Immune mechanisms of Concanavalin A model of autoimmune hepatitis

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Abstract
As a chronic inflammatory disease of the liver, the pathogenic mechanisms of autoimmune hepatitis (AIH) have not yet been elucidated, with prognosis and diagnosis remaining unsatisfied. Currently the only viable treatments of AIH are immunosuppressant application and liver transplantation. It is considered that lack of good animal AIH models is the main reason for the shortage of a simple and efficient cure. The Concanavalin A (Con A) model is a typical and well established model for investigating T-cell and macrophage dependent liver injury in mice, which closely mimics the pathogenesis mechanisms and pathological changes of patients, and is regarded as the best experimental model for AIH research so far. In this paper we elucidated the pathogenic mechanisms of AIH and the evolution of relative animal models. We go on to further focus on Con A-induced liver injury from the point of immunological mechanisms and the change of cytokine levels. Finally, we manifested the clinical significance of the AIH animal models and the challenges they would meet during their future development.

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Key words: Autoimmune hepatitis; Animal models; Concanavalin A

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INTRODUCTION
Autoimmune hepatitis (AIH) is a chronic inflammatory disease of the liver, characterized by a loss of self-tolerance leading to the appearance of autoantibodies, pathological changes and dysfunctions (the detailed pathogenic mechanisms of which still remain vague). According to different antibodies profiles, AIH is classified into three categories: AIH type 1 is characterized by the presence of antibodies to nuclear antigens (ANA) and/or anti-smooth muscle antigen (SMA) antibodies; AIH type 2 is characterized by anti-liver kidney microsomal (LKM)-1 and low level of LKM-3 antibodies (with or without ANA or SMA antibodies); AIH type 3 is characterized by autoantibodies against soluble liver...
antigen/liver pancreas (with or without ANA or SMA antibodies)\[1\].

Around the world, the incidence of AIH is 0.1-1.9 cases out of 100 000 persons per year, which is not very high\[2\]. However, the prevalence of autoimmune hepatitis in Europe is in the range of 11.6-16.9 cases per 100 000 persons\[2\], and in the United States, the proportion of hepatitis among patients with liver cancer is about 11%\[3\]. Incidence is also different between men and women. It was reported that women are more vulnerable to AIH\[2,4,5\].

Unfortunately, we do not have any better choice of medicines other than immunosuppressants, which can be classified into four generations\[6\]. In the 1950s, the first generation immunosuppressants were limited to azathioprine and steroids, which were enriched by polyclonal anti-lymphocyte and anti-thymocyte globulins in the 1960s\[7\]. For this generation, 70%-80% patients might relapse after withdrawal of treatment\[8\]. More seriously, they have many side effects\[9\]. Corticosteroids, Tacrolimus and Cyclosporine are typical of the second generation\[10\]. In the early 1990s a broad range of third-generation immunosuppressants emerged\[6\], most of which are monoclonal anti-lymphocyte and anti-thymocyte globulins followed by the fourth generation, such as the IL-2 monoclonal antibody with its highly specific sites of action\[10,11\]. The second and the third generation immunosuppressants are in most cases successfully used for treatment of AIH\[10,12\]. But long term applications of these immunosuppressive drugs carries serious risks\[13\] and sustained remission\[14\], even at low doses. Non-system steroids may be the best candidates\[15\]. Patients with liver failure or fulminant presentation who fail to improve under immunosuppressive therapy should be considered as candidates for liver transplantation. Without treatment, nearly 50% of patients with severe autoimmune hepatitis die in approximately 5 years\[16\]. Taking this into consideration, it is significantly important to develop new specific drugs. Animal models are the basis of drug discovery and development. Up to the time of writing, there are still no universal animal models of AIH which can be used as pathogenic models as well as therapeutic ones.

As the most important AIH research model, the Con A animal model plays a key role in AIH drug development. In this article we attempt to review the evolution of the Con A animal model of AIH, to sum up the mechanisms of Con A-induced liver injury, and to illustrate its statute in AIH drug development. Furthermore, the future challenges of the animal model are also discussed.

