Inorganic polyphosphate modulates leukocyte accumulation and vascular endothelial cell permeability and ameliorates cecal ligation and puncture-induced lethality

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ABSTRACT — Inorganic polyphosphates with an average degree of polymerization of 150 (polyP 150) have been shown to improve mortality in a lipopolysaccharide model of sepsis in mice. We aimed to investigate the effects of polyP 150 in a mouse model of cecal ligation and puncture (CLP) peritonitis, which accurately reflects clinical sepsis, and elucidate its mechanism of action and suitability as a candidate for sepsis treatment. The present study demonstrated that treatment with polyP 150 significantly improved survival rate in mouse model of CLP peritonitis. polyP 150 inhibited a CLP-mediated increase in pulmonary vascular permeability as demonstrated by Evans blue dye assay. Pretreatment of polyP 150 in human vascular endothelial cells, HMEC-1 cells, showed inhibition of tumor necrosis factor-α-induced monocytic THP-1 cell adhesion and intercellular adhesion molecule 1/CD54 gene expression. These results suggest that polyP 150 ameliorates fatal sepsis by inhibiting expression of the cell adhesion molecule and the accumulation of leukocytes in the vascular endothelium, thereby suppressing the increase in vascular permeability. Our results in this study suggest that polyP 150 could be a candidate for novel sepsis treatments.

Key words: Sepsis, Inorganic polyphosphates, Cecal ligation and puncture, Vascular permeability

INTRODUCTION

Sepsis is defined as a life-threatening dysfunction of multiple organs caused by a dysregulated host response to infection (Singer et al., 2016). The activation of pattern recognition receptors (PRRs) by infection triggers the induction of pro-inflammatory cytokines and tissue damage by inflammation. Endogenous damage-associated molecular patterns released from inflammatory tissues further activate the PRRs, resulting in overproduction of cytokines (hypercytokinemia). In addition, hypercytokinemia results in uncontrolled immune stimulation and suppression, inflammatory responses, and activation of coagulation and fibrinolysis, leading to increased vas-
cicular permeability, disseminated fibrin, decreased blood pressure, hypercoagulation, and circulatory failure. These pathologies in septic shock eventually cause tissue inability to metabolize oxygen, multiple organ damage, and death (Gyawali et al., 2019; Hotchkiss et al., 2016; McConnell and Coopersmith, 2016).

Currently, it is estimated that one person dies from sepsis every few seconds worldwide (Nishida et al., 2018). Various treatments for sepsis have been investigated to date, but no medication has been established. Antimicrobial therapy is the primary treatment for the underlying disease, but has been plagued by the emergence of drug-resistant bacteria and stagnation of antibiotic development (Nishida et al., 2018). Other therapies targeting PRRs and cytokines as well as immunoglobulin therapy have been explored, but have not shown clear improvement in prognosis (Alejandria et al., 2013; Bernard et al., 2014; Opal et al., 2013). These situations call for a different approach to the development of therapeutic agents for sepsis.

Inorganic polyphosphate, a compound of phosphoric acid that has undergone linear polymerization, is present ubiquitously in all biological species and is known to have various biological functions depending on its molecular length (Harada et al., 2013a, 2013b). Inorganic polyphosphate (polyP) with an average polymerization degree of 150 (polyP150) has been shown to improve organ damage and lethality in a mouse model of lipopolysaccharide (LPS)-induced endotoxin shock (Terashima-Hasegawa et al., 2019). However, the LPS model is considered to correspond to the end-stage in sepsis, and the basic clinical features of sepsis such as high cardiac output and hypermetabolic state have not been able to be reproduced (Gahhos et al., 1981; Wicherman et al., 1980). Therefore, studies on agents for sepsis treatment that appeared promising in endotoxin models have not proven efficacious in human clinical trials (Buras et al., 2005; Deitch, 1998; Opal et al., 2013).

In the present study, we aimed to investigate polyP150 in a mouse model of cecal ligation and puncture (CLP) peritonitis, which displays the typical signs of septic shock, ranging from a high cardiac output state with tachypnea, hyperglycemia, and hyperinsulinemia to a state of reduced cardiac output at the end of life (Li et al., 2018; Rittirsch et al., 2009; Wicherman et al., 1980). We also aimed to elucidate the mechanism of action of polyP150 in a mouse model CLP sepsis model and whether polyP150 is a suitable candidate for novel sepsis treatments.

