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IL-6 induces hepatic inflammation and collagen synthesis *in vivo*

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SUMMARY

IL-6 regulates the synthesis of a broad spectrum of acute phase proteins in the liver. Also, it is involved in the pathogenesis of many fibrogenic diseases. To study the inflammatory effects of IL-6 on the liver *in vivo*, human rIL-6, produced in *Escherichia coli*, was injected intraperitoneally into rats (25 μg/100 g body weight). The major fraction of injected IL-6 was accumulated in the liver within 40 min, and the number of platelets was increased during 72 h after injection. After 5 weeks of injection, the levels of serum glutamine pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) were not changed, but they were significantly elevated at 13 weeks of treatment. Meanwhile, serum albumin levels were slightly decreased compared with those of controls. The same phenomena were observed in carbon tetrachloride-treated rats. Collagen synthesis was increased in the liver tissues and in the culture supernatants of hepatic lipocytes isolated from the rats treated with IL-6 for 13 weeks. Histological analysis correlated well with biochemical analysis. At 5 weeks of treatment, only mild pathological changes were observed, but severe hepatocyte necrosis and the accumulation of fibres in necrotic area were developed in the liver of IL-6-treated rats after 13 weeks of treatment, confirming that hepatic inflammation and fibrosis were developed. IL-6 activities in the sera and in the culture supernatants of lipocytes from IL-6-treated rats were elevated compared with those in controls. These biochemical and pathological data indicate that IL-6 can induce hepatic inflammation, and it has important roles in the pathogenesis of fibrosis and diseases of the liver *in vivo*. In addition, these results will provide useful information for the clinical trials of IL-6.

Keywords IL-6 collagen hepatic inflammation lipocytes

INTRODUCTION

Many cytokines are involved in maintaining biochemical and physiological homeostasis in the liver. They affect protein synthesis as well as lipid and carbohydrate metabolism in the liver [1]. Epidermal growth factor (EGF) [2–4], transforming growth factor-alpha (TGF-α) [5], fibroblast growth factor (FGF) [6,7], and hepatocyte growth factor (HGF) [8,9] are known to participate in liver development and regeneration after liver injury. In contrast, TGF-β inhibits DNA synthesis in regenerating liver and acts as a potent negative regulator of proliferating normal and neoplastic hepatocytes [10,11].

IL-6 is a pleiotropic cytokine produced by various types of cells, and acts on many kinds of target cells in immune, haematopoietic and inflammatory systems. It is involved in B cell differentiation, T cell activation, chronic inflammation, autoimmune diseases, and malignancies [12–14]. In the liver, IL-6 induces hepatic acute phase protein synthesis *in vivo* [15] like IL-1, tumour necrosis factor-alpha (TNF-α), and leukaemia inhibitory factor (LIF), but it is able to induce the broadest range of acute phase proteins. High levels of IL-6 were detected in the sera of the patients with alcoholic liver cirrhosis [16], hepatitis B virus infection [17,18], and acute hepatitis [19]. IL-6 also increased the numbers of platelets [20] and neutrophils [21], and hepatocyte mitosis in synergy with IL-1 [22]. Furthermore, *in vitro* treatment of hepatocytes with IL-6 induces the production of phospholipase A2, one of the major inflammatory mediators [23].

When ^{125}I-IL-6 was injected into rats intravenously, it was accumulated in the liver within 20 min [24]. It was then released from the liver and finally degraded in skin, although the liver acts as the main scavenger for cytokines such as IL-1, IL-3, TNF-α, and TGF-β [1]. The cDNA for hepatic IL-6 receptor has been cloned [25], and the expression of its receptor was up-regulated by IL-6, IL-1, and dexamethasone treatments [26,27]. Recently, nuclear factors, NF-IL-6 [28] and NF-IL-6β [29], were identified to bind to the promoter regions of IL-6 and several acute phase protein genes. They have homologous sequences with C/EBP which is expressed in the liver specifically. NF-IL-6 and NF-IL-6β genes are induced by lipopolysaccharide and inflammatory cytokines such as IL-1 and IL-6. According to these results, one can consider that IL-6 is one of the important

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factors regulating immune and inflammatory responses in the liver.

