Hyaluronic acid conjugation facilitates clearance of intracellular bacterial infections by streptomycin with neglectable nephrotoxicity

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Abstract
Antibiotics such as β-lactams and aminoglycosides are often subtherapeutic to intracellular infections due to their high hydrophilicity, resulting in low effectiveness against intracellular pathogens and the emergence of antibiotic resistance. Here we reported that an endogenous aminoglycan, hyaluronic acid could be an effective carbohydrate carrier of the aminoglycoside antibiotic, streptomycin against intracellular pathogens. This conjugation could enhance phagocytic activity, and facilitated the entry of streptomycin into host cells via a CD44-mediated pathway. It appeared that this conjugate could clear intracellular bacteria in phagocytic or nonphagocytic cells in a short-term therapy (4 h) at a lower effective dose. In addition, this conjugate was more efficient in reducing bacteria burden in an in vivo acute infection model than streptomycin did. Interestingly, subcutaneous injection of this conjugate at an excess amount had undetectable side effects such as nephrotoxicity. These results suggested that hyaluronic acid might be an efficient Trojan horse for the delivery of hydrophilic antibiotics to deal with intracellular infections.

Key words: CD44, hyaluronic acid, intracellular bacterial infections, streptomycin

Introduction
Intracellular bacterial infections are especially difficult to eradicate because intracellular pathogens fight for their survival using several ingenious mechanisms: resistance to attack by lysosomal enzymes, oxygenated compounds and defensins of host macrophages, inhibition of the phagosome–lysosome fusion, and escape from the phagosome into the cytoplasm (Pinto-Alphandary et al. 2000). The crucial feature of intracellular pathogen is their capacity to stably infect and have evolved strategies for coping with the pressures encountered inside host cells (Kauffman 1993).

The antibiotic treatment of infections caused by intracellular bacteria presents a lot of pharmaceutical challenges. Many clinic
antibiotic exhibit concentration-dependent bactericidal activity (Peloquin et al. 2004) and are readily incorporated into phagocytes when these cells are in contact with the antibiotic for prolonged periods. This raises a critical issue that prolonged treatment with high doses of prescribed antibiotic might raise the risk of adverse outcomes to patients, such as ototoxicity and nephrotoxicity (Forge and Schacht 2000; Rybak et al. 1999). In this study, a conjugate was synthesized through the covalent coupling between partially N-deacetylated hyaluronic acid (dHA) and streptomycin. The intracellular killing capacity of this conjugate was explored in vitro and in vivo.

Results

Synthesis and characterization of H–S conjugate

After hydrazinolysis of hyaluronic acid (HA) under controlled reaction conditions, dHA with the deacetylation degree of 28.4% was obtained as described (Figure S1A) (Zhang et al. 2014). The conjugate, designated as HA–streptomycin conjugate (H–S) was achieved with a high yield of 95% by reductive amination between free amino groups in dHA and aldehyde groups in streptomycin (Figure 1). The successful conjugation between streptomycin and dHA was evidenced by \(^1\)H NMR analysis, in which the weak signals at 9.66 ppm (aldehyde proton) disappeared while the strong signals at 1.22 ppm (methyl protons) were observed in the extensively dialyzed polymer. In addition, one novel signal at approximately 2.8 ppm corresponding to the CH\(_2\) group generated from aldehyde group of streptomycin was observed in the NMR spectrum of H–S, compared to that of dHA.

The streptomycin contents in H–S conjugates was further determined as 15% (w/w) through quantification of guanidyl groups (Pretorius et al. 1991). The molecular weight of H–S was estimated as 253 kDa were used by high-performance gel permeation chromatography (HPGCP) (Figure S2).

