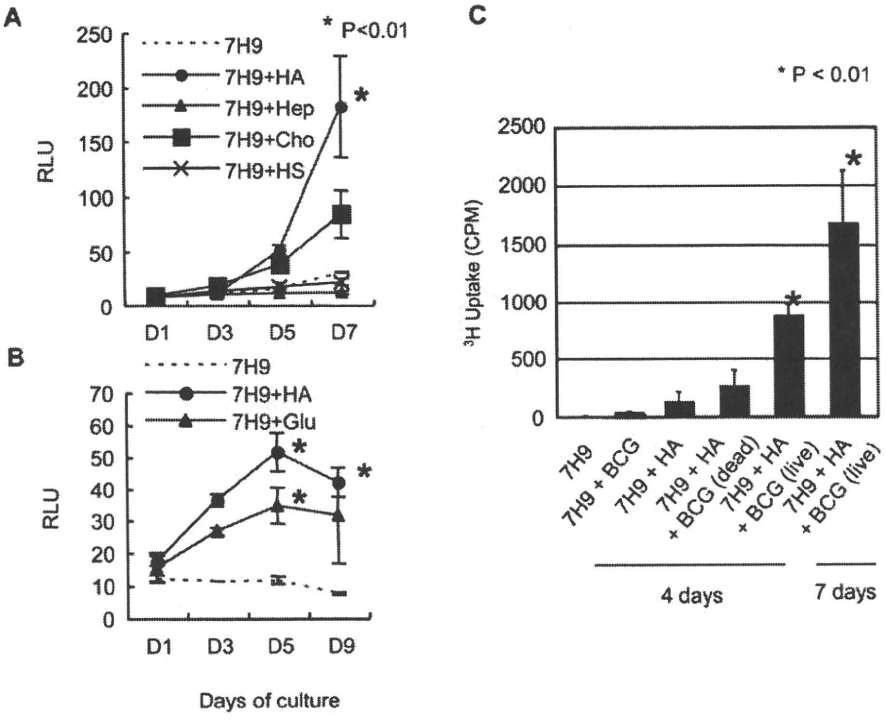

Figure 2. Effect of hyaluronan on BCG growth in carbon-starved 7H9 media. (A) (B), BCG-Luc was cultured in carbon-starved 7H9 media (7H9), or carbon-starved 7H9 media supplemented with 500 μg/ml of HA (7H9+HA), heparin (7H9+Hep), chondroitin sulfate C (7H9+Cho), heparan sulfate (7H9+HS), or glucose (7H9+Glu) at 37°C. Growth of BCG was monitored by luciferase activity. The results are expressed as mean±the standard deviation ($n = 3$). For statistical analysis, a two-way ANOVA with Bonferroni Post tests were used to obtain *P*-values for each time point, comparing the various growth conditions to the control. *$P<0.01$. (C), Uptake of ^{3}H-hyaluronan (HA) by BCG in carbon-starved 7H9 media. Live and heat-killed BCG cells were cultured in carbon-starved 7H9 media in the presence or absence of ^{3}H-labeled hyaluronan for 4 or 7 days. The uptake of ^{3}H-laveled hyaluronan was measured by a gamma counter.
doi:10.1371/journal.ppat.1000643.g002

complete media, which contains Tween 80, glycerol, and dextrose as carbon sources and BSA. We found that 25 μM Vcpal did not change the growth rate of BCG in 7H9-ADC complete media, while it abolished the growth of BCG in the media containing hyaluronan as the sole carbon source (Figure 4B).

We also examined the effect of Vcpal on the growth of *M. tuberculosis*. *M. tuberculosis* H37Rv was cultured in the media with or without Vcpal (50 and 100 μM). Vcpal suppressed the growth of *M. tuberculosis* in the media containing hyaluronan as a sole carbon source but not the growth in conventional 7H9-ADC media (Figure 4C). Other hyaluronidase inhibitors, such as apigenin and quercetin [32], also inhibited hyaluronan dependent growth of *M. tuberculosis* as shown in Figure S1. These results indicate that hyaluronidase activity is essential for both BCG and *M. tuberculosis* when utilizing hyaluronan as a carbon source.

Vcpal blocks growth of BCG after attachment to A549 cells

We next examined whether Vcpal suppresses the enhancing effect of hyaluronan on the growth of BCG after attachment to A549 epithelial cells. After exposure to BCG-Luc, hyaluronan was added with or without Vcpal (25 μM) into the culture and growth of BCG was monitored by measuring luciferase activity. After 6 days culture, RLU values of BCG-Luc increased to 36.6±7.5 RLU or 52.6±18.7 RLU in the absence or presence of hyaluronan, respectably. Adding Vcpal abrogated the enhanced effects of hyaluronan (29.3±2 RLU), demonstrating that BCG utilized exogenously added hyaluronan as a carbon source after infection to A549 cells.

BCG and *M. tuberculosis* efficiently utilize hyaluronan synthesized by HAS1 and HAS3

This work so far on the growth of mycobacteria has been performed with hyaluronan purified from human umbilical cord (Sigma). In order to elucidate whether mycobacteria can use hyaluronan actually synthesized *in situ* by mammalian cells, we employed the previously established stable human HAS1–3 expressing rat 3Y1 fibroblasts [15]. 3Y1 rat fibroblasts do not produce detectable hyaluronan themselves but each transfectant produces different sized hyaluronan. Both HAS1 and HAS3 transfectants secret hyaluronan with broad size distributions with molecular masses between 2×10^5 to $\sim2\times10^6$ Da, while the HAS2 transfectant secretes extremely large hyaluronan at an average molecular mass of $>2\times10^6$ Da [15]. We analyzed the level of hyaluronan production by utilizing a hyaluronan-binding protein (HABP)-based ELISA assay and confirmed that the HAS2 transfectant produced high levels of hyaluronan (235.7 μg/mL in the culture media), while the HAS3 transfectant synthesized the smallest amount of hyaluronan (15.9 μg/mL). The HAS1 transfectant produced moderate levels of hyaluronan (85.3 μg/mL), and the empty vector transfectant did not produce detectable amounts of hyaluronan.