Hepatic fibrosis is a common hepatic inflammation characterized by the deposition of extracellular matrix and hepatocellular necrosis. In this study, human recombinant IL-6 (hrIL-6) was administered to rats and the effects of *in vivo* IL-6 treatment on the liver were compared with CCl_4-induced hepatic fibrosis.

MATERIALS AND METHODS

Materials

Carrier free $Na^{125}I$ was purchased from Amersham International (Braunschweig, Germany). Stractan (arabinogalactan) was purchased from Sigma Chemical Co. (St Louis, MO). Anti-desmin antibody and anti-collagen type I antibody were purchased from Southern Biotechnology (Birmingham, AL), and anti-phosphotyrosine MoAb were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

IL-6

Human recombinant IL-6 was expressed in *Escherichia coli* and purified (specific activity 10^8 U/mg) [30]. Specificity was confirmed by B9 bioassay and immunoblotting using anti-IL-6 MoAb (Dr T. Kishimoto, Osaka University, Osaka, Japan). There was no detectable endotoxin contamination when tested using an E-TOXATE endotoxin-detection kit (Sigma). *In vivo* toxicity was tested by comparing body weights of rats treated with IL-6 or PBS intraperitoneally. There were no differences in body weights between two groups.

Treatments of rats

Four male Sprague-Dawley rats weighing about 200 g were treated with 25 μg IL-6 or 0·05 ml CCl_4/100 g body weight intraperitoneally twice a week. As controls, the same volume of PBS was injected intraperitoneally. For the study of the fate of IL-6 in the rats, 1×10^6 ct/min of ^{125}I-IL-6 (2×10^7 ct/min per μg) labelled using Iodobeads [31] was injected intraperitoneally. At indicated times, blood, livers, and other organs were removed for the appropriate assays. Data represent two different sets of experiments.

Histological and blood analysis

Immediately after removal, small pieces of the liver were fixed with 10% formalin in 0·1 M sodium phosphate buffer pH 7·4. Fixed liver samples and blood samples were analysed at the

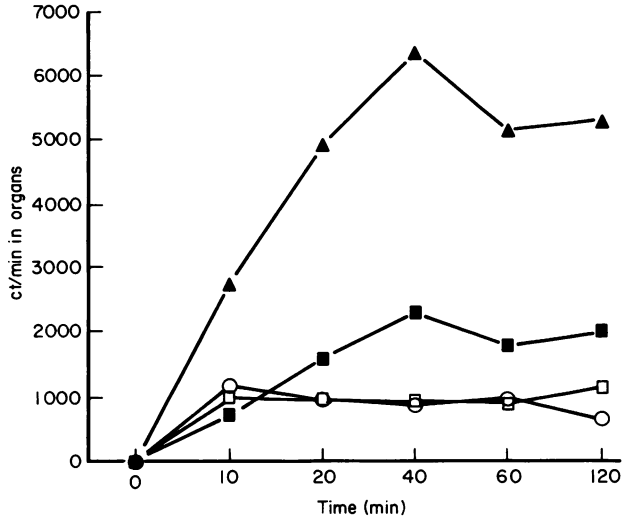

Fig. 1. Accumulation of ^{125}I-IL-6 into the liver *in vivo*. ^{125}I-IL-6 (1×10^6 ct/min per rat) was injected intraperitoneally. At indicated times, each organ was removed. Incorporated radioactivity was counted using a γ-counter. Data represent the averages of two different experiments. ■, Spleen; ○, lung; ▲, liver; □, blood.

