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RESEARCH ARTICLE

Comparison of Intravenous Versus Intraperitoneal Interleukin-10 Gene Delivery in Mouse Model of Sepsis

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Summary

The most novel approach utilizing IL-10 in sepsis is IL-10 gene delivery in experimental model of sepsis. In our study, we aimed to compare kinetics of intravenous versus intraperitoneal delivery of IL-10 gene transfer in early stages of sepsis. This is a prospective controlled experimental study. Six groups were gathered with 20 Swiss-Albino female mice. Intra-abdominal sepsis was induced by cecal ligation and puncture (CLP). Animals had either intraperitoneal or intravenous IL-10 liposomal gene transfer. Animals were sacrified 24 h after injections, followed by harvest of lung,liver,spleen,vena cava tissues. Immunostaining revealed more prominent staining in liver after intraperitoneal delivery. All endothelial tissues stained with intravenous delivery. There was striking difference between tissue expressions of transgene of animals in CLP intravenous group when compared to other groups. Our results point out that pro-inflammatory action of IL-10 is prominent in intravenous gene delivery which shows itself with induction of zyon. IL-10 still may harbor therapeutical potential which still needs to be explored.

Keywords: *Interleukin-10, Interleukin-6, Tumor Necrosis Factor-alpha, Sepsis, Gene therapy*

Farelerde İntraabdominal Sepsis Modelinde İntravenöz ve İntraperitoneal İnterlökin 10 Lipozom Aracılı Gen Tedavisinin Karşılaştırılması

Özet

Sepsiste IL-10 kullanımına yönelik en yaratıcı yöntem IL-10'un deneysel modelde gen tedavisi şeklinde verilmesidir. Biz çalışmamızda IL-10'un erken sepsiste intravenöz ve intraperitoneal kinetiğini karşılaştırdık. Araştırmamız prospektif kontrollü çalışmadır. Yirmi adet Swiss-Albino dişi fare kullanılarak altı grup oluşturulmuştur. İntra-abdominal sepsis çekal ligasyon ve ponksiyon (ÇLP) yöntemiyle ortaya çıkarılmıştır. Deney hayvanlarına ya intraperitoneal veya intravenöz olarak IL-10 lipozomal gen transferi yapılmıştır. Hayvanlar enjeksiyon sonrası 24. saatte sakrifiye edilmişlerdir. Bunu akciğer, karaciğer, dalak, vena kava dokularının çıkarılması izlemiştir. İmmünhistokimyasal boyamada intraperitoneal verilişten sonra karaciğer boyanmasının daha belirgin olduğu görülmüştür. İntravenöz verilişten sonra tüm endotelyal dokuların boyandığı görülmüştür. Transgenin doku ekspresyonunun ÇLP ve intravenöz enjeksiyon yapılan grupta diğer gruplara göre çok daha belirgin olduğu görülmüştür. Sonuçlarımız aynı zamanda göstermiştir ki IL-10'un pro-inflamatuar etkisi intravenöz verilişte daha belirgindir ve kendisini IL-6 indüksiyonuyla göstermektedir. IL-10 'un halen keşfedilmeyi bekleyen tedavi edici özelliği vardır.

Anahtar sözcükler: *İnterlökin-10, İnterlökin-6, Tümör nekroz faktör alfa,Sepsis, Gen tedavisi*

INTRODUCTION

Sepsis is a systemic illness caused by microbial invasion of parts of human body during the course of bloodstream infection [1]. The classical methods of sepsis treatment include maintenance of systemic perfusion and eradication of

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infectious sources [2].

Systemic inflammatory response syndrome induced by sepsis is characterized by orchesterated release of TNF-α, interleukin (IL) 6, IL-8, and IL-1 [3].

Immunomodulatory therapeutic approaches targeting TNF-α and IL-1 to control inflammatory cytokine response were not proven to be beneficial in humans although demonstrated to be valuable in different rodent models [4,5].