H–S conjugate effectively kills intracellular bacterial in infected host cells

Although H–S containing 15% (w/w) streptomycin had no advantage in bactericidal activities, as evidenced by the minimum inhibitory concentrations (MIC) of planktonic bacteria (Table S1), H–S could kill more *Listeria monocytogenes* and *Staphylococcus aureus* in RAW264.7 cells at 100 or 200 μg/mL than streptomycin alone (Strep) or the mixture with equivalent amount of HA and streptomycin (H+S) did (Figure 2 and Figure S3). To see whether H–S was able to clear intracellular bacteria in nonphagocytic cells, a VERO cell line was tested. Similarly, H–S was more effective in killing intracellular *L. monocytogenes* and *S. aureus* in VERO cells when compared to that of streptomycin alone or the mixture with equivalent amount of HA and streptomycin (Figure 2).
Possible mechanism involved in the potent intracellular killing activity of H–S conjugate

Generally, it has long been assumed that the physical interaction between antibiotic and intracellular bacteria is the beginning of antibiotic-mediated bacteria death (Kohanski et al. 2010). This implied that the entry of antibiotics into the infected cells was indispensabled. In fact, H–S could enhance phagocytic activity of RAW264.7 cells in a dose-dependent manner at the test concentrations (Figure S4). To see whether H–S conjugate could enter host cells, actin was visualized by TRITC phalloidin. Meanwhile, by using a polyclonal antibody to streptomycin produced in rabbit and a second Dylight 488-conjugated goat anti-rabbit IgG, streptomycin residing in RAW264.7 cells was visualized. As shown in Figure 3, HA conjugation facilitated streptomycin entrance into macrophages.

The CD44 antigen is a cell-surface glycoprotein and a receptor for HA involved in cell–cell interactions, cell adhesion and migration (Gee et al. 2004). CD44 mRNA was upregulated extensively in RAW264.7 cells at 1 h after infected with *L. monocytogenes* at an MOI (multiplicity of infection) = 2 (Figure 4A). The pretreatment of infected cells with free HA or a monoclonal antibody (IM-7) that blocks binding between HA and CD44 molecules, resulted in a reductive killing capacity of H–S against intracellular *L. monocytogenes* (Figure 4B), implying that surficial CD44 might involve in the entry of H–S into infected cells. The CD44 blocking antibody (IM-7) or free HA greatly suppressed the entrance of H–S conjugate into RAW264.7 cells, suggesting that the H–S uptake was mediated by CD44 molecules (Figure 4C and D).

**H–S conjugate promotes clearance of intracellular bacteria with neglectable nephrotoxicity in vivo**

In order to evaluate bactericidal efficiency of H–S against intracellular bacteria in vivo, mice were infected intraperitoneally (i.p.) with log-phase *L. monocytogenes* (~10⁵ CFU/mouse). After 24 h, mice received a daily subcutaneous injection of H–S (10 mg/kg), streptomycin alone (Strep) or the mixture with equivalent amount of N-deacetylated HA and streptomycin (H+S), or PBS for 3 days. There was a significant improvement in the reduction of bacteria burden in the spleen and kidney for H–S conjugate (Figure 5).

The significant clinical toxicities of aminoglycoside antibiotics are ototoxicity, nephrotoxicity and less often neuromuscular toxicity (Mingeot-Leclercq and Tulkens 1999). In kidney, streptomycin is retained in the epithelial cells lining the proximal tubules after glomerular filtration. It is attached to the brush-border membrane in its cationic form (Rougier et al. 2003). A daily subcutaneous injection of streptomycin at a high dose (300 mg/kg) for 10 days resulted in
Fig. 3. The conjugation promoted streptomycin entrance into macrophages. RAW264.7 cells were incubated with H–S conjugate (15% streptomycin), equivalent streptomycin (Strep) alone, and the respective mixture (H + S) for 4 h. PBS was used as control. Cytoskeletal protein was labeled with TRITC (red) and H–S was labeled with anti-streptomycin antibody (green). Quantification of immunoreactivity of streptomycin by using Image Pro Plus. Scale bar represented 50 μm. This figure is available in black and white in print and in color at *Glycobiology* online.

