Effects of the Antimicrobial Peptide LL-37 and Hyperthermic Preconditioning in Septic Rats

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Background: The authors tested the effects of LL-37 prophylaxis or therapy on the outcome after intraabdominal sepsis and examined whether hyperthermic preconditioning plus LL-37 therapy augments host immune response and improves survival.

Methods: A rat model of peritoneal contamination and infection (PCI) with human stool was used to simulate clinical conditions. In trial 1, the authors compared (1) PCI, (2) LL-37 prophylaxis (0.5 mg/kg, 12 h before PCI), and (3) LL-37 therapy (0.5 mg/kg, 1 h after PCI). In trial 2, the authors compared (1) PCI, (2) LL-37 therapy, (3) hyperthermic preconditioning (41°C for 1 h, 24 h before PCI), and (4) LL-37 therapy and hyperthermic preconditioning. The primary endpoints were mortality at 120 h. In trial 2, secondary endpoints were systemic levels of tumor necrosis factor α, interleukin 6, macrophage inflammatory protein 2, and heat shock protein 70; leukocyte counts; and neutrophil granulocyte phagocytosis.

Results: In trial 1, 30% of the control group compared with 70% of the LL-37 therapy group survived, but 55% after LL-37 prophylaxis survived (P = 0.038). In trial 2, 38% of the controls, 67% of the LL-37 therapy, 59% of the hyperthermic preconditioned, and 90% of the hyperthermic preconditioned plus LL-37 therapy group survived (P = 0.01). LL-37 therapy plus hyperthermic preconditioning reduced proinflammatory cytokine concentrations after sepsis; specifically compared with controls, macrophage inflammatory protein-2 and interleukin-6 levels were 1.5 ± 1.5 versus 11 ± 6 pg/ml (P = 0.028) and 13 ± 8 versus 86 ± 31 pg/ml (P = 0.015), respectively.

Conclusions: In this model of intraabdominal sepsis, LL-37 therapy improved outcome. Hyperthermic preconditioning per se was not successful, but in combination with LL-37 therapy, the survival rate after sepsis was increased and the proinflammatory cytokine response was downregulated.

ACTIVATED polymorphonuclear granulocytes (PMNs) attack microbes with an array of antimicrobial peptides, including defensins and cathelicidins, which act much like endogenous antibiotics. The term cathelicidins describes a bipartite molecule containing a cathelin domain and a C-terminal antimicrobial peptide domain. The only known human cathelicidin, LL-37/hCAP-18 (peptide starts with two leucines and comprises 37 amino acid residues/cathionic antimicrobial peptide of 18 kd), was isolated first from bone marrow. Cathelicidins are usually stored in the granules of PMNs, and are in addition expressed by epithelial cells, monocytes, natural killer cells, B cells, and γδ T cells in response to inflammation or injury. LL-37 stimulates host defenses against microbes, reduces proinflammatory cytokine responses, and promotes wound healing. As might therefore be expected, LL-37 is effective in certain types of lung infection, skin diseases, intraabdominal sepsis, and endotoxemia.

There is strong evidence in animals that fever-range hyperthermia improves outcome from serious infection. Specifically, PMN function may be enhanced, which is the most important host defense against bacterial pathogens. In rats, for example, hyperthermia increases PMN counts by attenuation of apoptosis. Furthermore leukocyte trafficking is improved, which has also been associated with tumor regression. Therefore, even without conclusive evidence of benefit in humans, hyperthermia is increasingly being used therapeutically not only in alternative or complementary medicine, but also in clinical oncology as an adjunctive treatment to radiochemotherapy and for various inflammatory diseases.

To date, there are no data examining the potentially beneficial effects of LL-37 prophylaxis for sepsis. Therefore, we tested LL-37 as prophylaxis or therapy in septic rats. Furthermore, besides the restriction of hyperthermia treatment to a fever-range level, a potential approach is to combine it with other therapeutic modalities, such as antimicrobial peptides, because both alter cytokine responses and may improve PMN function. Therefore, we also examined the outcome of hyperthermic preconditioning plus LL-37 therapy after intraabdominal sepsis.