**EVOLUTION OF AIH MODEL**

AIH models have evolved from crude liver homogenates and adjuvants to the genetic engineering level, which can be classified into five phases\[16\]. The first phase was in 1972 when Buschenfelde et al\[17\] induced chronic active hepatitis in rabbits immunized with human liver proteins combined with complete Freund's adjuvant. This work built a solid foundation for AIH models. The second phase began in 1983, when Mihas et al\[18\] established transient hepatitis in mice by immunization with syngeneic liver proteins together with the polysaccharide of Klebsiella pneumoniae. In the third phase, taking place from 1987 to 1990, many scientists used inbred or neonatal thymectomy mice to establish the T-cell reactive AIH model. They induced transient hepatitis by immunizing C57BL/6 mice with the supernatant of liver syngeneic liver homogenates with complete Freund's adjuvant and used adoptive transfer technology to study the roles of T-cell, which allowed studies of the pathogenesis of AIH\[19\]. The fourth phase, from 1992 to 2003, had endotoxin and plant lectin-induced hepatitis models receive extensive attention. Three types of inducers were wildly used during this period: Con A\[20\], D-galactosamine (GalN) with low dosage of lipopolysaccharides (LPS)\[21\], and high dosage of LPS\[22\]. In the fifth phase, from 2002 to 2008, the application of genetic engineering technology accelerated the development of AIH model\[23\]. From one aspect, gene knockout and transgenic animals facilitated the study of the functions of certain genes\[24\]. From the other, production of designated antibodies using genetic engineering methods made it possible for scientists to get specific types of autoantibodies\[25\], and also made it possible for the Con A models to mimic a specific subtype of AIH. Significantly, the production of designated autoantibodies is based on known antigens. Scientists have now clarified the antigens to the following autoantibodies: the antigen to LKM-1 is cytochrome P450 2D6\[26-29\], the antigen to LKM-2 is cytochrome P450 2C9\[30\], the antigens to Liver Microsomal are cytochrome P450 1A2 and cytochrome P450 2A6\[31\]. The animal models of type 2 AIH\[32\] have been reported, but obviously type 1 animal models have more clinical significance than type II\[3\]. As is widely known, it is difficult to find the antigen of autoantibodies, which is the limitation of the gene engineering AIH model. The features and parameters of the three models are listed in Table 1\[32\].

From the information in Table 1, it is obvious that the Con A-induced hepatitis model possesses more advantages than the other two. Firstly, the Con A model includes only one inducer, making it easier to be established compared with the GalN/LPS model. Secondly, there is no significant change of the level of transaminase, which is considered a valid index of the severity of liver injury, in the LPS model, while such change is remarkable in the Con A model. Thirdly, in the Con A model, the serum level of many cytokines relevant to inflammation change dramatically, which is favorable for the study of the pathogenic mechanisms of AIH\[33\]. Furthermore, besides AIH, Con A animal models with different parameters are adaptable to many clinical diseases, such as fulminant hepatitis\[34\], virus hepatitis\[35\], hepatotoxin\[32,33\] and alcoholic liver diseases\[34\]. In summary,
Con A AIH model is easy, convenient, inexpensive and repeatable, as well as a T-cell activated model and could greatly facilitate the study of the mechanisms of AIH-induced liver injury.

**IMMUNOLOGICAL MECHANISMS OF CON A-INDUCED LIVER INJURY**

Con A is one kind of lectin, which is purified from *Canavalia brasiiliensis*. Tiggs et al. injected Con A, Succinyl Con A with no agglutination activity, and *Vicia faba* lectin with strong agglutination activity to nuclear magnetic resonance imaging mice via tail vein, respectively. The results showed that only Con A could induce liver injury, which indicated that the *in vitro* agglutination activity of this lectin does not correlate with its hepatotoxic potential *in vivo*. They also studied the correlation between the hepatotoxic potential of Con A and its sugar-binding site. Con A has specific sugar-binding sites, whose ligands are α-D-mannoside, methyl α-D-mannopyranoside, α-D-glucose, and methyl-α-D-glucose. They co-administered Con A with α-D-mannoside or methyl α-D-mannopyranoside to mice, which prevented the induction of hepatic injury by the lectin. This suggested that free sugar-binding sites are indispensable for the induction of liver injury by lectin. Sato et al. confirmed that Con A/glycogen multilayer films can be decomposed by exposing them to sugar solutions (D-glucose, D-mannose, methyl-alpha-D-glucose and methyl-alpha-D-mannose), as a result of the displacement of sugar residues of glycogen from the binding sites of Con A by the free sugar added in the solution. This suggested that sugar-binding sites are prerequisites of activated Con A. But among Con A, Succinyl Con A and *Vicia faba* lectin, which have the same sugar-binding site, only Con A can lead to high level of transaminase. These two results indicated that the hepatotoxic potential of Con A is not determined by its agglutination activity or sugar-binding site. Other mechanisms may exist.