IL-10 is a biphasic immunomodulator cytokine which inhibits Th1 type immune response and pro-inflammatory cytokines like TNFα and IL-1 [6]. IL-10 is first defined as T helper cell derived factor inhibiting cytokine synthesis. IL-10 inhibits T helper cytokines like interferon and IL-12 as well as pro-inflammatory cytokines like TNFα, IL-1 and nitric oxide. IL-10 is considered to have a substantial role in acute illnesses because of these antiinflammatory effects [7].

In the last decade, there has been a substantial effort to identify factors in the host response to infection that could be used for therapy. In this prospective controlled experimental study we aimed to compare kinetics of intravenous (IV) versus intraperitoneal (IP) delivery of IL-10 gene transfer in early stages of sepsis.

MATERIAL and METHODS

Animals and Surgical Procedures

The study was approved by Hacettepe University Ethics commitee with approval number of 2007/42.

There were six groups containing a total of 20 Swiss Albino female mice with weight of 22±5 g. Intraabdominal sepsis was induced by cecal ligation and puncture (CLP) with 22 G needle.

The control group consisted of three sham operated mice where only laparotomy was performed. CLP procedure was performed on the 15 mice in three treatment groups. Nothing other than cecal ligation and puncture (CLP) was performed on mice in group two (n=3). Mice in group three (n=6) had CLP and ip injection of IL-10 plasmid through right lower abdominal quadrant. Animals in group four (n=6) had CLP and iv injection via tail vein. Fifth group had one mouse which received ip injection only. Sixth group had one mouse which received iv injection only.

Injections were applied 24 h after initiation of sepsis with CLP. Animals were sacrified 24 h after injections, followed by harvest of lung, liver, spleen, vena cava tissues. Tissues were divided into 4 pieces for mRNA, protein, DNA and reverse transcriptase polymerase chain reaction (RT-PCR) analyses. National Center for Biotechnology Information numbers of primers used for TNFα and IL-6 were NM_013693 and NM_031168 respectively.

All animals were kept at room temperature and 12-h day/night cycle, having access to food and water ad libitum. All animal procedures were performed according to an institution approved protocol and under strict biological containment.

RNA Isolation and Quantitative Gene Expression Analysis

75-90 mg of sampled tissue was disrupted in a 2ml screw-cap tube containing ceramic beads and 1 ml of Trizol reagent (Invitrogen™). The tissues were rapidly disrupted using a bead-beater and kept at -80°C. The total RNA extractions from all tissues were performed simultaneously upon manufacturer's recommendations and the RNA integrity was assessed and documented via denaturing agarose gel electrophoresis. Following RNA extraction and quality assessment, reverse transcription was accomplished using Improm II reverse transcriptase (Promega™). Briefly, 0.5 µg of oligo dT primers are hybridized to 1 µg of total RNA following an initial denaturation at 70°C and reverse transcribed into cDNA following manufacturer's recommendations.

The amplification of the target genes were achieved using the quantitative PCR technique using the SYBR green dye incorporation method using the primer design and amplification strategy described before. The expression of the genes of interest were normalized to the expression of the beta actin gene and the primers and conditions regarding the technique were available upon request by e-mail. The PCR reactions were carried out on a Rotor gene 6.000 real-time PCR instrument (Corbett Life Sciences™) using Jumpstart SYBRgreen mix (Sigma™) according to manufacturer's protocols.

Plasmids and Gene Transfer

The human IL-10 cDNA (Genebank accession: NM_000572) is purchased as a GeneStorm vector (Invitrogen, Carlsbad, CA) and subcloned into pCDNA3.1 expression vector together with a C terminal fused 14 aminoacids long V5 epitope to help to discriminate from the endogeneous IL-10. The open reading frame of the expression plasmid is sequenced and amplified in to yield sufficient amount. Injection volumes were 200 µl per mice each containing IL-10 gene carrying pcDNA3.1/GS plasmid vector and antiV5-HRP antibody (hIL-10pcDNA3.1/GS transformed into GeneHogs® *E.coli*) bought from Research Genetics CO. The expression was driven via the cytomegalovirus promoter and contained a 14-amino acid V5 fusion peptide tag at the 3´ end, which assists to trace the transgene expression *(Fig. 1)*. N-(1-[2,3-dioleoyloxyl]propyl) NNNtrimethylammoniummethylsulfate: cholesterol (1:1 molar ratio) liposomal transfection reagent (Sigma Aldrich©, St. Louis, MO) was used according to manufacturer's recommendations to achieve the IP and IV gene transfer 24 h after CLP procedure.

