Interleukin-6 is necessary, but not sufficient, for induction of the human C-reactive protein gene *in vivo*

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We have investigated the involvement of interleukin-6 (IL-6) in the induction of the gene encoding the acute-phase protein human C-reactive protein (hCRP). In transgenic mice the hCRP gene can be induced by lipopolysaccharide (LPS), but not by IL-6. In contrast, hCRP was inducible by IL-6 in primary human hepatocytes and in primary hepatocytes isolated from transgenic mice. To further evaluate the role of IL-6, we introduced the hCRP transgene into animals lacking endogenous IL-6 (IL-6-negative mice). Here, hCRP was not inducible by LPS, but was induced by a combination of LPS and IL-6. These results clearly demonstrate that IL-6 is necessary, but not sufficient, for the induction of hCRP expression. These animal models will allow further dissection of the cytokine network responsible for the regulation of the major human acute-phase reactant CRP.

INTRODUCTION

The acute-phase response (APR) is characterized by rapid and dramatic alterations in the expression patterns of proteins that are mostly produced by the liver during tissue damage, inflammation and infections [1–3]. The proteins whose serum concentrations are increased during the APR are referred to as positive acute-phase proteins (APPs) [4]. The major human APP is human C-reactive protein (hCRP), which is present in only trace amounts in healthy individuals (below 1 mg/l), but rises to concentrations of more than 300 mg/l upon inflammatory stimulation [5,6]. Therefore hCRP is a useful marker for monitoring the course of diseases such as cancer, bacterial infections, heart attack, rheumatic disease and chronic inflammation [7,8].

Macrophages are a source of cytokines that are implicated in APR regulation [9,10]. Of these cytokines, interleukin-6 (IL-6) is largely responsible for the induction of hCRP in hepatoma cells, although other cytokines, such as IL-1 and tumour necrosis factor-α, may also participate in this process [11–15]. Furthermore IL-6 has been identified as a potent regulator of a broad panel of acute-phase genes in hepatocytes of mice and other mammals [16–23]. Other than its effect on APPs, IL-6 has a wide range of biological activities, including regulation of B-cell differentiation, growth stimulation of hybridoma and plasmacytoma cells, activation of T cells and co-stimulation of thymocyte proliferation [24–27]. Despite these physiological functions, side-effects of IL-6 administration in mice have not been described. Even a broad overexpression of murine IL-6 (mIL-6) in several organs of transgenic mice did not affect the vitality and fertility of the animals [28]. However, specific expression of human IL-6 (hIL-6) in the B cells of transgenic mice resulted in the induction of plasmacytomas [29].

In contrast with the situation in humans, CRP can be induced only slightly in mice, and is not considered to be an APP in this species [30–32]. Nevertheless, the regulation of hCRP expression in transgenic mice is similar to its regulation in humans. A low basal level was greatly increased after induction of an acute phase. Expression of the transgene was strictly liver-specific, and was regulated at the transcriptional level [33,34]. *Cis* regulatory regions conferring *in vivo* inducibility were identified by deletion analyses of a 31 kb genomic clone in transgenic mice [35]. These mice were used successfully to express the simian virus 40 T-antigen in an inducible and liver-specific manner [36]. Lipopolysaccharide (LPS) has been used in these studies as the inducing agent. Although this substance is a very potent inducer of an APR, it has severe side-effects on the metabolism and immune system of the animals. In addition, long-term treatment causes LPS tolerance, requiring LPS dose escalation to maintain comparable levels of induction [37].

We have investigated the effects of IL-6 on the regulation of hCRP expression in transgenic animals. Surprisingly, we found that IL-6 did not induce hCRP in these animals, although it induced the gene encoding the endogenous mouse APP serum amyloid A protein (SAA). In order to evaluate the role of IL-6 in the regulation of hCRP expression, we investigated the inducibility of this APP in mice lacking endogenous IL-6. We showed that, in these animals, hCRP expression was abolished upon LPS injection.

MATERIALS AND METHODS

Animals and treatment

hCRP transgenic and IL-6$^{-/-}$ mice have been described previously [3,35,38]. Female transgenic animals aged 8–10 weeks were used in experiments. They were maintainend in standard conditions with a 12 h/12 h dark/light cycle.

LPS was purchased from Sigma (cat. no. L3254), resuspended in sterile PBS and injected intraperitoneally at a dose of 100 μg/mouse in a total volume of 100 μl. Recombinant mIL-6 and hIL-6 were purchased from IC Chemikalien (Ismanig, Germany), diluted in sterile PBS and injected intraperitoneally at a dose of 2.5×10^4 units/mouse. Blood was collected from the

Abbreviations used: APR, acute-phase response; APP, acute-phase protein; (h)CRP, (human) C-reactive protein; IL-6 (etc.), interleukin-6 (etc.); mIL-6, murine IL-6; hIL-6, human IL-6; LPS, lipopolysaccharide; SAA, serum amyloid A protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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retro-orbital cavity before, 9 h after and/or 24 h after treatment. The injection of 100 μl of PBS and the retro-orbital puncture by itself did not affect the hCRP serum level.

