INGESTIGATION OF SIDE EFFECTS AFTER RENAL GENE THERAPY (RNAi) AND LUPUS NEPHRITIS IVIG THERAPY ON MOUSE MODELS

PhD thesis

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Introduction

Loss of renal function in a short time leads to acute renal failure, which has several etiologic factors, and may lead to chronic renal failure. The therapeutic possibilities in these cases are still poor. In this study we investigated the side effects of therapies potentially used in nephrology using mouse models.

RNA interference is an ancient gene silencing method that protects the genome from viral infections and transposons. The basic molecule of RNAi is the double-stranded short interfering (si) RNA. During viral replication dsRNS-s are often produced [1], and the intracytoplasmic presence of them leads to the activation if interferon (IFN) response. In the first years of siRNS experiments is was believed that siRNA-s are too small for activating the IFN response. Sledz and co-workers found that siRNA-s triggered the IFN response *in vitro* [2]. In our experiment we assessed the response of the whole body to siRNAs on the level of genes (gene expression), cell (enzyme activity, cytokine levels), and tissue (histology), to answer the question of in vitro toxicity of siRNAs.

In another experiment of ours, we applied a previously used therapeutic agent in systemic lupus erytematodes (SLE). SLE is one of the most common autoimmune diseases, affecting 0.5% of population in Western countries [3].Today, therapy of SLE is non-specific and toxic [4], thus, in order to minimize side-effects, new therapeutic agents should be used rather to regulate than to suppress the immune response. Intravenous immune globuline (IVIG) belongs to these agents, and it has previously been used in many autoimmune diseases with amelioration skin manifestation, also in lupus [5]. It is worth to mention that during the use of IVIG nephrotoxicity occoured in some cases. Histology assessments revealed the vacuolization and swelling of proximal tubular epithel cells, and this phenomenon was named osmotic nephrosis or hydropic degeneration [6]. According to previous investigations, the case of osmotic nephrosis was the sugar component is IVIG [7], that was used to stabilize tertiary structure of IgG. In our experiment we investigated the effect of long-term, high-dose, glycine stabilized IVIG on mouse lupus, and to assess nephrotoxicity.

Aims

We aimed to investigate the side effect of RNA interference, a gene silencing method, that is used widely in animal and human experiments, too. We wished to answer the following questions:

1. **Do short dsRNAs activate the infereron response of the whole body if injected hydrodynamically?**

   We were investigating whether the interferon response occurs after systemic injection also, since according to previous publications, we know that siRNAs induce interferon response in vitro

2. **If yes, what kind of gene (STAT1 and OAS1), and cytokine (TNFα, IL-6, IL-12p40 and IFNβ) expression profile is it characterized by?**

   In our experiments we investigated the expression profile of interferon response genes (RT-PCR) and the blood level of inflammatory cytokines, most often participating in interferon response (ELISA).

   3. Does the low volume hydrodynamic injection cause so called non-specific side effect compared to high volume hydrodynamic injection?

   According to previous data, we supposed the injury of abdominal parenchymal organs (liver, spleen and kidney), thus, we examined the histology and organ functional parameters (ALAT, ASAT, BUN) of these organs.

In another experiment we examine the effects of long-term, high-dose IVIG therapy in SLE, and we wished to answer the following questions:

1. **How does the long-term, high-dose IVIG therapy affect the skin and renal manifestation of the SLE of MRL/lpr mice?**

   According to previous data, IVIG therapy had different effect on skin and renal lesions. In our experiment we investigated the effect of long-term IVIG therapy on skin and kidney disease in MRL/lpr mouse strain.

2. **Can we see organ specific difference (renal function, autoantibody titer, histology parameters) after long-term, high-dose, glycine stabilized IVIG therapy?**

We compared the semi quantitative histology scores of IVIG and saline treated mice iin skin and the kidney, together with serum autoantibody levels.