Materials and Methods

Our study was performed with permission of the regional animal welfare committee in Gießen, Hessen, Germany. We used 148 male Wistar rats, 220–300 g (Charles River Wiga, Sulzfeld, Germany), in two separate trials. They were given standard diet (Altromin, Lage, Germany) and water ad libitum. Two independent trials were performed: In the first, the survival rates of (1) peritoneal contamination and
infection (PCI) only (controls) were compared with (2) LL-37 prophylaxis (0.5 mg/kg, administered 12 h before PCI) or (3) LL-37 therapy (0.5 mg/kg, administered 1 h after PCI). In the second, we evaluated the outcome of (1) PCI only (controls) compared with (2) LL-37 therapy or (3) hyperthermic preconditioning (41°C for 1 h, applied 24 h before PCI) or (4) hyperthermic preconditioning plus LL-37 therapy.

Protocol

The rats were deprived of food 12 h before surgery. In the designated rats, a bolus of 0.5 mg/kg LL-37 (LLGDFFRK-SKEKIKKFKRIVQIKDFLRNLVPRTES-COOH, chemically synthesized at the Charite, Berlin, Germany) was given intravenously as prophylaxis, 12 h before surgery, or therapeutically 1 h after surgery and PCI. Control groups were given equal volumes of a placebo consisting of lactated Ringer’s solution. One hour before surgery, the animals were anesthetized with 0.08 mg/kg fentanyl and 4 mg/kg droperidol (Janssen-Cilag, Neuss, Germany), both given intraperitoneally. Ventilation was spontaneous. Subsequently, a tail vein was cannulated, and 2 ml lactated Ringer’s solution was given. All animals received intravenous antibiotic prophylaxis with 10 mg/kg cefuroxime Ringer’s solution. One hour before surgery, the animals received food and water ad libitum. At the end of each trial (after 120 h), survivors were killed by inhalation of carbon dioxide.

Trial 1. Animals were assigned by simple random permutation to three groups using ear marks (n = 20 rats/group): (1) control (PCI only), (2) LL-37 prophylaxis, or (3) LL-37 therapy.

Trial 2. Animals were assigned by simple random permutation to four groups (n = 22 rats/group): (1) control (PCI only), (2) LL-37 therapy, (3) hyperthermic preconditioning before surgery, or (4) hyperthermic preconditioning plus LL-37 therapy. The surgeon was blinded to the preoperative temperature management. Core temperature in the hyperthermic preconditioning group was maintained at 41°C ± 1°C for 1 h, 24 h before surgery by surface warming of anesthetized animals using an infrared heating lamp.

Measurements

Animals were weighed the day before surgery. In animals subjected to hyperthermic preconditioning, a digital thermometer was inserted 3 cm into the rectum for continuous core temperature measurement throughout hyperthermia. Nine rats, randomly selected from each trial 2 group, had 1.5 ml blood taken from the retroorbital venous plexus (after supplemented analgesia with fentanyl–droperidol) 1 h before and 1 h after PCI; the blood was replaced intravenously with 3 ml lactated Ringer’s solution. By using only nine rats from each group, half of each group was left unstressed by blood sampling. For cytokine determinations, blood was immediately centrifuged, and the resulting plasma was stored at −70°C until assayed. An enzyme-linked immunosorbent assay technique was used to determine interleukin-6 (IL-6), tumor necrosis factor-α, macrophage inflammatory protein-2 (MIP-2), and heat shock protein-70 concentrations (rat enzyme-linked immunosorbent assay from Pharmingen/Becton Dickinson, Heidelberg, Germany; Biosource, Camarillo, CA; and Stressgen, Ann Arbor, MI). In addition, 24 h after infection, heparinized whole blood was used to determine leukocyte counts of three animals from each trial 2 group with an automated blood cell Coulter optimized for rat blood (Coulter Max-M; Krefeld, Germany) and to analyze the phagocytosis rate of fluorescein isothiocyanate opsonized Escherichia coli by phagocytes using flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ) including a commercial kit (Phagotest®; Orpegen Pharma, Heidelberg, Germany).

Statistical Analysis

The primary endpoint for both trials was survival of rats at 120 h after surgery. A sample size of 20 rats/group for trial 1 and a sample size of 22 rats/group for trial 2 were calculated with the formula of Friedman20 estimating a 35% survival difference between control and treatment/combination groups with an α error of 0.025 and a power of 0.8. Survival rates were analyzed with the chi-square test, and Kaplan-Meier survival curves were depicted and analyzed with the log-rank test. Ordinal data were analyzed with the Kruskal-Wallis test using SPSS® software (SPSS Inc., Chicago, IL). Post hoc testing included a Bonferroni-Holm correction. Ordinal data are presented as mean ± SEM; P values less than 0.05 were considered statistically significant.