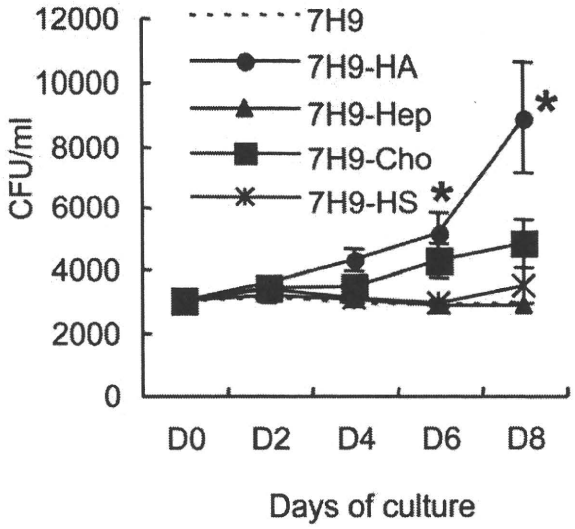

Figure 3. Effect of GAG on the growth of *M. tuberculosis* in carbon starved media. *M. tuberculosis* H37Rv was cultured in carbon-starved 7H9 media (7H9), or carbon-starved 7H9 media supplemented with 500 μg/ml of HA (7H9+HA), heparin (7H9+Hep), chondroitin sulfate C (7H9+Cho), or heparan sulfate (7H9+HS) at 37°C. Bacterial numbers were monitored by determining CFU at each time point. The results are expressed as mean±the standard deviation ($n=3$). For statistical analysis, a two-way ANOVA with Bonferroni Post tests were used to obtain *P*-values for each time point, comparing the various growth conditions to the control. *$P<0.01$.
doi:10.1371/journal.ppat.1000643.g003

Each human HAS transfectant was exposed to BCG-Luc and the growth kinetics of the bacteria were monitored by luciferase activity. The results showed that BCG grew after attachment to 3Y1 cells transfected with HAS1 and HAS3 but not with HAS2 or empty vector (Figure 5A). In addition, we found that hyaluronidase treatment of HAS1 transfected cells enhanced the growth of BCG (Figure 5B). These results suggest that shorter sized chains of hyaluronan are preferential for BCG growth.

We also monitored the growth of *M. tuberculosis* H37Rv after infection to these HAS transfectant cells. Along with the case of BCG, HAS1 and HAS3 but not HAS2-tranfectants supported the growth of *M. tuberculosis* (Figure 5C).

Production of hyaluronan in *M. tuberculosis*-infected lungs

To see if hyaluronan is present at the site of infection of *M. tuberculosis*, we assessed the expression of hyaluronan synthases (HAS1, HAS2, and HAS3) in the lungs of BALB/c mice infected with the *M. tuberculosis* H37Rv strain, using the low-dose aerosol infection model. Total RNA was extracted from the lungs after 1, 3, 5, 7, 14, and 21 days of infection, and analyzed for HAS1, HAS2, and HAS3 mRNA transcription by reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 6A). The data showed that HAS1 mRNA expression increased after infection and was maintained at all time points (Figure 6A).

We next determined if hyaluronan is present in alveoli using biotin-conjugated hyaluronan-binding protein (HABP) and histochemical analysis. Before infection, hyaluronan was located on the surface of the airways and alveoli (Figure 6B). After *M. tuberculosis* infection, hyaluronan levels were profoundly increased and accumulated in the granulomatous legion (Figure 6B). Taken together, these data indicate that the major hyaluronan synthase in the lungs is HAS1 both before and after *M. tuberculosis* infection and hyaluronan accumulates in the tuberculosis lesion.

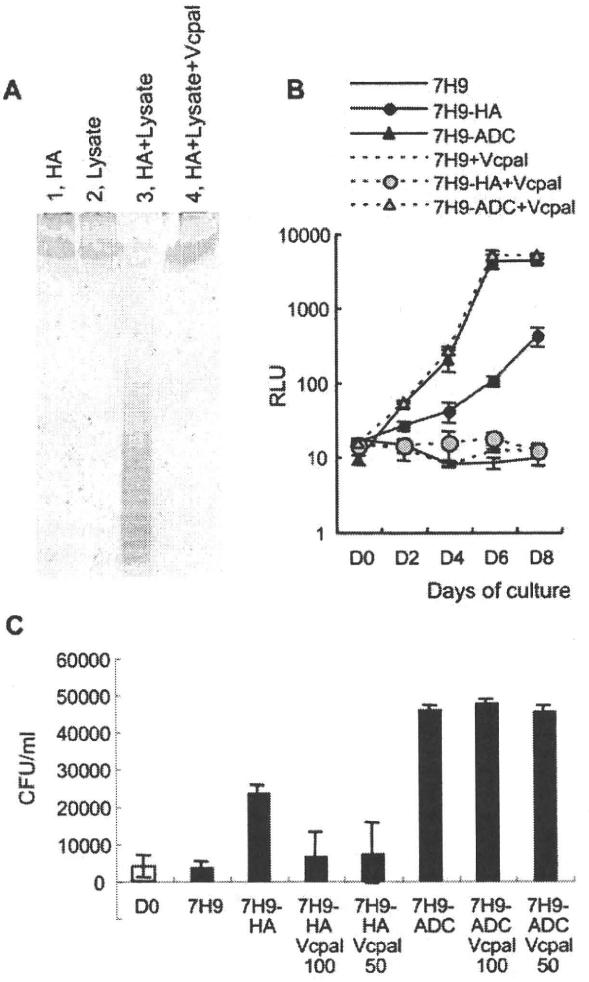

Figure 4. Hyaluronidase activity in mycobacteria and the effect of hyaluronidase inhibitor on hyaluronan-dependent growth of BCG and *M. tuberculosis*. (A), One mg/ml of hyaluronan and 700 μg/ml of BCG cell lysate was mixed and incubated for 3 days in the presence (HA+Lysate+Vcpal) or absence (HA+Lysate) of ascorbic palmitate (Vcpal), an inhibitor of hyaluronidase. As controls, hyaluronan alone (lane 1, HA) or BCG cell lysate alone (lane 2, Lysate) was treated in the same way. Hyaluronan was precipitated by ethanol after phenol extraction and resolved in water. Then hyaluronan was fractionated by PAGE gel electrophoresis and visualized by staining with alcian blue. (B), BCG-Luc (0.01 OD at 630 nm) was cultured in carbon-starved 7H9 media (7H9), media containing hyaluronan (500 μg/ml) as a sole carbon source (7H9-HA), or complete 7H9-ADC media (7H9-ADC) in the presence or absence of 25 μM Vcpal (+Vcpal), an inhibitor of hyaluronidase. The growth of bacteria was monitored by luciferase activity. RLU, relative luciferase unit (RLU). The results are expressed as mean±the standard deviation ($n=3$). (C), The effect of Vcpal on the growth of *M. tuberculosis*. *M. tuberculosis* H37Rv was cultured in carbon starved 7H9 media (7H9), media containing 100 μg/ml hyaluronan as a sole carbon source (7H9-HA), or conventional 7H9-ADC media (7H9-ADC) with or without 50 (50) or 100 (100) μM of Vcpal for 8 days (closed bars). Bacterial number was determined by plating dilutions for CFU on 7H9-OADC agar and compared to that of Time 0 (D0, open bar).
doi:10.1371/journal.ppat.1000643.g004