3. **Does the long-term, high-dose, glycine stabilized IVIG therapy possess in vivo toxicity compared to the sugar induced positive controls?**

We investigated the effects of glycine stabilized IVIG therapy on renal disease (histology and renal function) compared to saline and sugar treated negative and positive controls.

Materials and Methods

In our first experiment we treated NMRI mice with two naked siRNAs, and saline according to low volume HDI (50 μg/1 ml). Positive controls received 50 μg polyinosinic-polycytidilic acid (poly I:C). To investigate the liver damaging effect of high volume hydrodynamic injection (HVHDI), we treated one group of mice with saline of 10% of body weight. Six and 24 hours after treatment, we harvested the mice, and measured the liver and renal function (alanine amino transferase, aspartate amino transferase, blood urea nitrogen), the gene expression of STAT1 and OAS1 in liver, kidney and spleen, and the levels of interleukin-6 (IL-6), interleukin-12 (IL-12), tumor necrosis factor alpha (TNFα) and interferon beta (IFNβ) in the plasma. The latter measurements were performed 1.5, 2, 6, and 9 hours after LVHDI.

Results

Low volume hydrodynamic injection (LVHDI) caused low-scale elevation of STAT1 and OAS1 gene expression, and was not further elevated after injection of siRNAs

Six hours after LVHDI of saline we detected moderate elevation of STAT1 and OAS1 gene expression in liver, kidney and spleen, although, siRNA injection did not cause significant elevation compared to saline treated mice.

Low volume hydrodynamic injection did not cause significant elevation of inflammatory cytokine levels (IFNβ, TNFα, IL-6 and IL-12)

Plasma level of IFNβ elevated only 2 hours after injection of poly I:C (210.85±164.28 pg/ml), but it was not elevated in any other group. Plasma level of TNFα was measureable only 1.5 hours after treatment and only in positive controls, and then returned to normal level. Although, plasma levels of IL-6 in siRNA and saline treated mice elevated after injection, but this elevation was significantly lower than that of positive control group. Plasma IL-12

concentration did not elevate significantly after siRNA treatment, but it was high in positive controls, compared to saline treated mice (p=4.32E-08).

Poly I:C induced organ damage associated interferon response, but siRNAs did not cause organ damage

In liver, kidney and spleen of siRNA and saline injected mice we saw normal structure both at 6 and at 24 hour group.

High volume hydrodynamic injection (HVHDI) caused liver damage that was not present in LVHDI group, where the tissue damage was mild and transient

ALAT activity of untreated mice was 13.33±0.85 mmol/l. Activity of ALAT doubled after LVHDI (1 ml), compared to untreated controls, and returned to normal level by 24 hours. On the contrary, HVHDI (2.5 ml) we saw irreversible hepatocyte necrosis with prominent ALAT elevation.

Systemic high dose IVIG therapy alleviated skin disease

Physiologic salt treatment of MRL/lpr mice led to skin ulceration by harvest, while IVIG treatment significantly reduced macroscopic and microscopic skin damage in the interscapular region and on the ears. Scoring of histology changes also revealed the alleviation of skin disease after IVIG injection (Table 1.).

	IVIG (n=8)	Salina (n=10)	P value
Macroscopic			
Skin	0.75±0.34	1.3±0.4	<**0.03**
Ear	025±0.12	0.8±0.2	<**0.05**
Microscopic			
Dermal changes	058±0.47	1.21±0.58	**0.001**
Basal membrane changes	041±0.2	0.95±0.54	**0.001**
Hyper-and/or parakeratosis	0.39±0.1	0.66±0.28	**0.02**
Thickening of the stratum spinosum	0.23±0.11	0.61±0.28	0.212 (ns)
Ulcerations	0.11±0.33	0.4±0.51	0.089 (ns)
Inflammatory infiltration	0.35±0.21	0.75±0.42	0.123 (ns)
Summarized microscopic scores	0.34±0.29	0.76±0.56	**0.05**

Table 1.