Cell culture

Human hepatocytes were isolated after liver biopsy or tumour surgery from healthy liver tissue by collagenase treatment (collagenase IA from Sigma; cat. no. SI C9891) and prepared as described elsewhere [39]. The cells were seeded on a collagen-gel-covered cell culture dish in Williams E medium (Gibco BRL) supplemented with 2 mM glutamate, 100 units/ml each of penicillin and streptomycin, 197 units of insulin, 17.27 μg/ml glucagon, 10 nM dexamethasone (Sigma) and 10 % (v/v) fetal calf serum (Boehringer), referred to as hepatocyte medium, and were incubated for 2–4 h at 37 °C and 5 % CO_2. The cells were then washed, covered with a collagen gel, re-fed and incubated again as described. Medium was exchanged daily. At 3 days after isolation, the hepatocytes were incubated for 9 h with recombinant hIL-6 as indicated and subsequently harvested for RNA preparation.

Mouse hepatocytes were collected from 18.5-day embryos derived from matings between wild-type female and transgenic male mice. The embryonic livers were excised and treated with 0.5 mg/ml collagenase H solution (Boehringer) for 10 min at 37 °C. The cells were washed three times, resuspended in hepatocyte medium and treated as described above for human hepatocytes, except that mIL-6 was applied.

RNA analysis

Mice were killed by cervical dislocation, and RNA was isolated from livers by the guanidinium isothiocyanate method [40] and subjected to Northern blot analysis as described previously [35]. ^{32}P-labelled cDNA fragments for hCRP, mouse SAA-2, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rat GAPDH were used as probes.

Protein analysis

Sera were prepared from blood and stored at −20 °C until use. Titertek ELISA plates were coated with rabbit anti-hCRP (DAKO; A073) at 4 °C overnight, washed with PBS/0.05 % Tween and incubated with 0.2 % (w/v) gelatin solution at 37 °C for 1 h to block unspecific binding sites. An hCRP standard (DAKO; X923) and diluted samples were incubated for 1 h at room temperature, washed and incubated for 1 h with a peroxidase-conjugated rabbit anti-hCRP antibody at room temperature. For substrate reaction, *o*-phenylenediamine dihydrochloride (Sigma; P8287) was used according to the supplier's instructions. Absorbance was measured at 492 nm.

RESULTS

IL-6 and hCRP expression in transgenic mice

We have investigated the expression pattern of the hCRP gene in transgenic mice carrying a 31 kb genomic CRP fragment. This fragment contains the entire hCRP gene, 17 kb of 5′ and more than 11 kb of 3′ flanking regions [33]. To ensure that these animals were not in an acute-phase state before experimental induction, blood was taken by retro-orbital puncture and the serum concentration of hCRP was determined by ELISA. This test is specific for hCRP. The serum levels in uninduced animals did not exceed 0.1 μg/ml. After intraperitoneal injection of 100 μg of LPS, the serum level of hCRP increased 400-fold to 40 μg/ml, which is in agreement with previous results [33].

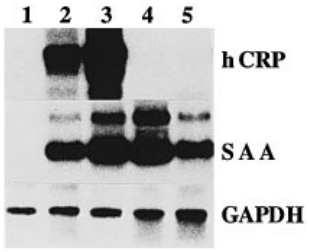

Figure 1 hCRP RNA expression pattern in transgenic mice

A Northern blot analysis of liver RNA from transgenic mice is presented. The livers were isolated after injection of PBS as a negative control (lane 1), after injection of 100 μg of LPS as a positive control (lanes 2 and 3) or after injection of 2.5×10^4 units of IL-6 (lanes 4 and 5). The filters were hybridized sequentially with probes for hCRP, mouse SAA-2 (SAA) and GAPDH.

One major regulator of the hepatic APR is IL-6 [16]. We therefore analysed hCRP expression in transgenic mice 24 h after the intraperitoneal injection of 2.5×10^4 units of mIL-6. Surprisingly, the administration of this cytokine did not lead to an increase in hCRP serum levels. To exclude the possibility of a more rapid induction of hCRP protein by IL-6 compared with LPS, we also analysed sera at earlier time points. However, we were unable to detect any hCRP protein at any of the time points (results not shown).