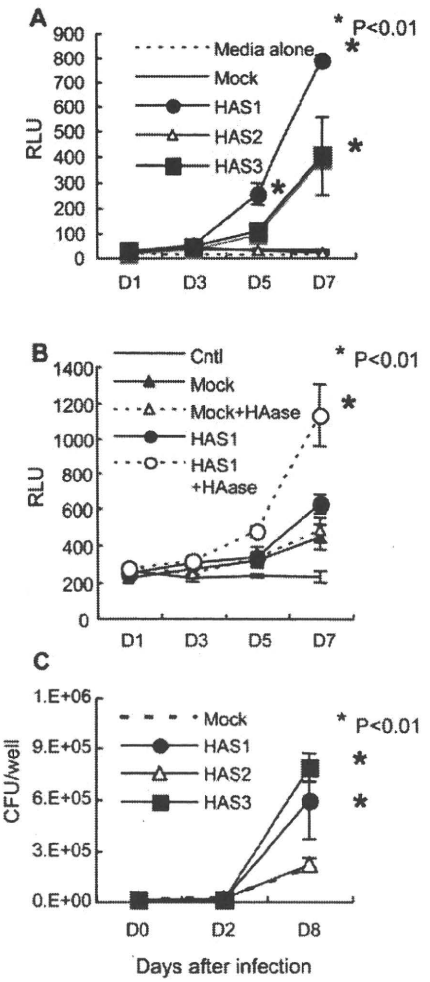

Figure 5. The effect of 3 hyaluronan synthases on the growth of BCG and *M. tuberculosis*. (A), Established transfectant cells (Rat 3Y1 fibroblasts) with control vector (Mock) or vector to express hyaluronan synthase 1 (HAS1), HAS2 (HAS2), or HAS3 (HAS3) were cultured in the presence of BCG-Luc or media alone. The growth of bacteria was monitored by luciferase activity. RLU, relative luciferase unit. The results are expressed as mean±the standard deviation ($n=3$). For statistical analysis, a two-way ANOVA with Bonferroni Post tests were used to obtain *P*-values for each time point, comparing the various growth conditions to the control. *$P<0.01$. (B), Hyaluronidase (HAase) treatment enhances the growth of BCG after infection to HAS1-tranfected cells. After 16 hours exposure of BCG-Luc to transfected cells with control vector (Mock) or vector expressing HAS1 (HAS1), unbound bacteria were washed and cultured in the presence or absence of 2 units/ml of hyaluronidase (HAase). Bacterial growth was monitored by the luciferase activity (RLU). Cntl, HAS1-transfectant cells without infection of BCG-luc. The results are expressed as mean±the standard deviation ($n=3$). For statistical analysis, a two-way ANOVA with Bonferroni Post tests were used to obtain *P*-values for each time point, comparing the various growth conditions to the control. *$P<0.01$. (C), The growth of *M. tuberculosis* H37Rv after infection to transfectant 3Y1 fibroblasts with control vector (Mock) or vector to express hyaluronan synthase 1 (HAS1), HAS2 (HAS2), or HAS3 (HAS3) was monitored by CFU. The results are expressed as mean±the standard deviation ($n=3$). For statistical analysis, a two-way ANOVA with Bonferroni Post tests were used to obtain *P*-values for each time point, comparing the various growth conditions to the control. *$P<0.01$.
doi:10.1371/journal.ppat.1000643.g005

Detection of hyaluronan in the lungs of rhesus monkeys that died of tuberculosis

M. tuberculosis-infected mice had numerous sites of granulomatous inflammation in their lungs but in primates, tuberculosis granulomas are well-organized and tighter. We next studied hyaluronan in the lung granuloma of *M. tuberculosis* H37Rv-infected rhesus monkeys by staining with alcian blue, which is commonly used dye to detect GAG. The dye stained the surrounding region of well-organized granuloma (Figure 7A) and the staining was largely abolished by treatment with hyaluronidase (Figure 7B), showing that hyaluronan is a major GAG surrounding granuloma. Acid-fast bacilli (arrow heads in Figure 7C) were located in alcian blue stained areas, thus suggesting a strong correlation between the localization of the tubercle bacilli and hyaluronan.

Vcpal suppresses mycobacterial growth *in vivo*

Finally, we addressed the effect of Vcpal on the growth of BCG in BALB/c mice. Mice were infected with BCG intravenously through their tail veins. One day after BCG challenge, the hyaluronidase inhibitor Vcpal (0.4 or 1.64 mg/dose) was injected every day thorough the tail veins for 14 days. Two days after the final injection, the mice were euthanized and viable bacteria counts were determined by the CFU assay. As a positive control, we also treated mice with amikacin (Amk), which kills extracellular but not intracellular mycobacteria, by an intramuscular injection. The results showed that Vcpal apparently suppressed growth of BCG in the lungs, similar to Amk (Figure 8).

Discussion

Although hyaluronan is crucial for both structural and physiological properties in the alveolar spaces, its role in mycobacterial infection was previously unknown. We demonstrated before that hyaluronan is the major attachment site of both BCG and *M. tuberculosis* in the infection of A549 cells, which itself produced hyaluronan [1] probably depending on HAS3 and HAS2 (Figure S2). In this study, we further extended our research and studied the role of hyaluronan after infection was established.

First, we examined the effect of hyaluronan on the growth of BCG after infection of A549 cells. BCG is an attenuated strain of the virulent *M. bovis* and is a live vaccine against tuberculosis. Because BCG bacilli share biological and pathological characteristics [33] and over 99.5% of their genome with that of *M. tuberculosis* [34], BCG is frequently utilized for the analysis of virulence of *M. tuberculosis*.

Utilizing BCG, we first found that exogenously added hyaluronan enhances the growth of BCG after incubation with A549 cells. We found that gentamicin treatment abrogated the growth of both BCG and *M. tuberculosis*, showing that these mycobacteria grow outside A549 cells. By contrast, this BCG strain (Pasteur) and *M. tuberculosis* H37Rv grew inside J774 mouse macrophages. These data apparently suggest that intracellular spaces in A549 cells are not suitable for the growth of mycobacteria.

Mycobacteria are intracellular pathogens and survive in macrophages by blocking phagosome-lysosome fusion (P-L fusion) at the stage of Rab5–Rab7 conversion [35–37]. Mycobacteria can infect non-professional epithelial cells in addition to alveolar macrophages. However, the exact mechanisms of how mycobacteria invade and persist or are killed in epithelial cells are unknown. Clemens and Horwitz demonstrated that mycobacterial phagosomes acquired Rab7 in HeLa epithelial cells, suggesting that P-L fusion is not efficiently blocked. Furthermore, Takeda's group recently found that type II pneumocytes produce antimi-