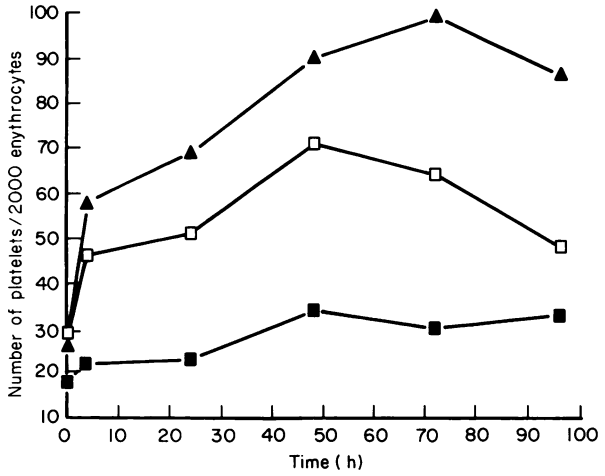

Fig. 2. Changes of platelet number by IL-6 treatments *in vivo*. Human recombinant IL-6 (25 μg/100 g body weight) was injected into rats intraperitoneally. At indicated intervals, blood was collected and platelets were counted. Data represent the averages of three different counts. ■, Control; □, CCl_4; ▲, IL-6.

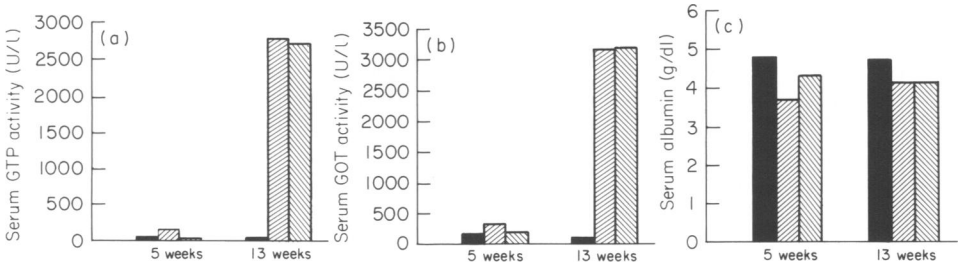

Fig. 3. *In vivo* regulation of serum glutamic pyruvic transaminase (GPT), (a), glutamic oxaloacetic transaminase (GOT), (b), and albumin (c) by IL-6. IL-6 was injected into rats twice per week for various times. Sera were collected and analysed for GPT, GOT, and albumin as described in Materials and Methods. ■, Control; ■, CCl_4; ■, IL-6.

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Collagen analysis

Quantification of collagen content in liver tissue was assessed by measuring hydroxyproline amount [32]. Released collagen from cultured lipocytes isolated by a Stractan density gradient centrifugation [33] was measured by ELISA using anti-collagen type I antibody.

RESULTS

IL-6-induced hepatic inflammation

To assess the effects of IL-6 on the liver *in vivo*, recombinant human IL-6 which had no apparent toxic effects (see Materials and Methods) was injected into rats intraperitoneally. IL-6 accumulated in the liver up to 40 min after injection, when the fate of IL-6 was monitored by using ^{125}I-IL-6 (Fig. 1). Comparing i.v. injection of IL-6 in previous study [24], IL-6 accumulated slowly into the liver (10 min *versus* 40 min) and disappeared