Renal damage was not deteriorated IVIG therapy, no hydropic degeneration was present

Untreated MRL/lpr mice developed proliferative hypercellular glomerular lupus nephritis, the severity of which was similar both in IVIG and saline treated mice. Blood urea nitrogen level was also similar. Tubulointerstitial changes were almost the same in the two groups (2.42±0.33 vs 2.22±0.446, p=0.269): the most prominent histologic change was degeneration and necrosis of tubular epithelium. The typical picture os osmotic nephrosis developed in sacharose injected positive control mice was not seen either in IVIG or in saline treated mice.

Dermal and renal complement-3 (C3) deposition

Complement 3 deposition in the skin was observed in the vasculature (endothelial cells). IVIG treatment significantly reduced C3 positivity in the skin (p=0.02), but this profile was not seen in the kidney (0.387±0.289 vs 0.384±0.267; p=0.975).

IVIG treatment did not alter plasma autoantibody level in MRL/lpr mice

Optical density of anti-Sm, anti-DNA and anti-histone autoantitbodies was similar in both groups. Positivity and the pattern of anti-nuclear antibody (ANA) was also the same in both groups: in most of the cases it was homogenous (IVIG n=6, saline, n=5), in some of the cases granular pattern was also observed (IVIG n=3, saline n = 4).

Conclusions

According to our data, we can claim that there were no side effects in our mouse models using siRNAs or IVIG. We concluded the following points:

1. **DsRNAs injected according to the protocol of low volume hydrodynamic injection do not induce the interferon response.**

RNA interference is one of the most interesting field of modern gene therapy. Though, side effects, such as interferon (IFN) response, activation of immune system, or organ damage are still not well understood. In our experiment we found that systemic injection of short RNAs did not activate the immune system in vivo. RT-PCR measurements showed, that siRNAs did not induce the activation of Jak/STAT signaling pathway. Histology assessments showed no sign of tissue injury in any of the investigated organs (liver, kidney, spleen).

2. **The expression level of STAT1 and OAS1 genes and blood level of TNFα, IL-6, IL-12p40 and IFNβ cytokinesdid not elevate after systemic injection of siRNAs**

After low volume hydrodynamic injection (LVHDI) we measured the expressions of STAT1 and OAS1, and cytokine concentrations, and did not see significant elevations. We did not see histology changes.

3. **Low volume hydrodynamic injection has non specific side-effects, that are mild compared to those of high volume hydrodynamic injection.**

LVDHI caused slight elevation of alanine aminotransferase (ALAT) and mild hepatocyte injury, while HVHDI led to very high levels of ALAT and hepatocate necrosis. LVHDI itself caused weak increase in the gene expression of STAT1 and OAS1. Thus, LVHDI might lead to mild interferon response..

Intravenous immunoglobulin (IVIG) has many positive effects in the therapy of autoimmune diseases. IVIG has also been used in SLE, but due to the stabilizing sugar component, tubular damage was observed leading to acute renal failure. Thus, IVIG therapy was stopped. Nowadays, sugar components are replaced by amino acids, like glycine, with less prospensitiy to renal failure. In our experiment we examined the long-term, high-dose use of glycine stabilized IVIG on the mouse model of SLE.

1. Long-term, high-dose, glycine stabilized IVIG therapy alleviated skin lupus in MRL/lpr mouse strain.

We injected MRL/lpr mice once/week with glycine stabilized IVIG or saline (0,2 ml/10 g) from the age of 6 weeks to 5 months. At harvest we saw that IVIG therapy significantly reduced skin disease. Even microscopic investigation showed the same.

2. IVIG therapy did not change the outcome of renal disase

Histology examinations and complement-3 immunohistochemical reaction also showed the amelioration of skin disease after IVIG therapy. Kidney disease showed no difference between the two groups.

3. Glycine-stabilized IVIG therapy did not have renal toxicity in our model

Histology examintations of IVIG or saline treated mice showed no hydropic degeneration, while 5% sacharose and 10% maltose treated mice did.

Our data confirmed, that IVIG therapy ameliorated the skin disease without affecting renal disease or causing renal toxic event

List of publications

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