**MATERIALS AND METHODS**

**Animals**

Seven- to eight-week-old male ddY mice were obtained from Sankyo Labo Service Co. (Tokyo, Japan). They were housed 5 per cage in plastic cages in a temperature-controlled room (22 ± 1°C) with a humidity of 40–60% and kept in a 12 hr light/dark cycle with free access to standard chow diet (F-2, Sankyo Labo Service Co.) and water. All procedures of the animal experiments were conducted according to the National Institutes of Health guidelines for the management and use of laboratory animals and approved by the Animal Care and Use Committee of Showa University (approval #, 28049). Every effort was made to minimize the number of mice used and their suffering. Mice were allowed to acclimate for a week before start of experiments.

**Survival rate in CLP mouse model**

Standardized protocol was followed to establish a CLP peritonitis mouse model (Murando et al., 2019; Rittirsch et al., 2009). The cecum was ligated at 50% of its total length and then perforated by a single puncture midway between the ligation and the tip of the cecum with a 21G needle under anesthesia using medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). The experiment was conducted in two sessions: one in which polyP150 (0.1 mmol/kg, RegeneTiss, Okay, Japan) or saline (10 mL/kg) was intraperitoneally administered a day before CLP and one in which either polyP150 (0.1 mmol/kg) or saline (10 mL/kg) was intraperitoneally administered a day after CLP. In both sessions, polyP150 (0.1 mmol/kg) or saline (10 mL/kg) was administered once daily after CLP until the end of the session. Therefore, the only difference between these two sessions was whether polyP150 was administered before CLP or not. Survival of the animals was observed for 14 days after CLP. In the sham animals, the cecum is exteriorized but neither ligated nor punctured.

**Vascular permeability in CLP mouse model**

Vascular permeability was assessed using the Evans blue dye assay (Radu and Chernoff, 2013; Wick et al., 2018). Mice were subjected to CLP and 300 μL of 0.5% Evans blue (Fujifilm, Tokyo, Japan) was administered at the tail vein under isoflurane anesthesia 72 hr later. The chest was opened 30 min after dye injection under anesthesia using medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg), and the lung tissue was removed and weighed. A part of the weighed tissue was dried at 150°C for 24 hr, and the wet/dry weight ratio...
was calculated. The other portion of the lung tissue was homogenized with 1.5 mL of formamide and the homogenate was incubated at 55°C for 24 hr. After centrifugation at 15,000 rpm/4°C for 30 min, the optical density of the supernatant was determined using a dual-wavelength spectrophotometer (620/740 nm). The extravasated dye concentration in the lung homogenate was calculated against a standard curve (μg Evans Blues dye/g lung).

Cell culture

Immortalized human dermal microvascular endothelial cells-1 (HMEC-1) and human acute monocytic leukemia THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and Riken Cell Bank (Ibaraki, Japan), respectively. HMEC-1 cells were cultivated in MCDB131 medium (Sigma-Aldrich, St Louis, MO, USA) containing 10 ng/mL recombinant murine epidermal growth factor (rmEGF; Fujifilm), 1 μg/mL hydrocortisone (Fujifilm), 10 mM L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). THP-1 cells were cultivated in RPMI 1640 (Nissui, Tokyo, Japan) containing 10 mM L-glutamine, 20 mM HEPES, 10 units/mL penicillin 100 μg/mL streptomycin, and 10% FBS. Cells were maintained at a logarithmic growth phase in a humidified incubator at 37°C and 5% CO₂.