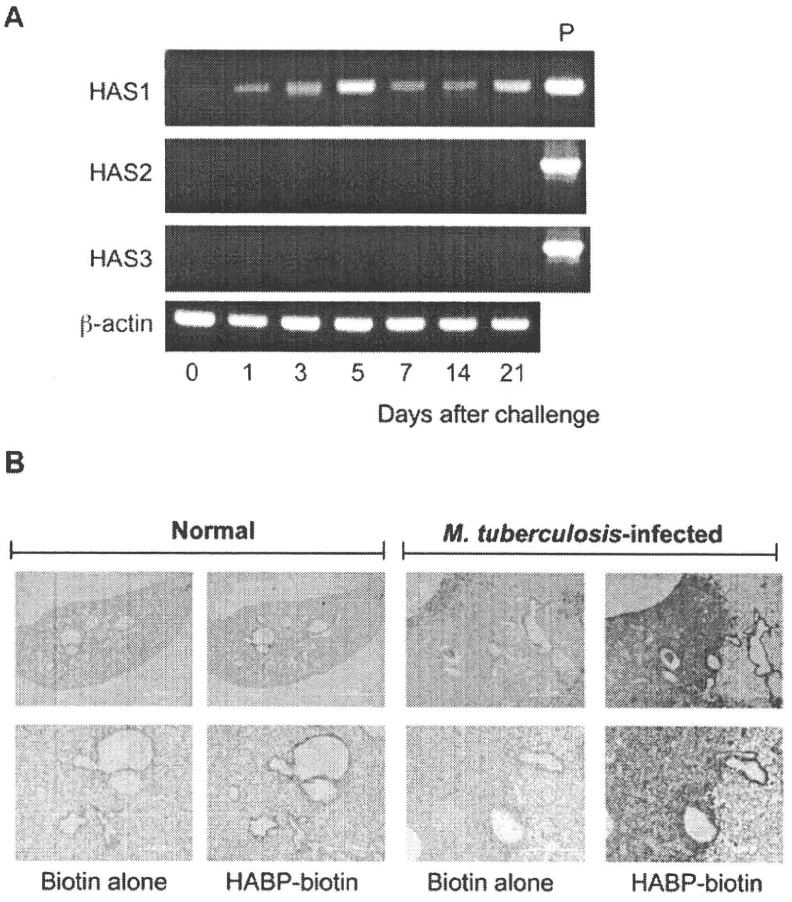

Figure 6. Production of hyaluronan during *M. tuberculosis* infection in mice. (A), BALB/c mice were aerogenically infected with *M. tuberculosis* H37Rv (around 10 CFU/lung). At the indicated periods, mice were euthanized and total RNA was extracted from the lungs. Transcription of each gene encoding HAS1, HAS2, HAS3 and beta-actin was analyzed by RT-PCR. Three mice were analyzed for each time point and representative data are presented. P, positive control of PCR employing the cDNA clone of each HAS gene as a template. (B), After euthanized, lungs from uninfected mice (Normal) or mice 21 days after infection with *M. tuberculosis* H37Rv (*M. tuberculosis* infected) were removed and histological sections were made by standard methods including formalin fixation, dehydration, and embedding in paraffin. Biotinylated hyaluronan-binding protein (HABP-biotin) was used to stain the hyaluronan in the lungs. Biotin alone was used as control straining (Biotin alone). Avidin-conjugated alkaline phosphatase and chromogen as the substrate were used to generate a red reaction product. Digital images of representative sites were acquired at ×20 (upper pictures) or ×100 (lower pictures) magnification. Experiments were performed at least three times using 5 mice for each group.
doi:10.1371/journal.ppat.1000643.g006

crobial peptides, secretory leukocyte protease inhibitor and Lipocalin 2, which have potent anti-mycobactericidal activities [5,6]. Such bactericidal molecules may contribute to the inhibition of intracellular growth of mycobacteria within type II pneumocytes. These data suggest that intracellular trafficking of mycobacteria-containing vacuoles and intracellular states of mycobacteria are different from that in macrophages.

We found that both BCG and *M. tuberculosis* grew in the media containing hyaluronan as the sole carbon source (Figure 2A and 3). In addition to hyaluronan, mammals synthesize several GAGs, but hyaluronan most strongly supported the growth of BCG among GAGs and is comparable with glucose (Figure 2). By contrast, environmental mycobacteria, such as *M. smegmatis* and *M. avium*, failed to use hyaluronan as a carbon source. These data help us to understand why pathogenic mycobacteria have the ability to adhere to hyaluronan and metabolize it. It is reasonable to assume that this property is a great advantage, allowing them to grow in the hyaluronan-rich respiratory organs of their hosts.

Because hyaluronan is a long carbon chain, we considered that cleavage must be an essential step for its use as a carbon source, and indeed found hyaluronidase activity in BCG (Figure 4). Although certain other species of bacterial pathogens, such as *Streptococcus*, *Staphylococcus*, and *Streptomyces*, produce hyaluronidases [38], there has been no report of hyaluronidase of mycobacteria. This is the first report showing hyaluronidase activity in mycobacteria.

There are two main groups of hyaluronidases identified to date. One group is endo-β-*N*-acetyl-hexosaminidase or endo-β-glucuronidase, which degrades hyaluronan by hydrolysis [39]. These enzymes are distributed in some vertebrates including mouse and human. Others are lyase type hyaluronidase that degrade hyaluronan by β-elimination [39]. Bacterial hyaluronidases are lyases, which are unstable but have stronger activity than those of vertebrates, and generate unsaturated products, which is more suitable for energy supply than saturated hyaluronan. Therefore, it is reasonable to consider that mycobacteria have the lyase type of

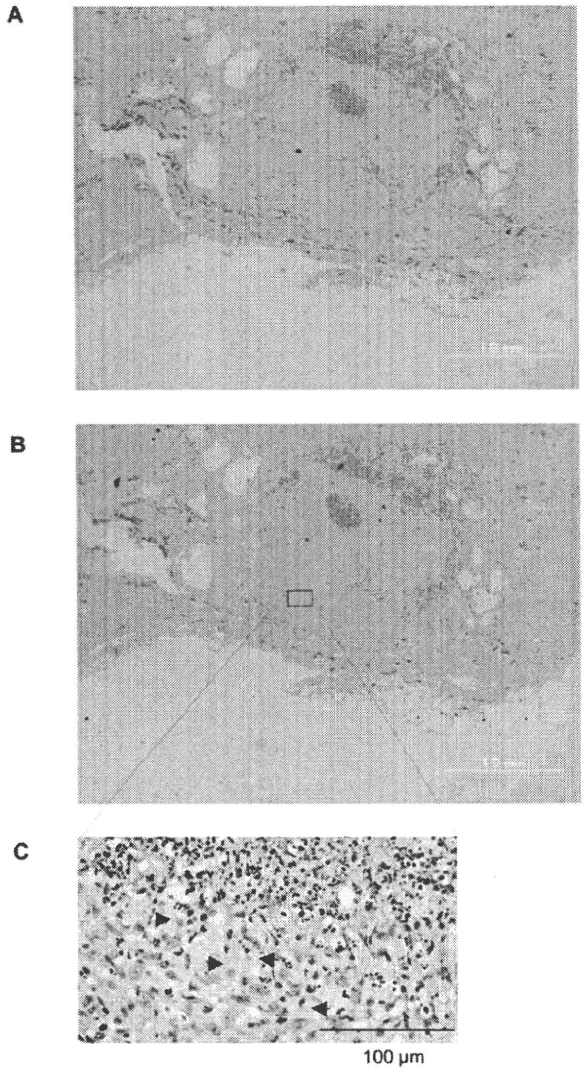

Figure 7. Presence of hyaluronan in the lungs of rhesus monkeys that died from tuberculosis. The lung sections were obtained from rhesus monkeys that had died of tuberculosis after challenge with 3,000 CFU/lung of *M. tuberculosis* H37Rv intratracheally. The sections were stained with alcian blue with (B) or without (A) pretreatment of hyaluronidase and counterstained with nuclear fast red. The section was also stained with Ziehl-Neelsen to demonstrate the presence of acid-fast bacilli (arrow heads) (C).
doi:10.1371/journal.ppat.1000643.g007