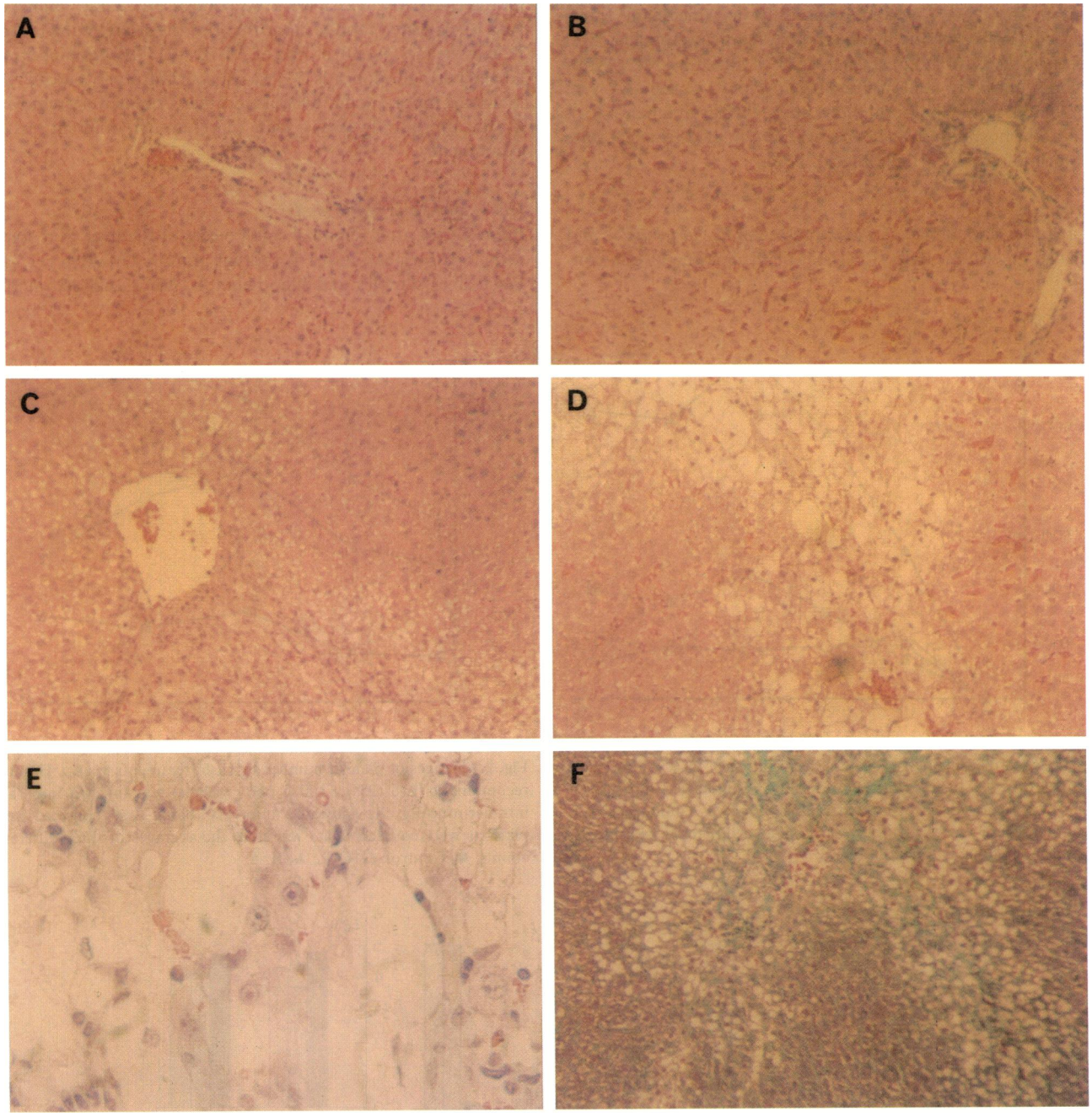

Fig. 4. Histological analysis of liver tissues from rats treated with IL-6. Rat livers were fixed with 10% formalin in 0·1 M phosphate pH 7·4 and embedded in paraffin. Cut sections were stained with haematoxylin–eosin staining (a–e) or Masson's trichrome staining (f). (a) Control (5 weeks, × 100). (b) Control (13 weeks, × 100). (c) CCl_4 (5 weeks, × 100). (d) CCl_4 (13 weeks, × 100). (e) IL-6 (13 weeks, × 400). (f) IL-6 (13 weeks, × 100).

slowly. It could explain why i.p. injection of the same amount of IL-6 induced acute phase protein expression to a greater extent [24]. Up to 70 h after IL-6 injection, the number of platelets was increased (Fig. 2). After 13 weeks of initial injection, the levels of serum glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) were elevated in IL-6-treated rats compared with those of PBS-treated rats. CCl_4, which can induce hepatic inflammation and fibrosis, also showed the same effects (Fig. 3). The level of serum albumin, however, was slightly decreased in IL-6- or CCl_4-treated rats. These blood analyses indicated that hepatic inflammation was induced by IL-6 treatment, as in the case of CCl_4 treatment.