The mechanisms of the Con A model have interested many scientists. Previews papers describe that the aminotransferase of mice in thymus and CD4 neutralized groups decreased significantly compared with the control group, while the CD8 neutralized group show no significant change. What is more, after injection of Con A, the blood level of interleukin 2 (IL-2), IL-4 and interferon gamma (IFN-γ) all increased dramatically. This suggested that the CD4⁺ T helper (Th) cell was involved in the liver injury. It is reported that CD4⁺-positive Th cells recognize the Con A-modified major histocompatibility complex (MHC) structures of macrophages and become activated, followed by an inflammation reaction and the release of IL-1 and IL-2 to the blood. In the experiment of CD8 neutralization, there was a minor decrease of the transaminase level, which suggested that the target cell lysis by cytotoxic CD8⁺ T lymphocyte (CTL) also contributes to liver injury, but not as the major factor. In conclusion, the main mechanism of the Con A model is that Th cell activation increases the relevant cytokine level, which leads to liver injury. Meanwhile, the CTL-mediated target cell lysis may be the secondary mechanism.

In the liver, lymphocytes, sinusoid endothelial cells (SECs), Kupffer cells (KCs) and stellate cells are all involved in the immune response. Lymphocytes can be classified into two groups, exogenous and endogenous. Exogenous lymphocytes originate from the thymus, bone marrow, intestinal tract, spleen and lymph gland, and enter the liver through circulation. Endogenous lymphocytes are enriched in the portal area of the liver, which count for 25% of non-parenchyma cells in the liver. The endogenous lymphocytes are mainly T cells, while B cells only count for 5% of them. This is why lymphocyte infiltration is mainly focused in the portal area.

For a long time, there have been debates about whether KCs or SECs plays a major role in immunological liver injury. Knolle et al. established the spontaneous and LPS activated cell model, and found that SECs and KCs both secreted IL-1 and IL-6, which suggested that SECs are also key cells in liver injury. It has been found that fifteen minutes after intravenous injection of Con A, Con A binds to SECs first; 4 h later, Con A begins to bind to the KCs. Using Scanning Electron micrograph, it is clearly seen that 4 h after intravenous injection of Con A, blood cell endothelium attaches to the SECS first. Then lymphocytes or neutrophils are trafficked into the hepatocytes, leading to inflammation. We can conclude that SECs and KCs are both important, but they play their roles in the different phases. After injection, Con A binds to the mannose gland in the SECS surface first, leading to the breakdown of the SECS membrane, bleb formation and cytoplasm disappearance. SECS detachment facilitates the binding of Con A to the KCs. CD4⁺ Th cells recognize the MHC class II and T cell receptor of KCs modified by Con A and are then activated. Such liver injury is mainly mediated by T helper cells, including Th1 and Th2 cells.

**Table 1** The features of the autoimmune hepatitis model induced by endotoxins and plant lectins

| Animal          | Inducer | Dosage | Application method | Transaminase level (max) | Con A/LPS/LPS [29] |
|-----------------|---------|--------|--------------------|--------------------------|--------------------|
| BALB/c-mice     | Con A   | 20 mg/kg| Tail vein          | 8 h                      | BALB/c-mice       |
| (6-8 wk)        | GalN/LPS| 5 μg/kg|                    | 8 h                      | (6-8 wk)           |
|                 | LPS     | 10 mg/kg|                    | 8 h                      | (6-8 wk)           |

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Some major cytokines involved in the Con A-induced liver injury are IFN-γ, IL-2, IL-4, IL-6 and tumor necrosis factor α (TNF-α), of which TNF-α and IFN-γ are the major ones.

Figure 2 shows the time when different cytokines reach their peak level in the plasma and liver. In the plasma, TNF-α and IL-10 first reach their peak level after 1 h, followed by IL-4 after 2 h. IFN-γ, IL-2 and IL-6 reach their peak after 3 h, followed by IL-12. However, in the liver, TNF-α, IFN-γ, IL-4 reach their peak level in 1 h, followed by IL-2 and IL-12. There is no significant change for IL-6 and IL-10 in the liver. Especially, the level of IL-10 is very low in the liver compared with that in the plasma, which suggested that IL-10 might originate from other tissues, such as the spleen. But one previous paper reported that IL-10 expression in the liver is higher than that in the spleen. As yet, where IL-10 originates remains unanswered.