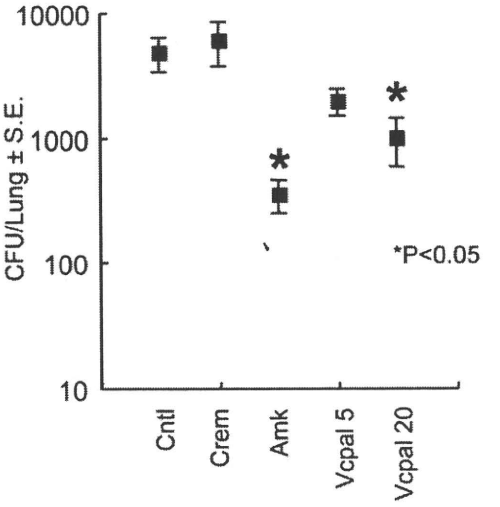

Figure 8. Vcpal suppresses the growth of mycobacteria in mouse lungs. BALB/c mice were infected with 10^6 CFU of BCG (Pasteur) intravenously. One day after the challenge, mice were treated with amikacin (Amk) and Vcpal every day for 14 days. Two days after final treatment, mice were euthanized and their lungs were homogenized. Lung pastes were serially diluted and plated in duplicate on Middlebrook 7H11 OADC agars. After incubation for 3–4 weeks at 37°C, colonies were counted and the number of CFU was calculated per lung. For statistical analysis, a two-way ANOVA with Bonferroni Post tests were used to obtain *P*-values to determine the effect of Vcpal and amikacin on bacterial growth to the control. *$P<0.05$. Cntl, control mice without treatment.
doi:10.1371/journal.ppat.1000643.g008

hyaluronidase. Although hyaluronidase is not yet described in the genome of either *M. tuberculosis* [33] or BCG [34], there are approximately 40 lyases. One of these lyases may be responsible for degradation of hyaluronan. Defining which enzyme is responsible for cleavage of hyaluronan is next important issue. Most hyaluronidases in mammals and bacteria display redundancy in recognition of their GAG substrates. Our data show that chondroitin sulfate also supported the growth of BCG (Figure 2). This may imply that hyaluronidase(s) of BCG cleave chondroitin sulfate as well.

Hyaluronan possesses many properties *in vivo* and it is believed that these biological activities are dependent on its size [40–42]. Although hyaluronan is composed of simple repeating disaccharides, its secondary structure is flexible. It is affected by the numbers of intramolecular hydrogen bonds, their location, and hydrophobic interactions [43,44], all of which are increased as the size of the chains increase. Dynamic laser light-scattering analysis showed that the rod-like structure of low molecular weight hyaluronan changes to a stiff coil structure beyond a molecular weight of 1×10^5 Da [45]. Taken together, it is conceivable that hyaluronan synthesized by HAS1 and HAS3 exhibits a different structure from that synthesized by HAS2. Employing HAS transfectants, we found that both BCG and *M. tuberculosis* utilize hyaluronan synthesized only by HAS1 or HAS3 for multiplication (Figure 5A and 5C).

The fact that BCG and *M. tuberculosis* grow when co-cultured with HAS1 and HAS3 but not HAS2 transfected cells (Figure 5A and 5C) suggests that HAS1 and HAS3-synthesized hyaluronan supports the growth of mycobacteria in the human body. We founds that HAS1 is the major hyaluronan synthase in *M. tuberculosis*-infected mouse lungs (Figure 6A). HAS1 is expressed in immune cells, such as dendritic cells and T cells [46]. To clarify what kind of cell expresses HAS1 during mycobacterial infection is the next important issue.

In spite of the importance of hyaluronan in host protection in the lungs, its role in mycobacterial diseases had not been elucidated. In this study, we demonstrated that BCG and *M. tuberculosis* can utilize it as a carbon source. Hyaluronan was observed in the granulomatous region of mice lungs infected with *M. tuberculosis* (Figure 6). Furthermore, *M. tuberculosis* bacilli were residing in the region where hyaluronan was located in the lungs of monkeys that had died from tuberculosis (Figure 7). We also showed that blocking hyaluronidase inhibited *in vivo* multiplication

of BCG (Figure 8). These results suggest that pathogenic mycobacteria have evolved to exploit the intrinsically host-protective molecule, hyaluronan as a nutrient to grow. Similar behavior of pathogenic mycobacteria was observed during infection of macrophages, that is, BCG is phagocytized in a cholesterol-dependent manner [47] and utilizes cholesterol as a carbon source to survive in activated macrophages [48]. It is likely that mycobacteria developed several strategies to obtain nutrients under nutrient-limited conditions.

After digestion of hyaluronan, it must be incorporated into mycobacteria through specific receptors or membrane proteins. Based on our results and consideration, hyaluronidase and a potential transporter of fragmented hyaluronan of pathogenic mycobacteria are potential drug targets.

Materials and Methods

Animal studies

All animals were maintained under specific pathogen-free conditions in the animal facilities of Osaka City University Graduate School of Medicine and in a biosafety-level-3 facility at The Research Institute of Tuberculosis according to the standard guidelines for animal experiments at each institute.

Culture medium and reagents

RPMI 1640 media, L-glutamine, fetal bovine serum, HEPES, hyaluronan from human umbilical cord, heparin from porcine intestinal mucosa and heparan sulfate from bovine kidney were purchased from Sigma-Aldrich (St. Louis, MO). Chondroitin sulfate A and C were purchased from Calbiochem (Gibbstown, NJ). For conventional culture of mycobacteria, Middlebrook 7H9 medium (Becton Dickinson) supplemented with 0.085% NaCl, 10% albumin-dextrose-catalase (BD Biosciences), 0.2% glycerol, and 0.05% Tween 80 (7H9-ADC) or 7H11-agar supplemented with 0.085% NaCl, 10% oleic acid-albumin-dextrose-catalase (BD Biosciences), and 0.2% glycerol (7H11-OADC) were used. 7H9 medium (Becton Dickinson) supplemented with 0.085% NaCl and 0.1% albumin was used as a carbon-starved 7H9 medium.