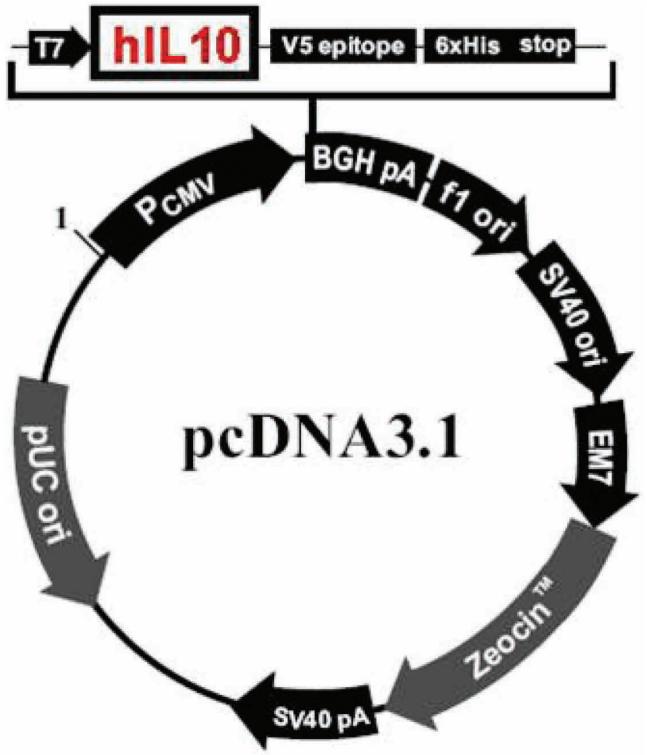


Fig 1. Schematic presentation of the gene construct delivered in injections to mice

ŞekIL-1. Hayvanlara enjekte edilen genin şematik yapısı

Immunostaining

The immunostaining was performed on the animals killed 24 h after IP and IV injections to localize and assess the expression of the IL-10 transgene in lung, liver, spleen, vena cava tissues. These tissues were fresh-frozen after the necropsy, and the standard immunostaining procedure was employed with a primary horseradish peroxidase-conjugated monoclonal anti-V5 antibody coupled to the secondary DIG-conjugated anti-horseradish peroxidase for localization. Apart from IL-10 staining, TNF-α, IL-6 gene expression levels were identified.

RESULTS

IL-10 Transgene Expression in Tissues

Immunostaining revealed more prominent staining in liver after i.p delivery while all the endothelial tissues stained with IV delivery. The signal for anti-V5 epitope antibody was used as tracer in tissues. The immunostaining images are presented in *Fig. 2* and *Fig. 3*.

IL-6 Transgene Expression in Tissues

There was a striking difference between tissue expressions of transgene of animals in CLP IV group when compared to other groups. In this group, spleen tissue showed highest expression and liver showed lowest expression *(Fig. 4)*.

CLP ip group animals had highest expression in their spleens while lowest staining was seen in lung tissues. The difference in expression when compared to CLP only group was statistically significant (P<0.05, ANOVA).

TNF-α Transgene Expression

Highest transgene expression was seen in CLP only group. In this group, spleens showed highest amount of staining whereas livers and lungs had almost equal amounts of stainings. All tissues in CLP IP group had higher expressions than CLP IV group *(Fig. 5)*. The difference was statistically significant (P<0.05, ANOVA).