Comparing the acute and chronic animal models, the expression profiles of IL-10 are quite different. For example, in the acute model induced by Con A, TNF-α, IFN-γ and IL-12 levels increased to 2.11, 1.92 and 8.30 times of their normal level, respectively, after neutralization of IL-10. Reversely, administration of recombinant IL-10 prior to injection of Con A decreased by 47%, 47% and 80% of TNF-α, IFN-γ and IL-12 expression levels respectively. IL-10 is considered to be an anti-inflammatory cytokine in a murine model of Con A. Kato et al. described that the IL-10 level is increased at 12 h after the Con A injection. After neutralizing antibodies to IL-10, it was intraperitoneally injected into animals of the same model at 6 h before Con A treatment, with serum alanine aminotransferase level being significantly higher than in the control group. Histological studies showed spotty necrosis in the group treated with anti-IL-10 antibodies. These results suggest that IL-10 has an inhibitory effect on liver injury in a murine model of Con A-induced experimental liver injury mediated by cellular immunity. These studies suggested that both endogenous and exogenous IL-10 can protect the liver from acute injury.

However, there is evidence indicating that IL-10 could accelerate liver injury in the chronic model. When Con A was administrated intravenously to BALB/c mice once a week, the IL-10 expression level in plasma increased to 7 times higher 20 wk later. Accordingly, in this model, inflammatory infiltration also lasted for 20 wk and activated stellate cells also dramatically increased. All these results suggested that IL-10 aggravated liver injury in the chronic Con A model.

Paradoxically, IL-10 does not play the same role in all chronic models. For example, in the CCl4 chronic model, IL-10 slows down the process of fibrosis. This may be due to the fact that the mechanisms of liver injury in these two models are different, and the latter does not involve T cell activation. In the acute Con A model, IL-10 may inhibit macrophages and Th1 cells from releasing inflammatory cytokines, which explains why it plays an anti-inflammation role in the acute model. Though IL-10 can inhibit the secretion of anti-inflammatory cytokine, secretion of IFN-γ is also inhibited. Some previous studies reported that, to some extent, IFN-γ may relieve liver fibrosis. Therefore, a long duration of IFN-γ deficiency may aggravate fibrosis. As for the CCl4 model, liver injury is mediated only by free
radicals, which is not relevant to the activation of the immune response and the release of inflammation cytokines. In conclusion, the expression profiles in different models, even with the same inducer, are not the same. The various mechanisms, cell types and micro-environments should be taken into consideration in experimental design and execution.

CON A MODEL AND NEW DRUG DEVELOPMENT

In recent years, based on the Con A animal model, many new therapeutic antibodies or proteins have been developed to attenuate liver injury in experimental models (Table 2) [63-66].

Fan et al. [63] humanized a murine monoclonal antibody 23C3 against human osteopontin by a complementary-determining region grafting method based on computer-assisted molecular modeling, denoted as Hu23C3. They demonstrated that Hu23C3 could have the potential for attenuating Con A-induced liver injury through the nuclear factor kappa B (NF-κB) pathway.

Nakano et al. [64] intraperitoneally injected a polyclonal antibody against histone H1 immediately after Con A injection; they found that injection of anti-histone H1 antibodies could reduce Con A-induced liver damage, also via the NF-κB pathway.

It is reported that Con A-induced hepatitis was attenuated by the administration of apolipoprotein A-II, which is the second major apolipoprotein of high-density lipoprotein [63]; this inhibited leukocyte infiltration and the expression of T-cell-related cytokines and chemokines.

The survival rate of mice was markedly enhanced by the administration of CpG-containing oligodeoxynucleotides (CpG ODN) [66]. This is because CpG ODN pretreatment inhibits the DNA binding ability of NF-κB, leading to the decrease of systemic/liver levels of TNF-α and IFN-γ. These results suggest that CpG ODN pretreatment protects the mice from Con A-induced liver injury, also via NF-κB pathway.

CONCLUSION

In this article we reviewed the evolution of the AIH model and emphasized the importance of the Con A AIH model. Based on the previous papers, we summarized the mechanisms of Con A-induced liver injury, its pathogenic changes and cytokines expression levels. The Con A animal model, which is a typical T cell dependent model, can mimic the mechanisms of clinical AIH diseases. Therefore, we think that it is a good and convenient model for studying the mechanisms of AIH and developing new therapeutic drugs.

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