IL-6-induced hepatic fibrosis

Histological analysis of the liver treated with IL-6 or CCl_4 showed centrilobular necrosis of the liver with infiltrated inflammatory cells in both cases. Accumulation of fibre in necrotic area was also observed, as demonstrated by Masson's trichrome staining (Fig. 4). Mild hepatic fibrosis was observed at 5 weeks after IL-6 or CCl_4 treatments. At 10–13 weeks of treatments, a gradual increase of hepatic necrosis and the accumulation of extracellular matrix were observed. Total collagen synthesis of liver tissue was assessed by measuring hydroxyproline content. As shown in histological analysis of the liver, collagen synthesis was elevated in the liver from IL-6-treated rats (Fig. 5a). There are several different types of liver cells producing extracellular matrix proteins, such as lipocytes, fibroblasts, hepatocytes, and endothelial cells. Among them, lipocytes are known to be important effectors of hepatic fibrosis and major producers of collagen. In the case of CCl_4-induced fibrogenesis in rats, mRNAs for collagen type I and III in lipocytes were increased significantly [34,35]. Lipocytes were purified by a Stractan density gradient centrifugation according to the method of Friedman & Roll [33]. Lipocytes were efficiently separated from other non-parenchymal cells at the top of the gradient. Purity of lipocytes (>95%) was checked by staining with anti-desmin antibody as described [33]. Collagen synthesis by lipocytes from the rats treated with IL-6 or CCl_4 was much higher than that of controls (Fig. 5b). Collagen synthesis by lipocytes remained relatively constant for at least 12 days as it was observed previously [33]. These data indicate that

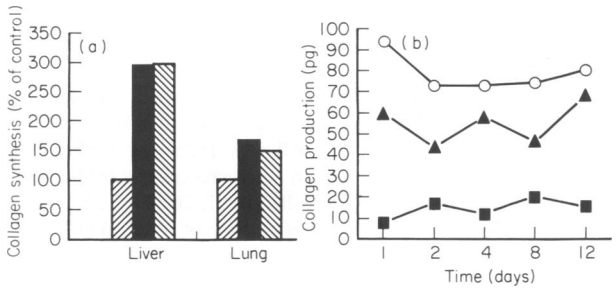

Fig. 5. IL-6-induced collagen synthesis by the liver tissues and lipocytes. (a) Liver or lung tissues (200 mg) were solubilized in HCl and collagen contents were quantified by the hydroxy proline method [32]. ■, Control; ■, CCl_4; ▧, IL-6. (b) Liver lipocytes were isolated by a Stractan density gradient centrifugation [33] and cultured in complete media for various periods. Released collagen was measured by ELISA using anti-collagen type I antibody. ■, Control; ○, CCl_4; ▲, IL-6.

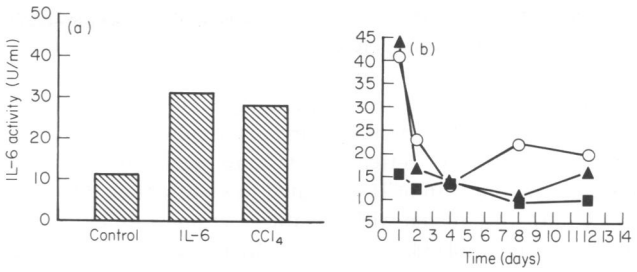

Fig. 6. IL-6 activities in the sera and in the culture supernatants of lipocytes. (a) Sera were collected from rats treated with IL-6 or CCl_4 for 13 weeks. IL-6 activities in the sera were assayed using B9 bioassay. (b) Isolated liver lipocytes from rats treated with IL-6 or CCl_4 for 13 weeks were incubated in complete media for various times. IL-6 activities in the culture supernatants were assayed using B9 bioassay. ■, Control; ○, CCl_4; ▲, IL-6.

IL-6 affects liver cells, especially lipocytes, and increases collagen synthesis, leading to hepatic fibrosis.