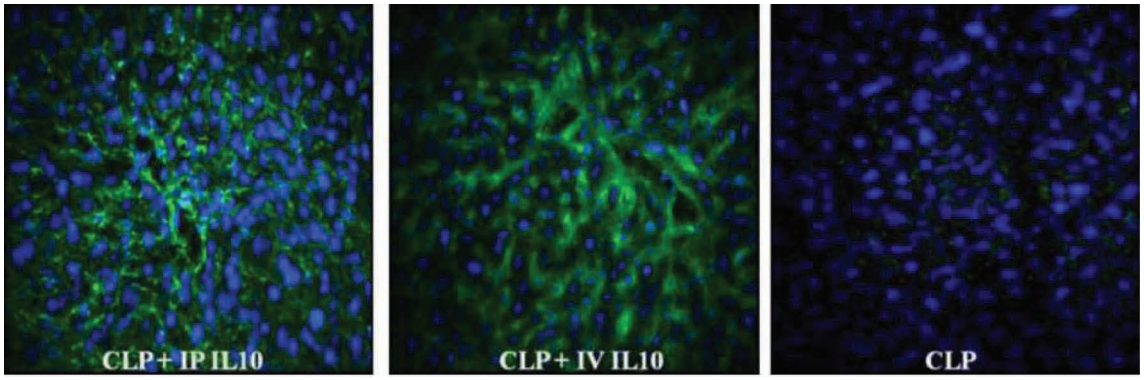


Fig 2. Liver parenchymal and sinusoidal staining after intraperitoneal and intravenous

delivery. Only CLP group staining was given for comparison (IL-10 in green, 4′,6-diamidino-2-phenylindole fluorescent stain in blue, CLP: liver specimen from cecal ligation and puncture only mouse, CLP+ IP IL10: liver specimen from mice which had intraperitoneal injection after cecal ligation and puncture, CLP+IV IL10: liver specimen from mice which had intravenous injection after cecal ligation and puncture)

Şekil 2. İntraperitoneal ve intravenöz enjeksiyon sonrası karaciğer parankimal ve sinüsoidal boyanmaları gösterilmektedir

Sadece çekal ligasyon ve delme yapılan farenin boyanması karşılaştıma amaçlı verilmiştir (IL-10 yeşil renk, 4′,6-diamidino-2-fenilindol floresan mavi renk, CLP: sadece çekal ligasyon ve delme yapılan farenin karaciğer boyanması, CLP+ IP IL10: çekal ligasyon ve delmeden sonra intraperitoneal enjeksiyon yapılan farenin boyaması, CLP+IV IL10: çekal ligasyon ve delmeden sonra intravenöz enjeksiyon yapılan farenin boyaması)

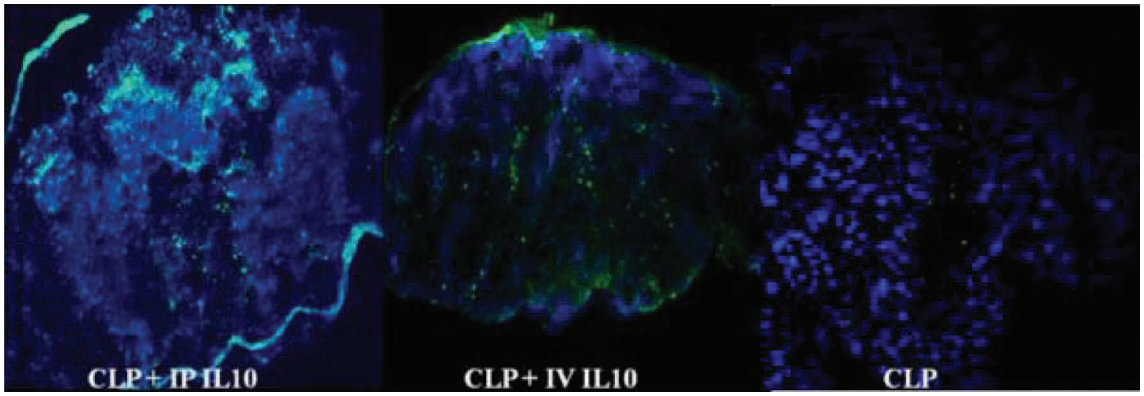


Fig 3. Spleen staining after intraperitoneal and intravenous delivery

Only CLP group staining is for comparison (IL-10 in green, 4',6-diamidino-2-phenylindole fluorescent stain in blue, CLP: spleen specimen from cecal ligation and puncture only mouse, CLP + IP IL10: spleen specimen from mice which had intraperitoneal injection after cecal ligation and puncture, CLP + IV IL10: spleen specimen from mice which had intravenous injection after cecal ligation and puncture)