Fig. 4. The conjugation facilitated the entry of streptomycin into host cells via a CD44-mediated pathway. (A) The relative fold change of CD44 gene expression levels of RAW264.7 cells infected with *L. monocytogenes* (MOI = 2). (B) *L. monocytogenes* infected RAW264.7 cells were incubated with Anti-CD44 (20 μg/mL) or free hyaluronic acid (100 μg/mL) for 1 h prior treatment with H–S (100 μg/mL) for 4 h. Data presented represent the average of three independent replicates. (C) Quantification of immunoreactivity of streptomycin by using Image Pro Plus. (D) RAW264.7 cells were incubated with Anti-CD44 (20 μg/mL) or free hyaluronic acid (100 μg/mL) for 1 h prior treatment with H–S (100 μg/mL) for 4 h, PBS was used as control. Cytoskeletal protein was labeled with TRITC (red) and H–S was labeled with anti-streptomycin antibody (green). Scale bar represented 50 μm. This figure is available in black and white in print and in color at *Glycobiology* online.
the tubule epithelial cell swelling and necrosis, as well as stenosis of the renal tubule (Figure 6) as expected. Instead, mice treated with a dose of 2 g/kg H–S which contain the same amount of streptomycin as above had a slight denaturalization could be seen on the renal tubule.

**Discussion**

Our laboratory is to improve therapeutic efficacy of antibiotics in treating infectious diseases, and previously we have described a strategy for streptomycin at a relatively low concentration to achieve the intracellular killing through introducing a cationic polysaccharide, chitosan as an efficient carrier (Mu et al. 2016). However, chitosan exhibited significant cytotoxicity at high concentrations (>500 μg/mL) (Huang et al. 2004). We ask whether the chitosan can be replaced by other polysaccharides with better biocompatibility as a carrier of antibiotics. HA is a natural and linear polymer composed of repeating disaccharide units of β-1,4-glucuronic acid and β-1,3-N-acetyl glucosamine (Tripodo et al. 2015). As one extremely ubiquitous extracellular matrix molecule, HA is widely distributed throughout connective, epithelial and neural tissues (Fraser et al. 1997). An H–S conjugate was synthesized by reduction of the resulting Schiff base formed by free amino groups in partially dHA and aldehyde groups in streptomycin (Figure 1).

Although H–S conjugate could not improve the bactericidal activity of streptomycin against planktonic bacteria (Table S1), it was more effective in killing intracellular bacteria in phagocytic or nonphagocytic cells in a short-term therapy (4 h) after infections. Apparently, this advantage decreased the risk of side effects including ototoxicity and nephrotoxicity due to prolonged treatment with high doses of aminoglycoside antibiotics (De Jager and Van Altena 2002). Unlike the chitosan–streptomycin (C–S) conjugate reported previously (Mu et al. 2016), H–S displayed an undetectable cytotoxicity to RAW264.7 or human kidney HK-2 cells especially at high concentrations (Figure S5), although C–S elicited a stronger bactericidal activity against the intracellular organism (Figure S6). There were several difference between H–S and C–S: (1) structurally, C–S was derived from polycationic polymer, chitosan and H–S was made from polyanionic polymer, HA; (2) the actin filaments were involved in the uptake process of C–S while H–S uptake was mediated by CD44 antigen; and (3) C–S, not H–S had higher bactericidal activity against planktonic bacteria than streptomycin did.

In vivo, subcutaneous injection of H–S could reduce bacteria burden in an intraperitoneal infection animal model. Pathological sections demonstrated that overuse of streptomycin could lead to swelling in renal proximal convoluted tubules (Cojocel et al. 1984), while H–S with equivalent antibiotic didn’t. Unfortunately, ototoxicity could not be examined since experimental conditions at our facility were not allowed.

It is well-known that HA is the key molecule involved in skin moisture due to its strong water-retaining ability, and this moisture-absorption properties of HA is largely dependent on its molecular mass and N-deacetylation degree (Zhang et al. 2013). HA with higher degree of polymerization had a stronger moisture-absorption capacity. In this study, we also observed the relatively high viscosity

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**Fig. 5.** H–S conjugate promotes clearance of intracellular bacteria in vivo. *L. monocytogenes* infected mice received a daily subcutaneous injection of H–S (10 mg/kg), streptomycin (1.5 mg/kg), the mixture with equivalent amount of hyaluronic acid and streptomycin or PBS (n = 5 mice each group) for 3 days. The CFU in spleen and kidney were counted. Data points represent each mouse, means ± SD from all mice. This figure is available in black and white in print and in color at Glycobiology online.