Results

The rats in all of the groups were of similar weight (250 ± 30 g). There were no complications related to surgery or hyperthermia. However, in trial 2, three rats died after anesthesia induction (one rat in each group 1 and 2 before surgery and one rat in group 4 before...
hyperthermia treatment). Data from these animals were not included in the analysis.

In trial 1, 30% (6 of 20) of the control group (PCI only) survived 120 h. After LL-37 prophylaxis, the survival rate was 55% (11 of 20), and with LL-37 therapy, the survival rate was 70% (14 of 20) after infection ($P = 0.038, df = 2$; LL-37 therapy vs. control: $P = 0.012, df = 1$; fig. 1).

In trial 2, the survival rate in the control group was 38% (8 of 21) compared with 67% (14 of 21) in the LL-37 therapy group ($P = 0.06$ vs. control) and 59% (13 of 22) in the hyperthermia group ($P = 0.1$ vs. control). However, LL-37 therapy combined with hyperthermic preconditioning improved the survival rate significantly to 90% (19 of 21) ($P = 0.01, df = 3$; versus control: $P = 0.001$; versus hyperthermia alone: $P = 0.02$; versus LL-37 therapy: $P = 0.08$; figure 2). The survival rate of the animals stressed by blood sampling did not differ significantly from that of rats that were otherwise treated comparably.

In none of the groups of trial 2 could we detect heat shock protein release in plasma. Preoperative cytokine concentrations of tumor necrosis factor-$\alpha$, IL-6, and chemokine MIP-2 levels were at the detection limit of the assays. Postoperative plasma tumor necrosis factor-$\alpha$ concentrations did not differ between groups (controls: 74 ± 41 pg/ml; hyperthermic preconditioning: 65 ± 31 pg/ml; LL-37 therapy: 15 ± 6 pg/ml; combination group: 12 ± 3 pg/ml). Plasma concentrations of IL-6 and MIP-2 were significantly lower in the combination group than in the single intervention groups, specifically, MIP-2 concentrations 1 h after PCI were 16 ± 11 pg/ml in the control group, 4 ± 1.5 pg/ml in the hyperthermic preconditioning group, 15 ± 20 pg/ml in the LL-37 therapy group, and 2 ± 1 pg/ml in the hyperthermic preconditioning plus LL-37 therapy group ($P = 0.028$); IL-6 concentrations were 88 ± 86 pg/ml in the control group, 9 ± 5 pg/ml in the LL-37 therapy group, 69 ± 50 pg/ml in the hyperthermic preconditioning group, and 24 ± 13 pg/ml in the hyperthermic preconditioning plus LL-37 therapy group ($P = 0.015$).

In the hyperthermic preconditioning alone group, blood was clotted; therefore, blood cell counts and phagocytosis rate of only three groups could be analyzed. The phagocytosis rate at 24 h after infection was 62% (40–85%) in the control group, 82% (33–92%) in the LL-37 therapy group, and 61% (35–76%) in the combination group. Leukocyte counts and %PMNs did not differ between groups, ranging from 3.3 to 4.9 × 10$^6$ cells/ml and 29% to 61%, respectively.

**Discussion**

The main finding of our first trial was that only LL-37 therapy improved survival rate after sepsis. Cathelicidins are one family of antimicrobial peptides characterized by conserved propeptide sequences. LL-37/hCAP-18 is the only human cathelicidin and is expressed in inflammatory and epithelial cells. Besides their direct antimicrobial function, cathelicidins have multiple roles as mediators of inflammation influencing diverse processes such as cell proliferation and migration, immune modulation, wound healing, angiogenesis, and the release of cytokines and histamine. Because it is known that LL-37 binds to apolipoprotein A-I, we may speculate that after prophylaxis, bioavailability of LL-37 was too low at the time of infection. Our results also support the findings of a recent study that demonstrated improved survival after cecal ligation and puncture-induced sepsis, with LL-37 treatment being superior to conventional antibiotic treatment. We can extend this observation in that we routinely use antibiotics in our sepsis model.
suggesting synergistic effects of antibiotics (cefuroxime/metronidazole) plus LL-37, although the mechanism of interaction needs further investigation. Antibiotics are routinely given for sepsis prophylaxis in clinical practice and may interact differentially with other mediators such as cytokines.