Şekil 3. İntraperitoneal ve intravenöz enjeksiyon sonrası dalak boyamaları

Sadece çekal ligasyon ve delme yapılan farenin boyanması karşılaştırma amaçlı verilmiştir (IL-10 yeşil renk, 4',6-diamidino-2-phenylindol floresan mavi renk, CLP: sadece çekal ligasyon ve delme yapılan farenin dalak boyanması, CLP + IP IL10: çekal ligasyon ve delmeden sonra intraperitoneal enjeksiyon yapılan farenin dalak boyaması, CLP + IV IL10: çekal ligasyon ve delmeden sonra intravenöz enjeksiyon yapılan farenin dalak boyaması)

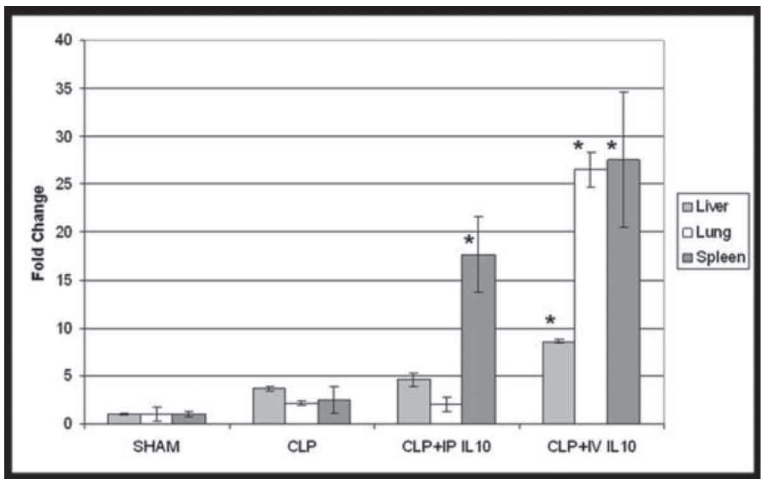
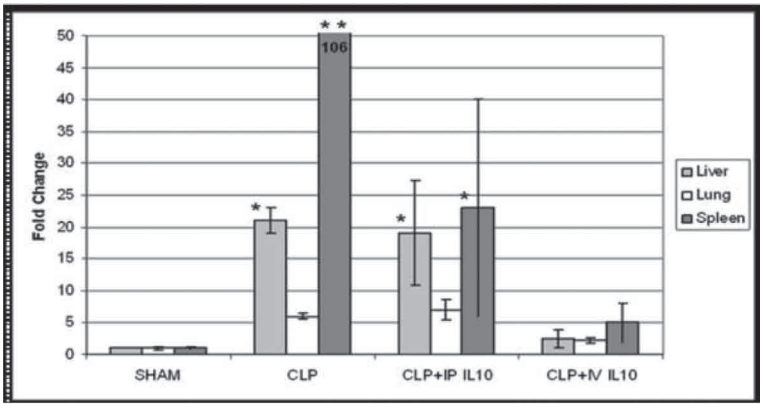


Fig 4. IL-6 transgene expression in groups (* P<0.05) (CLP: specimens from cecal ligation and puncture only mouse, CLP + IP IL10: specimens from mice which had intraperitoneal injection after cecal ligation and puncture, CLP + IV IL10: specimens from mice which had intravenous injection after cecal ligation and puncture)

Şekil 4. Gruplardaki IL-6 transgen ekspresyonu (* P<0.05 (CLP: sadece çekal ligasyon ve delme yapılan farelerden alınan dokuların analizi, CLP + IP IL10: çekal ligasyon ve delmeden sonra intraperitoneal enjeksiyon yapılan farelerden alınan dokuların analizi, CLP + IV IL10: çekal ligasyon ve delmeden sonra intravenöz enjeksiyon yapılan yapılan farelerden alınan dokuların analizi)