Effect of hyaluronan on extracellular growth of BCG and *M. tuberculosis* after infection to A549 cells

A549 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES and 5.5×10^{-5} M 2-mercaptoethanol (complete culture medium) at 37°C in an atmosphere of 5% CO_2. Cells were suspended at 2×10^5/ml in complete culture medium and 1 ml of cell suspension was dispensed into individual wells of a 24-well polystyrene plate (BD Biosciences, San Jose, CA). Plates were incubated at 37°C for 24 h and were washed with serum-free RPMI 1640 medium to remove nonadherent cells. Wells were then refilled with 1 ml of complete culture medium. *M. bovis* BCG or *M. tuberculosis* cell suspension was prepared as described previously [1]. The bacterial cell suspension was added to A549 cells at multiplicities of infection (MOI) of 10. After 16 (BCG) or 4 (*M. tuberculosis*) h incubation, unbound bacteria were removed by washing with serum-free RPMI 1640 three times. After adding 1 ml of fresh complete culture medium to each well, hyaluronan solution was added to final concentrations ranging from 5 to 500 μg/ml. Cells were collected periodically for luciferase or CFU assays.

Luciferase assays

Construction of BCG expressing luciferase was described previously [1]. Luciferase activity was measured using the luciferase assay system from Promega (Madison, WI) according to the manufacturer's protocol on a Wallac 1420 manager as described previously [14].

Effect of gentamicin on mycobacterial growth after infection to A549 cells

A549 cells in 96-well polystyrene plates (8×10^4/well) were infected with BCG-Luc or *M. tuberculosis* at MOI of 10 at 37°C. After 16 (BCG) or 4 (*M. tuberculosis*) h, the monolayers were washed three times with RPMI 1640 medium to remove extracellular bacteria. Fresh complete culture medium containing 1 mg/ml of hyaluronan and 50 μg/ml of gentamicin were added to each well (200 μl/well) and incubated at 37°C. Cells were collected periodically for detection of luciferase activity of BCG-Luc or CFU assay of *M. tuberculosis*.

Evaluation of glucose and GAG as carbon sources for growth of mycobacteria

BCG-Luc or *M. tuberculosis* was adjusted to a concentration of 1×10^4 CFU/ml in carbon-starved 7H9 medium described previously [14], and 200 μl of bacterial cell suspension was added to 96-well polystyrene plates. Heparin, heparan sulfate, chondroitin sulfate, hyaluronan or glucose was added to appropriate wells to a final concentration of 500 μg/ml. Plates were incubated at 37°C and bacterial cells were collected periodically for detection of luciferase activity of BCG-Luc or CFU assay of *M. tuberculosis*.

Evaluation of ingestion of hyaluronan into mycobacteria

BCG Pasteur was grown aerobically in 7H9-ADC medium at 37°C. Cells were then collected by centrifugation and half of the cells were heat-killed by heating at 65°C for 30 min. Then bacteria were washed, resuspended by carbon-starved 7H9 medium and adjusted to an optical density at 600 nm of 0.07. One hundred microliters of cell suspension was added to 100 ml of carbon-starved 7H9 with or without 6 mg of ^{3}H-labeled hyaluronan and 14 mg of non-labeled hyaluronan (final concentration of 100 mg/L of total hyaluronan). Cells were then incubated at 37°C. After incubation, cells were harvested by use of a Scatron Harvester (Scatron) onto a glass fiber filter. The incorporated radioactivity was measured in a gamma counter (ALOKA ARC-2000).

Effect of hyaluronan on mycobacterial growth

M. tuberculosis strain H37Rv, *M. smegmatis* strain mc^{2}155 and *M. avium* strain type4 were grown in carbon-starved 7H9 medium containing 0.5 mg/ml of hyaluronan, and the cultures were monitored periodically for their optical density at 600 nm (*M. tuberculosis and M. smegmatis*) or CFU (*M. tuberculosis* and *M. avium*).

Preparation of oligosaccharides from hyaluronan digested by crude extracts of BCG

BCG was grown in 7H9-ADC medium to mid-log phase. After incubation, bacterial cells were harvested, washed three times with ice-cold PBS (pH 6.0) and resuspended in the same buffer. To disrupt bacterial cells, the cell suspension was added to a screw-capped tube containing glass beads (diameter, 1.0 mm) and the tube was oscillated on a Mini-Bead Beater (Cole-Parmer). The tube was centrifuged at 10,000×*g* for 10 min, and the supernatant containing the bacterial protein extract was collected into a new tube. The protein solution was then mixed with 1 mg/ml of hyaluronan in PBS (pH 6.0) at 37°C. After incubation for 24 h, the solution was mixed with an equal volume of phenol to remove protein. The mixture was centrifuged at 10,000×*g* for 10 min and the supernatant was collected for PAGE analysis.

Polyacrylamide Gel Electrophoresis (PAGE) of hyaluronan

PAGE analysis of hyaluronan was performed as previously described by Ikegami-Kawai *et al.* [30] with minor modifications. The PAGE mini-slab gels contained 12.5% acrylamide, 0.32% *N, N'*-methylene bis-acrylamide in 0.1 M Tris-borate-1 mM Na_2EDTA (TBE, pH 8.3). For the electrophoretic run, samples containing hyaluronan were mixed with one-fifth volume of 2M sucrose in TBE and 10 µl of the mixtures was applied directly to the gel. Bromophenol blue in TBE containing 0.3 M sucrose was used as a tracking dye, but was generally applied to a well with no sample. The gels were electrophoresed at 300 V for approximately 70 min using TBE as a reservoir buffer. After electrophoresis, the gels were stained with alcian blue as described previously [30]. Briefly, the gels were soaked in 0.05% Alcian blue in distilled water for 30 min in the dark and destained in water for 30 min.

Inhibition of bacterial growth by hyaluronidase inhibitor

BCG-Luc or *M. tuberculosis* H37Rv was suspended in 7H9-ADC, carbon-starved 7H9 or carbon-starved 7H9 containing 0.5 mg/ml of hyalurona to a final concentration of 1×10^4 CFU/ml and 200µl of each suspension was added to 96-well polystyrene plates. Vcpal was added to each well. Bacterial cells were then incubated at 37°C and were collected periodically for detection of luciferase activity for BCG-Luc or CFU assay for *M. tuberculosis*. Similarly, *M. tuberculosis* H37Rv was incubated in the media containing 0.5 mg/ml hyaluronan in presence or absence of 0.1 or 0.5 mM of apigenin or quercetin. After incubation for 7 days, living bacterial number was determined by CFU assay.

RT-PCR

The expression of hyaluronan synthase genes in the lung tissues of mice aerogenically challenged with the virulent *M. tuberculosis* strain H37Rv was determined by RT-PCR. Seven-week-old of female BALB/c mice were aerogenically infected with the *M. tuberculosis* strain H37Rv (2×10^2 CFU/mouse) using a Glas-Col chamber. At different time points, 3 mice per group were euthanized and, the lungs were homogenized in PBS containing 0.05% Tween 80. The homogenates were centrifuged, and the pellets were processed to isolate total RNA using the RNeasy mini kit (QIAGEN, West Sussex, UK) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed using Super Script II RNase H reverse transcriptase (Invitrogen). The cDNA was then subjected to RT-PCR. The following primer pairs were used: β-actin, 5'-TGGAATCCTGTGGCATCCATGAAAC-3' (F) and 5'-TAAACGCAGCAGCTCAGTAACAGTCCG-3' (R); HAS1, 5'-GCTCTATGGGGCGTTCCTC-3' (F) and 5'-CACACATAAGTGGCAGGGTCC-3' (R); HAS2, 5'-TGGAACACCGGAAAATGAAGAAG-3' (F) and 5'-GGACCGAGCCGTGTATTTAGTTGC-3' (R); HAS3, 5'-CCATGAGGCGGGTGAAGGAGAG-3' (F) and 5'-ATGCGGCCACGGTAGAAAAGTTGT-3' (R). The amplification procedure involved initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing of primers at 57°C for 1 min and primer extension at 72°C for 3 min. After completion of the 35th cycle, the extension reaction was continued for another 7 min at 72°C.