Ciliberto et al. [33] showed that hCRP is regulated in transgenic mice at a transcriptional level. To ensure that the protein data reflected the situation at the RNA level, we prepared RNA from the livers of transgenic animals after injection of either 100 μg of LPS or 2.5×10^4 units of mIL-6. The RNA was subjected to Northern blot analysis and hybridized with probes specific for hCRP and for the endogenous APP SAA (Figure 1). No signals for hCRP or SAA were detectable in RNA isolated from uninduced animals. Upon treatment with mIL-6, the endogenous SAA gene was induced to nearly the same extent as after LPS injection. This demonstrated that the administered IL-6 was biologically active. Despite this, mIL-6 did not induce hCRP mRNA expression (Figure 1). Similar results were obtained using hIL-6 (not shown). To exclude integration-site-specific regulation of the hCRP gene, we analysed several other transgenic hCRP lines carrying different constructs integrated at different sites in the mouse genome. Although these hCRP transgenes were inducible with LPS, in no case did we observe induction by IL-6 (results not shown). Thus the hCRP gene cannot be induced by IL-6 in transgenic mice.

Expression profile of hCRP in primary human hepatocytes after IL-6 treatment

The finding that hCRP cannot be induced by IL-6 in transgenic mice is in clear contrast to results with hepatocyte-derived cell cultures. In the human hepatoma cell line PLC/PTF/5 and in primary human hepatocytes, the hCRP gene can be induced by IL-6 [15,18,41]. Although both cell types are of hepatic origin, hepatoma cells show considerable differences in growth behaviour and expression of acute-phase genes compared with hepatocytes [42]. Additionally, primary hepatocytes very rapidly

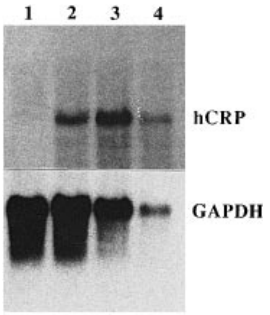

Figure 2 hCRP RNA expression in primary human hepatocytes

A Northern blot analysis of human primary hepatocytes is shown. The cells were kept in a collagen sandwich culture system, and RNA was isolated after a 9 h incubation with 0, 250, 500 or 1000 units/ml hIL-6 (lanes 1–4 respectively). The filter was hybridized sequentially with probes for hCRP and human GAPDH.

lose their natural bipolarity and specific functional characteristics when cultured under conventional conditions. A great improvement in the culture of primary hepatocytes is the sandwich culture system, in which the cells are placed between a double layer of collagen gels. In this system, primary hepatocytes maintain their bipolarity, and the lifespan of these cells in culture can be greatly prolonged [39], thus reflecting true physiological conditions more closely than is possible in conventional systems. We employed this system to analyse the expression of hCRP in primary human hepatocytes after treatment with hIL-6. Cells were cultured for at least 3 days to down-regulate the expression of acute-phase genes that may have been induced during isolation of the hepatocytes from the liver. After this time, cells were incubated for 9 h in the presence or absence of various concentrations of hIL-6. The abundance of hCRP transcripts was examined by Northern blot analysis. As shown in Figure 2, hCRP expression was undetectable without induction, but was highly induced upon hIL-6 treatment. Thus the hCRP gene is inducible by IL-6 in primary human hepatocytes.

Expression profile of hCRP in primary mouse hepatocytes from hCRP transgenic mice on incubation with IL-6

The difference in hCRP expression upon IL-6 treatment in transgenic mice and primary human hepatocytes could be caused by a species-specific response to IL-6, or by differences between *in vivo* and *in vitro* systems. To evaluate this latter possibility, we investigated the expression of hCRP in primary hepatocytes

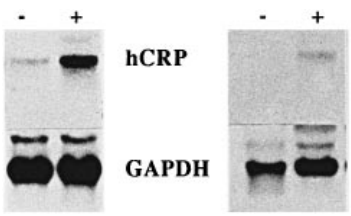

Figure 3 hCRP RNA expression in primary hepatocytes from transgenic mice

The cells were cultured on a collagen monolayer and RNA from untreated cells (—) and from cells after a 9 h incubation with 500 units/ml mIL-6 (+) was analysed on Northern blots. The expression pattern of hCRP in two independent experiments is presented. For a loading control, the filters were rehybridized with a GAPDH probe.

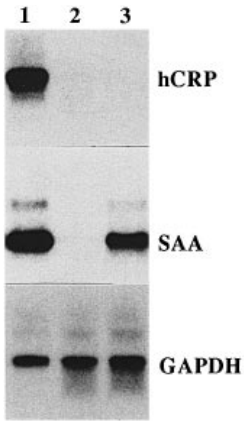

Figure 4 hCRP RNA expression in transgenic wild-type mice and in mice lacking IL-6

Liver RNA was isolated from hCRP transgenic IL-$6^{+/+}$ mice (after injection of 100 μg of LPS; lane 1) or from transgenic mice with a disrupted IL-6 gene without induction (lane 2) and after LPS injection (lane 3), and analysed on Northern blots. The filters were hybridized with probes for hCRP, mouse SAA and GAPDH.

from transgenic animals. Since mouse hepatocytes can be easily isolated from embryos shortly before birth and kept in culture for several days, we used primary hepatocytes of 18.5-day embryos. These cells were cultured for 4 days and then incubated in the presence or absence of 500 units/ml mIL-6 for 9 h.