Fig 5. TNF-α transgene expression in groups (CLP: specimens from cecal ligation and puncture only mouse, CLP + IP IL10: specimens from mice which had intraperitoneal injection after cecal ligation and puncture, CLP + IV IL10: specimens from mice which had intravenous injection after cecal ligation and puncture)

Şekil 5. Gruplardaki TNF-α transgen ekspresyonu (CLP: sadece çekal ligasyon ve delme yapılan farelerden alınan dokuların analizi, CLP + IP IL10: çekal ligasyon ve delmeden sonra intraperitoneal enjeksiyon yapılan farelerden alınan dokuların analizi, CLP + IV IL10: çekal ligasyon ve delmeden sonra intravenöz enjeksiyon yapılan yapılan farelerden alınan dokuların analizi)



DISCUSSION

In '90s, rise of IL-10 levels has been documented in sepsis and its plasma concentration was related to mortality and the severity of multi organ dysfunction. In various studies plasma levels of IL-10 and TNF-α were found to be correlated [8,9].

In early systemic inflammatory response stage of

sepsis, IL-10 is shown to be proinflammatory whereas in multi organ dysfunction stage it is known to exert anti-inflammatory function. IL-10 was thus called as "biphasic immune modulator". Stemming from these findings it was proposed that IL-10 administration would be beneficial before inflammation is triggered.

IL-10 infusion trials in humans were proven to be untolerable causing severe fever, tremor, myalgia and hypotension while it's half life changes between 2.5 and 4 h. One alternative approach to IL-10 protein administration is to synthesize the IL-10 *de novo* in the host body. The most novel of these approaches is achieving the IL-10 gene delivery which is known to enhance survival in experimental model of sepsis [10-12].

In the second half of '90s, phase I studies in humans showed that the infusion of IL-10 was not well tolerated [13,14]. Along with TNF-α and IL-6 blockage by IL-10, other immunotherapy options were also tried without any success [15]. After these studies IL-10 was deemed unuseful. After gene transfer and gene therapy techniques were utilized in medical sciences new interventions using IL-10 became possible.

When given exogenously, IL-10 molecule has a short bioefficacy and half life. But gene transfer of IL-10 provides de novo continous expression of IL-10 inside the cell which overcomes bioefficacy and half life problems. Our previous studies have pointed out the beneficial role of IL-10 gene transfer in CLP model of sepsis [5].

In a pre-treatment model, the animals conditioned with ip IL-10 supressed TNF-α expression following CLP and exhibited better survival rate [16]. The major drawback in this was the pro-inflammatory effect exerted by the viral vectors themselves while gene transfer by cationic liposomes was free of inflammatory side effects providing safe expression. This was the main reason why we chose to use liposomal vectors.

Intravenous administration is a true systemic approach with successful gene delivery to the endothelium which is the most vulnerable target organ in sepsis. Our study proved that IL-10 liposomal gene transfer via iv or ip routes are both efficient in maintaining adequate tissue levels including endothelium 24 h after delivery.

We propose that the early expression of IL-10 in peritoneum helps to suppress and delay the overt inflammatory reaction in this irreversable model of intra-abdominal sepsis. These results also suggest that the IL-10 gene delivery via ip route helps to diminish TNF-α induction in 48th h of sepsis but the iv route is more succsessful in reducing the TNF-α levels.

Our results point out that the pro-inflammatory action of IL-10 is prominent in IV gene delivery which shows itself with induction of IL-6. IL-6 was suppressed with ip delivery in liver and lung in our study.

It is known from the kinetics of this gene transfer that the transgene expression peaks at 36 to 48th h and diminishes following the 3rd day, thus suggesting a temporary de novo source for IL-10 [17]. Further immunological and experimental data is needed to elucidate the crosstalk between IL-10 and other cytokines in sepsis.

Our study highlights the fact while preventing mortality in CLP model of sepsis, IL-10 may also harbor a therapeutical potential which still needs to be explored.

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