Total RNA was extracted from A549 cells by RNeasy mini kit (QIAGEN) and then 1 µg of total RNA was reverse transcribed using Super Script II RNase H reverse transcriptase (Invitrogen). The cDNA was then subjected to RT-PCR. The following primer pairs were used: β-actin, 5'-GATCATTGCTCCTCCTGAGC-3' (F) and 5'-CACCTTCACCGTTCCAGTTT-3' (R); HAS1, 5'- ACTCGGACACAAGGTTGGAC -3' (F) and 5'- TGTACAGCCACTCACGGAAG -3' (R); HAS2, 5'- ATGCATTGTGAGAGGTTTCT -3' (F) and 5'- CCATGACAACTTTAATCCCAG -3' (R); HAS3, 5'- GACGACAGCCCTGCGTGT -3' (F) and 5'- TTGAGGTCAGGGAAGGAGAT-3' (R). The amplification procedure involved initial denaturation at 94°C for 10 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing of primers at 56°C for 1 min and primer extension at 72°C for 2.5 min.

Lung sections of rhesus monkeys that died from tuberculosis

The *M. tuberculosis* H37Rv challenge infection study of in rhesus male monkeys was performed previously [49]. The lung of non-vaccinated monkeys that died of tuberculosis 3 month after intratracheal challenge of 3,000 CFU/lung of *M. tuberculosis* H37Rv were immediately removed and fixed with 15% formalin for 10 days. Three animals' lungs were embedded in paraffin blocks and used in this study as well.

Histochemical staining for hyaluronan

After deparaffinization by washing with xylene and ethanol, the tissue sections were washed in TBS and incubated with fresh TBE containing 0.05 mM of Pronase K (Dako) for 60 min at room temperature. After washing with TBS containing 1% bovine serum albumin, the slides were incubated with 3% bovine serum albumin in TBS for 30 min at room temperature to block non-specific binding sites. The slides were then washed with TBS twice for 10 min and incubated with the biotinylated hyaluronan-binding protein (HABP) probe at a concentration of 2 mg/ml in TBS for 60 min at room temperature. Following washing in TBS, the slides were incubated with a streptavidin-peroxidase reagent and the staining developed using DAKO Cytomation LSAB-system AP (Dako). The slides were then washed with distilled water and counterstained with Mayer's hematoxylin. Paraffin sections were also stained with alcian blue (Sigma) pH 2.5 (3% acetic acid) for 5 min. The slides were counterstained with nuclear fast red (Biomeda) and mounted with Gel/Mount (Biomeda). For GAG digestion, 0.5 mg/ml (10 U/ml) *Streptomyces* hyaluronidase was added for 30 min at 37°C before alcian blue staining. The slides were stained by Ziehl-Neelsen technique using carbol-fuchsin and malachite green (Sigma).

Supporting Information

Figure S1 Apigenin and quercetin suppress growth of *M. tuberculosis* in the media containing hyaluronan as a sole carbon source. *M. tuberculosis* H37Rv was cultured for 7 days in carbon-starved media (7H9) or the media containing 500 µg/ml hyaluronan as a sole carbon source (7H9-HA). Apigenin or quercetin, inhibitors of hyaluronidase, were added to be 0.5 mM or 0.1 mM. CFU was determined at time 0 (open bar) and 7 days after culture (closed bars).
Found at: doi:10.1371/journal.ppat.1000643.s001 (0.08 MB TIF)

Figure S2 Analysis of transcription of HAS genes in A549 cells. Total RNA was extracted from A549 cells cultured in RPMI1640 media containing 10% FCS. Transcription of each gene encoding human HAS1, HAS2, HAS3 and beta-actin was analyzed by RT-PCR. Three samples were analyzed and representative data are presented. M, DNA markers.
Found at: doi:10.1371/journal.ppat.1000643.s002 (0.61 MB TIF)

Acknowledgments

We are grateful to Dr. Todd P. Primm (Sam Houston State University) for editing of the manuscript and Sara Matsumoto for heartfelt encouragement.

Author Contributions

Conceived and designed the experiments: Y. Hirayama, M. Yoshimura, S. Matsumoto. Performed the experiments: Y. Hirayama, M. Yoshimura, Y. Ozeki, I. Sugawara, T. Udagawa, S. Mizuno, A. Tamaru, S. Matsumoto. Analyzed the data: Y. Hirayama, M. Yoshimura, Y. Ozeki, I. Sugawara, T. Udagawa, S. Mizuno, K. Kobayashi, S. Matsumoto. Contributed reagents/materials/analysis tools: N. Itano, K. Kimata. Wrote the paper: M. Yoshimura, I. Sugawara, H. Ogura, K. Kobayashi, S. Matsumoto.