Monocyte-endothelial cell interactions

HMEC-1 cells were seeded onto 96-well plates (5 × 10⁴/well) and cultured for 48 hr until subconfluence was achieved. The medium was replaced with rmEGF (-) and FBS (-) MCDB131 medium and the cells were cultured for another 12 hr. They were pretreated with 10 μM polyP₁₅₀ or phosphate-buffered saline (PBS) as a control for 4 hr and then either treated with 25 ng/mL TNF-α or untreated for 4 hr. RNA was prepared from HMEC-1 cells according to the protocol for the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). cDNAs were prepared using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Primers (Thermo Fisher Scientific) used for human intercellular adhesion molecule 1 (ICAM-1) were: forward 5'-CCTACCAGCTCCAGACCTTT-3', reverse 5'-AAGGAGTCGTTGCCATAGGT-3'; those for human vascular cell adhesion molecule 1 (VCAM-1) were: forward 5'-GAAGGTGACGAATGAGGGGA-3' and reverse 5'-CTTGACTGTGTCGGCTTCC-3'; and those for human glyceraldehyde 3-phosphate dehydrogenase were: forward 5'-GCTCACTGGCATGGCCTTCCG-3' and reverse 5'-GTGGGCCATGAGGTCCACCAC-3'. Quantitative PCR (qPCR) was performed on a QuantStudio 3 (Thermo Fisher Scientific) according to the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) protocol. The mRNA levels were measured relative to glyceraldehyde 3-phosphate dehydrogenase mRNA expression in each run. All samples were measured in triplicate and analyzed by the ΔΔCT method.

Statistical analysis

All values are expressed as mean ± standard error of mean. Survival rates were analyzed by the log-rank test and presented as Kaplan-Meier survival curves. Multiple tests were performed using Dunnett’s test for comparisons against the respective control group. Statistically significance in all cases was defined at P < 0.05.
RESULTS

Effect of polyP\textsubscript{150} on survival rate in the CLP mouse model

We examined the effects of polyP\textsubscript{150} on the CLP mouse model in the present study. Based on preliminary experiments, the conditions of CLP were set as a single puncture with a 21G needle. Under these experimental conditions, 85% of animals in the CLP group treated intraperitoneally with saline died after 9 days of treatment in both cases which mice were treated with saline a day before CLP and thereafter as well as with saline after CLP for the observation period. These control groups did not differ significantly, therefore, were combined and are shown as a saline group in Fig. 1. In contrast, daily intraperitoneal polyP\textsubscript{150} administration at a dose of 0.1 mmol/kg from a day before and every day after CLP significantly improved the mortality rate; 60% of the animals were still alive after 14 days under the experimental conditions (Fig. 1, open square). Similar effect of polyP\textsubscript{150} was observed when administered after CLP treatment (Fig. 1, closed square).

Effect of polyP\textsubscript{150} on vascular permeability in CLP mouse model

Increased vascular permeability and disruption of its barrier function are largely responsible for the pathogenesis of CLP-induced septic shock (Jiang et al., 2020; Joffre et al., 2020). Therefore, the effects of polyP\textsubscript{150} on the increased vascular permeability in the CLP model, which has been known to occur in this animal model, was examined. polyP\textsubscript{150} was administered a day before and every day for 2 days after CLP, and vascular permeability in lung tissue was examined 72 hr after CLP using the Evans blue assay. Compared with the sham group, CLP increased Evans blue infiltration into lung tissue by approximately two-fold. In contrast, the administration of polyP\textsubscript{150} almost completely suppressed the CLP-induced accumulation of Evans blue in the lungs (Fig. 2a). Pulmonary edema was examined by determining the ratio of wet to dry weight of lung tissue. Under these experimental conditions, polyP\textsubscript{150} inhibited the CLP-induced increase in water content of the lungs (Fig. 2b). These results indicate that polyP\textsubscript{150} suppresses the vascular permeability and pulmonary edema in the CLP model.

![Fig. 1](image1.png)

**Fig. 1.** Effect of polyP\textsubscript{150} on survival rate in the CLP mouse model. Survival rate of mice administered saline (10 mL/kg, closed circle), polyP\textsubscript{150} (100 µmol/kg, i.p.) one day before and every day after CLP (pre, open square) or every day after CLP (post, closed square). Sham group (closed triangle) received sham operation and saline. Survival curves were compared by log-rank test (*P < 0.05, n = 10 in sham and polyP\textsubscript{150} groups and n = 20 in saline group).