However, a limitation of all sepsis models is reproducibility and variability especially when a specific survival rate is intended. Our PCI model using human stool is well validated and reproducible in that we always create a dose-response relation before entering the main trials. Stool preparation was identical for both of the trials presented, the pooled stool was microbiologically characterized, and antibiotic effectiveness was proved. Especially compared with the cecal ligation and puncture model, where a high variability in survival has been reported depending on the technique used (length of ligated cecum, needle size used, single or double puncture), in our model this variability does not exceed approximately 10%. Therefore, in the second trial with a higher survival rate in the control group (38% vs. 30% in trial 1), LL-37 therapy scarcely missed significance (P = 0.06) compared with controls; however, meta-analysis of both trials confirms efficacy of LL-37 therapy (P = 0.04).

The main finding of our second trial was that hyperthermic preconditioning (41°C for 1 h) per se could not increase the survival rate after sepsis (P = 0.1), but the combination with LL-37 therapy was effective as compared with controls (P = 0.001) and with hyperthermic preconditioning alone (P = 0.02), suggesting additive effects. The evolutionarily conserved febrile response during infection has been associated with improved survival in vertebrates and invertebrates, although its protective mechanism of action and especially that of the thermal component is one of the most poorly understood aspects of inflammation. Much of the hyperthermia effects on the immune system have been attributed to the expression of heat shock proteins in several organs, as shown, for example, after hyperthermia treatment of mice (39°–40°C over 6 h); because we could not detect heat shock protein release in our setting 24 h after hyperthermia treatment, the duration of hyperthermia (41°C) of only 1 h may not have been long enough. In addition, we did not measure or control glutamine levels in our animals, although glutamine-starved monocytes have a reduced thermoreistance which is associated with a reduced expression of the cytoprotective protein heat shock protein 70 in vitro. Temperatures within the range of physiologic fever also influence production and bioactivity of proinflammatory cytokines. Hyperthermia also attenuates sepsis-induced apoptosis; reduces lipopolysaccharide-induced hyposeriveness of platelets, promotes lymphocyte–endothelial adhesion, which may suppress tumor progression, and enhances PMNs. PMNs are recognized as key mediators of inflammation and may also play a crucial role in mediating the antitumor effects of fever-range whole body hyperthermia. Although there is strong evidence in animal models of infection supporting therapeutic use of hyperthermia, only a few prospective, randomized trials have been reported in humans focusing on metastatic tumors. For example, therapeutic use of fever-range whole body hyperthermia (39°–40°C) was first reported to be beneficial in a phase I study in patients with advanced solid tumors. Equivocally, others found that mild to moderate hyperthermia as an adjunct to radio/chemotherapy provides substantial benefit in a phase II study in 41 patients with metastatic colon cancer and in 16 patients with advanced gastric carcinoma.

Assuming hyperthermia is beneficial for various infectious conditions and tumors, its application in humans is problematic because unanesthetized humans precisely regulate core temperature. Efforts to induce hyperthermia thus provoke vigorous thermoregulatory reactions such as vasodilation and evaporation (sweating), both of which activate the sympathetic nervous system resulting in hypertension, tachycardia, and substantial increases in circulating catecholamine concentrations. Therefore, a fever-range, mild level of induced hyperthermia is preferred, which is generally well tolerated with only occasionally needed light sedation of patients. However, in contrast to others, we did not find an improved survival rate 120 h after intraabdominal sepsis in animals with the chosen degree and duration of hyperthermic preconditioning alone; Timing of hyperthermia may also be important, because in one recent report hyperthermia treatment after sepsis was found to be unfavourable.

A potential explanation for increased survival with LL-37 is that it reduced the systemic concentrations of proinflammatory cytokines: Like others before, we found that LL-37 plus hyperthermia suppressed the excessive release of the proinflammatory cytokine IL-6 and chemokine MIP-2 after sepsis. MIP-2 enhances PMN recruitment and migration into infected tissues. With all the restrictions of the small sample size we had available for phagocytosis determination in trial 2, data indicate that phagocytosis rate was increased after LL-37 therapy, but this must be confirmed in further studies. However, this effect seemed to be abolished in the combination group.

Current and previous work indicates that administration of LL-37 or its active motifs may improve host response to bacterial peritonitis and sepsis; however, more data on the mechanisms and possible side effects are needed before entering a clinical trial. Fever-range hyperthermic preconditioning per se did not improve outcome after a subsequent infectious challenge in this setting. However, given its clinical feasibility as an adjunctive pretreatment in surgical patients prone to infec-
tion, our results suggest that a combination with an immunomodulator such as LL-37 may be beneficial.

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