**Fig. 6.** H–S conjugate had neglectable nephrotoxicity in vivo. H&E staining of kidney from mice treated with high dose of streptomycin, not H–S showed the tubule epithelial cell swelling and necrosis, as well as stenosis of the renal tubule (triangle). Scale bar represented 50 μm. This figure is available in black and white in print and in color at Glycobiology online.
of H–S derived form 219 kDa HA at high concentrations, which is a disadvantage during preparation, storage and consumption. To solve this, H–S derived from HA oligosaccharides with low viscosity was under investigation.

**Conclusion**

Our data highlighted that the covalent coupling streptomycin to HA could improve intracellular bactericidal capacity in different cell types. This conjugate could readily be incorporated into host cells via a CD44-mediated uptake mechanism, and in vivo acute infection models indicated it elicited a better therapeutic effect on intracellular infection than streptomycin did. An excess dose of the conjugate had a negligible nephrotoxicity. These findings might open up a new avenue to fight against intracellular bacteria by streptomycin at a lower effective dose.

**Materials and methods**

**Bacterial strains**

*L. monocytogenes* (CMCC 54004) and *S. aureus* (ATCC 25923), which were purchased from the National Center for Medical Culture Collection (CMCC). The strains were grown in Tryptic Soy Broth (TSB) at 37°C.

**Cell culture**

RAW264.7 and VERO cells were cultured in RPMI 1640 (GIBCO) medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (GIBCO) at 37°C in a humidified 5% CO2 atmosphere.

**Synthesis of HA–streptomycin conjugates**

As described (Zhang et al. 2013), sodium hyaluronate (Aladdin Chemical Reagent Company, China) was treated with NH2NH2.H2O at 105°C for 3 h. Partially dHA were generated. A solution of streptomycin sulfate and NaCNBH3 was added into dHA solutions. The reaction mixture was stirred for 12 h in the dark and then dialyzed 72 h by using dialysis membrane (Mw cut off 3500 Da) and finally lyophilized. For 1HNRM spectral analysis, samples were dissolved in 500 μL D2O (10 mg/mL), and the spectra were carried out on a Bruker AM500 spectrometer in the FT mode at room temperature (Zhang et al. 2013). Streptomycin contents in conjugates were determined through quantification of guanidyl groups, and streptomycin sulfate was used as a standard.

**Determination of molecular weight**

The molecular weight were determined by high performance gel permeation chromatography (HPGPC) on three columns (Waters Ultrahydrogel 120, 250 and 1000; 30 cm × 7.8 mm; 6 μm particles) in series. The columns were calibrated with T-series Dextans (1, 5.2, 10, 48.6, 668 kDa). Sodium acetate (3 mM) was used as eluant and the flow rate was kept at 0.5 mL/min. A 50 μL aliquot was injected for each run. The calibration curve of Log (Mw) vs. elution time (t) is: Log (Mw) = −0.1869x + 12.061.

**MIC determination for planktonic bacteria**

The MIC of H–S, H + S, Strep and dHA against planktonic bacteria were performed using broth microdilution method. The final concentration of *L. monocytogenes* or *S. aureus* was 10^5 CFU/mL (Benincasa et al. 2015; Chaudhari et al. 2015).

**Assay of phagocytic activities**

This was measured by neutral red uptake assay. RAW264.7 cells were plated at a density of 1 × 10^5/well, followed by treatment with different concentration H–S for 12 h as described previously (Zhang et al. 2015). Culture media was removed and the cells were incubated with 100 μL of 0.075% (w/v) neutral red solution for 2 h. The supernatant was discarded and the cells were washed with PBS three times. Cell lysis buffer (1% glacial acetic acid: ethanol = 1:1, 200 μL/well) was added. After that, cells were incubated at 4°C overnight, and the absorbance was measured at 540 nm in microplate reader.