![Fig. 2](image2.png)

**Fig. 2.** Effect of polyP\textsubscript{150} on pulmonary vascular permeability in CLP mouse model. Mice were subjected to CLP and were administered either saline or polyP\textsubscript{150} (100 µmol/kg), 1 day before and every day after CLP for 2 days. Lung tissues were removed from the animals 3 days after CLP. (a) The amount of Evans blue permeated into the lung tissue is indicated per wet weight of the lung. (b) The wet to dry weight ratio of lung tissue is shown. *P < 0.05, ***P < 0.001 (Dunnett’s test compared with CLP group as a control, n = 4).
Effect of polyP_{150} on monocyte-endothelial cell interactions

Inhibition of vascular permeability by polyP_{150} may be one of the causes of improvement in the survival rate of the CLP mouse model. In general, the increased vascular permeability in sepsis is thought to be closely related to the fact that overproduced inflammatory mediators reduce the function of endothelial cell adhesion (Joffre et al., 2020). Meanwhile, it has also been shown that adhesion and infiltration of neutrophils to the pulmonary vascular endothelium play an important role in vascular permeability in the acute lung injury secondary to sepsis (Gill et al., 2015; Jiang et al., 2020; Marcus et al., 1997). Therefore, we investigated monocyte-endothelial cell interactions in vitro using monocytic cells that are recruited by a mechanism similar to neutrophil adsorption to the vascular endothelium (Ley et al., 2007; Nourshargh and Alon, 2014). In this experiment, we observed the adhesion of fluorescence-labeled monocytic THP-1 cells to vascular endothelium-derived HMEC-1 cells.

Compared with the control cells, treatment of HMEC-1 cells with TNF-α increased the relative fluorescence intensity approximately 2.4-fold. In contrast, pretreatment of HMEC-1 cells with 10 μM polyP_{150} significantly attenuated the TNF-α-mediated increase in fluorescence (Fig. 3a). These results indicate that polyP_{150} suppressed the TNF-α-induced monocyte adhesion to vascular endothelial cells. Conversely, when THP-1 cells were treated with polyP_{150} and TNF-α, there was no sign of suppression (Fig. 3b), suggesting that polyP_{150} acts on vascular endothelial cells. In addition, even when HMEC-1 cells were treated with polyP_{150} after TNFα, the adhesion of THP-1 cells to HMEC-1 cells was attenuated (Fig. 3c).

Next, series of experiments were conducted to observe the gene expression of ICAM-1 and VCAM-1 in HMEC-1 cells by qPCR. Compared with the control cells, the expression level of ICAM-1 was increased approximately 144-fold by TNF-α treatment. Pretreatment of the cells with polyP_{150} significantly reduced the effect of TNF-α by about half (Fig. 4a). In contrast, polyP_{150} had little effect on the induction of VCAM-1 by TNF-α treatment (Fig. 4b), indicating that polyP_{150} specifically suppresses ICAM-1 expression by TNF-α in HMEC-1 cells. Thus, our results suggest that polyP_{150}-mediated inhibition of the recruitment of monocytes to vascular endothelial cells is at least partly mediated through suppression of ICAM-1 gene expression.

**DISCUSSION**

This study expands our previous reports with the aim of testing the potential of polyP_{150} as a new therapeutic strategy for sepsis. Although we previously reported that polyP_{150} improves mortality and organ damage in a mouse
model of LPS sepsis (Terashima-Hasegawa et al., 2019), the present study shows that polyP150 also has a certain lethality-improving effect in a mouse model of CLP (Murando et al., 2019; Rittirsch et al., 2009; Wichterman et al., 1980), which resembles the clinical picture of sepsis more closely.

Our previous report showed that polyP150 protects against LPS-induced death and organ damage by modulating the recruitment of macrophages to tissues such as lung and liver (Terashima-Hasegawa et al., 2019). It has been suggested that activated inflammatory cells accumulate in the lungs under septic conditions and release cytokines and reactive oxygen species, disrupting pulmonary vascular endothelial and epithelial cells and increasing pulmonary vascular permeability (Gill et al., 2015; Jiang et al., 2020; Marcus et al., 1997). Excessive leukocyte accumulation may destabilize alveolar barrier function and increase vascular permeability, resulting in further disruption of alveolar barrier function. The major pathologies of acute respiratory distress syndrome secondary to sepsis are hyperpermeable pulmonary edema due to the damage to pulmonary vascular endothelial cells (Matute-Bello et al., 2008; Zhou et al., 2019). As an initial response, pro-inflammatory cytokines such as IL-1β and TNF-α produced by monocytes/macrophages and activated by endotoxins and other infectious factors lead to an increased expression of adhesion molecules such as ICAM-1 and VCAM-1 on pulmonary vascular endothelial cells (Idriss and Naismith, 2000; De Freitas Caires et al., 2018; Lee et al., 2011; Niu et al., 2020). These adhesion molecules are thought to cause migration and infiltration of leukocytes from the vessels to the interstitium, releasing activated proteases and reactive oxygen species and inducing tissue damage (Amin et al., 2006). In the present study, we also observed increased pulmonary vascular permeability in the early stages of CLP treatment, which is thought to be related to CLP-mediated mortality (Aziz et al., 2018). Furthermore, increased pulmonary vascular permeability was significantly attenuated by administration of polyP150, which reduced it to levels similar to those in the sham group. Thus, it is suggested that polyP150 may improve mortality in the CLP sepsis model, at least in part, by suppressing the hyperpermeability of the pulmonary vessels.