IL-6 levels in the sera and its production by lipocytes

IL-6 is produced by a variety of types of cells and is involved in inflammation. In alcoholic liver cirrhosis, high levels of IL-6 in the sera and increased production by peripheral blood mononuclear cells were detected [16]. IL-6 levels were elevated in the sera of the rats treated with CCl_4 or IL-6 (Fig. 6a). Also, IL-6 production by lipocytes from the same rats was increased (Fig. 6b).

DISCUSSION

Hepatic fibrosis is one of the chronic inflammatory liver diseases. It is a complex process that involves the deposition of extracellular matrix components, activation of cells capable of producing inflammatory mediators, cytokines, and tissue remodelling. Unbalanced production of cytokines and inflammatory mediators has been reported in chronic liver diseases. In cirrhotic liver, levels of IL-1, TNF, and IL-6 are increased. TGF-β is also involved in collagen synthesis and chronic liver diseases [36,37]. In CCl_4-induced rat liver fibrosis, the expression of TGF-β and procollagen type I, III, and IV was increased in the lipocytes which are major sources of overproduction of matrix synthesis during hepatic fibrogenesis [34,35].

To examine the *in vivo* effects of IL-6 on hepatic fibrosis, IL-6 was injected into rats for 13 weeks. Injected IL-6 accumulated in the liver during 40 min after injection, indicating that the primary target of injected IL-6 in the rats is the liver, as reported previously [24]. As an early response, IL-6 injection increased the number of platelets during 72 h. Serum GPT and GOT levels were not elevated at 5 weeks of IL-6 or CCl_4 treatment, but they were increased significantly after 13 weeks of treatment. Collagen synthesis of the liver analysed by measuring hydroxyproline content was increased by injecting IL-6 for 13 weeks, but collagen content of the lung from the same rats was not elevated significantly. Isolated lipocytes from IL-6-treated rats secreted about five times more collagen than those of controls. During the fibrosis process, lipocytes appeared to play the principal role in the synthesis of collagen. Further detailed study of the expression of IL-6 receptor or gp130 in the lipocytes will be interesting.

Histological observation further supported these biochemical analyses. At 5 weeks of treatment, only mild pathological

changes were noticeable, but severe hepatocyte necrosis in the centrilobular area was detected after 13 weeks of treatment. Also, the accumulation of fibres was developed after 13 weeks of treatment, as observed in collagen analysis of liver tissue.

The levels of IL-6 in the sera and in the culture supernatants of lipocytes from IL-6-treated rats were higher than those of controls. Serum IL-6 of IL-6-treated rats was about 300 pg/ml (30 U/ml). In patients with hepatitis, serum IL-6 levels were about 30–70 pg/ml [38,39], but much higher levels (40–1700 pg/ml) were reported in liver cirrhosis [16]. Based on the observations of our and other studies, it can be postulated that high levels of serum IL-6 are not only a possible marker for liver disease, but also one of the major causes of hepatic fibrosis. In CCl_4-treated rats, serum IL-6 levels were also elevated. One simple explanation can be that CCl_4 treatment may induce IL-6 production from IL-6 producer cells such as Kupffer cells, fibroblasts, and endothelial cells, IL-6 then acting on the target cells as in the case of IL-6 administration. The roles of other cytokines and soluble factors can not be ignored, however. As expected, the concentrations of anti-IL-6 antibody were also increased about two-fold in the sera of IL-6- or CCl_4-treated rats compared with those in controls (data not shown).

According to Shalaby *et al.* [40], injection of 30 μg/100 g body weight into mice had no lethal effects even after having sensitized them with actinomycin-D treatment, but the same amounts of IL-1β or TNF-α injection caused the death of the animals, indicating IL-6 is less toxic than IL-1β and TNF-α. In this study, injection of 25 μg/100 g body weight IL-6 into the rats did not cause the reduction of body weight, but it had severe influences on liver metabolism. These results suggest that the potential side effects of IL-6 on the liver should be considered when it is used clinically, especially at high doses.

In conclusion, *in vivo* administration of IL-6 into rats induced hepatic inflammation and fibrosis. IL-6 also induced collagen synthesis in the liver and lipocytes in the rats. To understand the role of IL-6 in liver diseases, more dissected studies on the individual event induced by IL-6 will be required.

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