References

1. Aoki K, Matsumoto S, Hirayama Y, Wada T, Ozeki Y, et al. (2004) Extracellular mycobacterial DNA-binding protein 1 participates in *Mycobacterium*-lung epithelial cell interaction through hyaluronic acid. J Biol Chem 279: 39798–39806.
2. Bermudez LE, Goodman J (1996) *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. Infect Immun 64: 1400–1406.
3. Hernandez-Pando R, Jeyanathan M, Mengistu G, Aguilar D, Orozco H, et al. (2000) Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. Lancet 356: 2133–2138.
4. Teitelbaum R, Schubert W, Gunther L, Kress Y, Macaluso F, et al. (1999) The M cell as a portal of entry to the lung for the bacterial pathogen *Mycobacterium tuberculosis*. Immunity 10: 641–650.
5. Nishimura J, Saiga H, Sato S, Okuyama M, Kayama H, et al. (2008) Potent antimycobacterial activity of mouse secretory leukocyte protease inhibitor. J Immunol 180: 4032–4039.
6. Saiga H, Nishimura J, Kuwata H, Okuyama M, Matsumoto S, et al. (2008) Lipocalin 2-dependent inhibition of mycobacterial growth in alveolar epithelium. J Immunol 181: 8521–8527.
7. Dannenberg AM, Jr. (1994) Roles of cytotoxic delayed-type hypersensitivity and macrophage-activating cell-mediated immunity in the pathogenesis of tuberculosis. Immunobiology 191: 461–473.
8. Gobin J, Horwitz MA (1996) Exochelins of *Mycobacterium tuberculosis* remove iron from human iron-binding proteins and donate iron to mycobactins in the *M. tuberculosis* cell wall. J Exp Med 183: 1527–1532.
9. Ernst JD (1998) Macrophage receptors for *Mycobacterium tuberculosis*. Infect Immun 66: 1277–1281.
10. Menozzi FD, Rouse JH, Alavi M, Laude-Sharp M, Muller J, et al. (1996) Identification of a heparin-binding hemagglutinin present in mycobacteria. J Exp Med 184: 993–1001.
11. Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, et al. (2001) The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. Nature 412: 190–194.
12. Soares de Lima C, Zulianello L, Marques MA, Kim H, Portugal MI, et al. (2005) Mapping the laminin-binding and adhesive domain of the cell surface-associated Hlp/LBP protein from *Mycobacterium leprae*. Microbes Infect 7: 1097–1109.
13. Pethe K, Aumercier M, Fort E, Gatot C, Locht C, et al. (2000) Characterization of the heparin-binding site of the mycobacterial heparin-binding hemagglutinin adhesin. J Biol Chem 275: 14273–14280.
14. Katsube T, Matsumoto S, Takatsuka M, Okuyama M, Ozeki Y, et al. (2007) Control of cell wall assembly by a histone-like protein in mycobacteria. J Bacteriol 189: 8241–8249.
15. Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, et al. (1999) Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. J Biol Chem 274: 25085–25092.
16. Shyjan AM, Heldin P, Butcher EC, Yoshino T, Briskin MJ (1996) Functional cloning of the cDNA for a human hyaluronan synthase. J Biol Chem 271: 23395–23399.
17. Weigel PH, DeAngelis PL (2007) Hyaluronan synthases: a decade-plus of novel glycosyltransferases. J Biol Chem 282: 36777–36781.
18. Weigel PH, Hascall VC, Tammi M (1997) Hyaluronan synthases. J Biol Chem 272: 13997–14000.
19. Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, et al. (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. J Clin Invest 106: 349–360.
20. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. Cell 61: 1303–1313.
21. Yang B, Hall CL, Yang BL, Savani RC, Turley EA (1994) Identification of a novel heparin binding domain in RHAMM and evidence that it modifies HA mediated locomotion of ras-transformed cells. J Cell Biochem 56: 455–468.
22. Bartolazzi A, Peach R, Aruffo A, Stamenkovic I (1994) Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumor development. J Exp Med 180: 53–66.
23. Hall CL, Yang B, Yang X, Zhang S, Turley M, et al. (1995) Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-ras transformation. Cell 82: 19–26.
24. Jameson JM, Cauvi G, Sharp LL, Witherden DA, Havran WL (2005) Gammadelta T cell-induced hyaluronan production by epithelial cells regulates inflammation. J Exp Med 201: 1269–1279.
25. Jiang D, Liang J, Fan J, Yu S, Chen S, et al. (2005) Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med 11: 1173–1179.
26. Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, et al. (2002) Resolution of lung inflammation by CD44. Science 296: 155–158.
27. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, et al. (2002) Oligosaccharides of hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med 195: 99–111.
28. Forteza R, Lieb T, Aoki T, Savani RC, Conner GE, et al. (2001) Hyaluronan serves a novel role in airway mucosal host defense. FASEB J 15: 2179–2186.
29. Jacobs WR, Jr., Barletta RG, Udani R, Chan J, Kalkut G, et al. (1993) Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. Science 260: 819–822.
30. Ikegami-Kawai M, Takahashi T (2002) Microanalysis of hyaluronan oligosaccharides by polyacrylamide gel electrophoresis and its application to assay of hyaluronidase activity. Anal Biochem 311: 157–165.
31. Botzki A, Rigden DJ, Braun S, Nukui M, Salmen S, et al. (2004) L-Ascorbic acid 6-hexadecanoate, a potent hyaluronidase inhibitor. X-ray structure and molecular modeling of enzyme-inhibitor complexes. J Biol Chem 279: 45990–45997.
32. Li MW, Yudin AI, VandeVoort CA, Sabeur K, Primakoff P, et al. (1997) Inhibition of monkey sperm hyaluronidase activity and heterologous cumulus penetration by flavonoids. Biol Reprod 56: 1383–1389.
33. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537–544.
34. Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, et al. (2007) Genome plasticity of BCG and impact on vaccine efficacy. Proc Natl Acad Sci U S A 104: 5596–5601.
35. Rink J, Ghigo E, Kalaidzidis Y, Zerial M (2005) Rab conversion as a mechanism of progression from early to late endosomes. Cell 122: 735–749.
36. Deretic V, Singh S, Master S, Harris J, Roberts E, et al. (2006) *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cell Microbiol 8: 719–727.
37. Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, et al. (1997) Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem 272: 13326–13331.
38. Girish KS, Kemparaju K (2007) The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. Life Sci 80: 1921–1943.
39. Stern R, Jedrzejas MJ (2006) Hyaluronidases: their genomics, structures, and mechanisms of action. Chem Rev 106: 818–839.
40. Hascall VC, Majors AK, De La Motte CA, Evanko SP, Wang A, et al. (2004) Intracellular hyaluronan: a new frontier for inflammation? Biochim Biophys Acta 1673: 3–12.
41. Jiang D, Liang J, Noble PW (2007) Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 23: 435–461.
42. Stern R, Kogan G, Jedrzejas MJ, Soltes L (2007) The many ways to cleave hyaluronan. Biotechnol Adv 25: 537–557.
43. Gribbon P, Heng BC, Hardingham TE (2000) The analysis of intermolecular interactions in concentrated hyaluronan solutions suggest no evidence for chain-chain association. Biochem J 350 Pt 1: 329–335.
44. Scott JE, Heatley F (1999) Hyaluronan forms specific stable tertiary structures in aqueous solution: a 13C NMR study. Proc Natl Acad Sci USA 96: 4850–4855.
45. Almond A, Brass A, Sheehan JK (1998) Deducing polymeric structure from aqueous molecular dynamics simulations of oligosaccharides: predictions from simulations of hyaluronan tetrasaccharides compared with hydrodynamic and X-ray fibre diffraction data. J Mol Biol 284: 1425–1437.
46. Mummert ME, Mummert D, Edelbaum D, Hui F, Matsue H, et al. (2002) Synthesis and surface expression of hyaluronan by dendritic cells and its potential role in antigen presentation. J Immunol 169: 4322–4331.
47. Gatfield J, Pieters J (2000) Essential role for cholesterol in entry of mycobacteria into macrophages. Science 288: 1647–1650.
48. Pandey AK, Sassetti CM (2008) Mycobacterial persistence requires the utilization of host cholesterol. Proc Natl Acad Sci U S A 105: 4376–4380.
49. Sugawara I, Sun L, Mizuno S, Taniyama T (2009) Protective efficacy of recombinant BCG Tokyo (Ag85A) in rhesus monkeys (Macaca mulatta) infected intratracheally with H37Rv *Mycobacterium tuberculosis*. Tuberculosis 89: 62–67.