Fig. 4. Effect of polyP150 on the gene expression of cell adhesion molecules induced by TNF-α in HMEC-1 cells. HMEC-1 cells were pretreated with PBS or 10 µM polyP150 for 4 hr and either untreated or treated with 25 ng/mL TNF-α for 4 hr. The expression levels of ICAM-1(A) and VCAM-1(B) in HMEC-1 cells were determined by qPCR and are shown as the relative gene expression. **P < 0.01 (Dunnett’s test compared with TNF-α group as a control, n = 3).
similar mechanisms (Ley et al., 2007; Nourshargh and Alon, 2014). Therefore, the present study examined the recruitment of THP-1 cells to HMEC-1 cells as an in vitro model (Choi et al., 2018; Zhao et al., 2017). Treatment of HMEC-1 cells with polyP_{150} before as well as after TNF-α inhibited the adhesion of THP-1 cells to HMEC-1 cells. This inhibition may involve, at least in part, the repression of ICAM-1 induction of HMEC-1 cells. The results obtained here are consistent with the results obtained with in vivo experiments where polyP_{150} was effective even when treated after CLP. In the present study, we also tested a system in which THP-1 cells were stimulated with TNF-α, but polyP_{150} had no effect on the induction of cell adhesion to HMEC-1 cells. These results indicate that polyP_{150} acts on the endothelium and has little effect on monocytes with respect to the cell-to-cell adhesion. Accumulated findings indicate that polyP with 60–100 phosphate residues released from platelets shows pro-inflammatory responses with activation of the coagulation pathway in vivo (Baе et al., 2012; Dinarvand et al., 2014; Müller et al., 2009). However, these studies used from 15 to 30 times greater dose of polyP (0.15–0.3 mg/g body weight, which mean approximately 1.5–3 mmol/kg body weight) compared to the current study. In addition, these values were calculated based on the number of moles in monomer, and considering that their studies used a lower molecular weight polyP than ours, the difference in dosage would be much larger. Furthermore, our preliminary study indicates that higher dose of polyP_{150} (1 mmol/kg) was ineffective to LPS-induced lethality (Terashima-Hasegawa et al., 2019). It has already been demonstrated that polyP shows biological effects in a chain length-dependent manner (Angelova et al., 2016). Indeed, bacterial long-chain polyP has been shown to potentiate pathogenicity by inhibiting phagosome maturation (Roewe et al., 2020) and interfering with macrophage function (Rijal et al., 2020). Therefore, it is suggested that the response of polyP to inflammation will be different depending on the dose and degree of polymerization.

In summary, the results of the present study demonstrated that polyP_{150} significantly inhibited vascular permeability and increased survival in the CLP animal model. In addition, it was suggested that a part of this mechanism may include the suppression of ICAM-1 expression, which inhibited leukocyte adhesion in pulmonary vascular endothelial cells, and protects the lung tissue from inflammatory response. Although this study focused on pulmonary vascular permeability, there may be other mechanisms involved in polyP_{150}-mediated improvement of septic mortality that is yet to be determined. We hope that further elucidation of the mechanism of polyP_{150} will lead to its clinical use in the future.

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**REFERENCES**


Y. Yamazaki et al.

phate amplifies proinflammatory responses of nuclear proteins through interaction with receptor for advanced glycation end products and P2Y1 purinergic receptor. Blood, 123, 935-945.