**Real-time quantitative PCR**

RAW264.7 cells were plated on six-well plate for 6 h. Then, cells were infected at an MOI = 2 for 1 h, washed thrice with PBS. Total RNA was extracted from cells using TRIzol reagent (Takara Biotechnology, Japan). Total RNA was reverse transcribed to cDNA, which was subsequently used to perform PCR amplification (Bio-Rad, America). Relative expression values were normalized using an internal GAPDH control. The fold change of relative gene expression levels was calculated following the formula: 2^−ΔΔCt (Livak and Schmittgen 2001; Qi et al. 2016). CD44 primers were designed from a GenBank-registered mouse CD44 sequence (NM009851.2). The sense primer is 5-AGCAGGAGAGCTGGTTT CAG-3 and the antisense primer is 5-TTGTCCCATATTGATG GAGA-3. GAPDH primers were designed from a GenBank-registered (NM001289726.1). The sense primer is 5-GAGGCCGGTGCT GAGATGCG-3 and the antisense primer is 5-GTGGTGCAAGAT GCATGTGCTA-3.

**In vitro infections**

As described (Donaldson et al. 2011), RAW264.7 cells and VERO cells were plated on 24-well plate for 6 h. Then, cells were infected at an MOI = 2 for 1 h. After washed thrice with PBS, cells were incubated in 1 mL fresh medium supplemented with compounds for different periods as indicated. Then cells were lysed with 0.25% Triton X-100 for 30 min. Serial dilutions were plated on TSB plates and colonies were counted the next day to determine CFU.

**Immunofluorescence**

RAW264.7 cells were plated on coverslips 12 h before incubation with H–S conjugate (15% streptomycin), equivalent streptomycin (Strep) alone, the respective mixture (H + S) or PBS for 4 h. Then the coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature then permeabilized with 0.25% Triton X-100 for 10 min and saturated with 1% BSA. Streptomycin was marked using rabbit anti-streptomycin ployclon (Abcam, Cambridge) and Dylight 488-conjugated goat anti-rabbit IgG (Jackson Immuno Research Inc., West Grove, MA). The actin was stained with TRITC-phalldin (YEASEN, China). For blocking experiments, cells were incubated with Anti-CD44 (20 μg/mL, ebiscience, CA) or free HA (100 μg/mL) for 1 h prior treatment with H–S.

**In vivo infection model**

Male Kunming mice (20 ± 2 g) were purchased from School of Medicine, Xi’an Jiao Tong University, China. All animals were acclimated for a week before experiments. Mice were infected intraperitoneally with 5 × 10^7 CFU of log phase *L. monocytogenes*. Next day, mice received a daily subcutaneous injection of H–S (10 mg/kg), streptomycin (1.5 mg/kg), the mixture with equivalent amount of HA and...
streptomycin or PBS (n = 5 mice each group) for 3 days. Then spleen and kidney were harvested, and CFU were counted by dilution plating.

Pathological experiments
Mice received a daily subcutaneous injection of H-S (2 g/kg), streptomycin (300 mg/kg) or PBS (n = 3 mice each group) for 10 days. Kidney were fixed in a 4% paraformaldehyde at room temperature for 72 h. H&E staining was performed and observed with an invert microscopy.

All experiments were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China.

Statistical analysis
All data were made using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Data were analyzed by t-tests and ANOVA were used to evaluate significant differences. Data represent means ± SD of three individual experiments.

Supplementary data
Supplementary data is available at GLYCOBIOLOGY online.

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Conflict of interest statement
None declared.

Abbreviations
CFU, colony-forming units; G-S, chitosan-streptomycin; dHA, N-deacetylated hyaluronic acid; HA, hyaluronic acid; H-S, hyaluronic acid-streptomycin conjugate; H+S, dHA and streptomycin mixture; MIC, minimum inhibitory concentrations; MOL, multiplicity of infection; PBS, phosphate buffer saline.

Reference