In two independent experiments, hCRP gene expression was investigated at the RNA level. In the first experiment, a low level of hCRP expression was present prior to induction. However, in both analyses, the hCRP gene could clearly be induced by IL-6, although in one experiment the level of expression of hCRP after mIL-6 treatment was very low (Figure 3). To rule out the existence of a short window of inducibility during embryo development, we tested the expression of SAA and hCRP in mouse embryos at day 17.5, 9 h after the injection of mIL-6 either into the mother or directly into embryos within the uterus. However, we did not detect transcripts of the acute-phase genes in any of the embryos by Northern blot analysis, although the mother expressed SAA strongly (results not shown).

These results indicate that differences in the induction of hCRP by IL-6 are more likely to be a consequence of cell culture conditions than a result of species-specific differences in its regulation.

Induction of hCRP by LPS is abolished in transgenic mice lacking endogenous IL-6

As IL-6 could not induce hCRP in transgenic mice, we addressed the question of whether this cytokine plays any role in the induction of this human major acute-phase gene in this organism. For this, we analysed hCRP transgenic mice containing an inactivated IL-6 gene derived by crossing hCRP transgenic and IL-$6^{-/-}$ mice. Transgenic mice lacking endogenous IL-6 were analysed for the inducibility of hCRP and SAA by LPS. In contrast with mice with an intact IL-6 gene, the IL-$6^{-/-}$ mice did not express hCRP after LPS treatment, although the endogenous SAA gene was strongly induced (Figure 4).

Additionally, the serum concentration of hCRP was measured in IL-$6^{-/-}$ mice both before and 9 h and 24 h after LPS injection. Again, in contrast with transgenic wild-type mice, hCRP levels

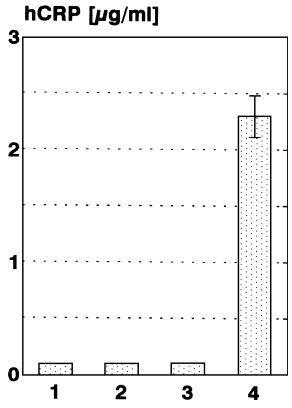

Figure 5 Serum levels of hCRP in transgenic mice lacking IL-6

Sera from transgenic mice with a disrupted IL-6 gene were analysed before (1) and 24 h after intraperitoneal injection of 100 μg of LPS (2), 2.5×10^4 units of mIL-6 (3) or 100 μg of LPS plus 2.5×10^4 units of mIL-6 (4). Results are means $\pm$ S.D. of results from seven (2) or three (3 and 4) animals.

did not increase in the sera of LPS-treated IL-6$^{-/-}$ mice, confirming the RNA results (Figure 5).

To address the question of whether the hCRP gene is inducible at all in IL-6$^{-/-}$ mice, we injected 100 μg of LPS together with 2.5×10^4 units of mIL-6. As expected, we observed an increase in the serum level of hCRP of about 20-fold (Figure 5). Thus IL-6 is necessary, but not sufficient, to induce the hCRP gene *in vivo*.

DISCUSSION

In this study we have investigated the role of IL-6 in the regulation of the hCRP gene in transgenic mice and primary hepatocytes. We have shown that IL-6, although considered to be an important regulator of the APR, failed to induce the hCRP gene in transgenic mice. However, in primary human hepatocytes and in primary mouse hepatocytes isolated from transgenic animals, the hCRP gene was clearly induced by IL-6.

To define a role for IL-6 in the regulation of hCRP expression in transgenic mice, we investigated the induction of this gene in IL-6$^{-/-}$ mice. In these animals the hCRP response to LPS was completely abolished, but could be reconstituted by co-injection of IL-6. These studies reveal that IL-6 is necessary, but not sufficient, for the regulation of hCRP expression.

Regulation of hCRP expression in hepatocyte cultures and transgenic mice

The finding that IL-6 did not induce the hCRP gene in transgenic mice was surprising, as this cytokine has been shown to regulate hCRP expression in hepatic cell culture [16,18,43]. The fact that the hCRP gene could also be induced by IL-6 in primary mouse hepatocytes from transgenic mice indicates that functional IL-6 responsive elements are present within the hCRP construct, and that the regulation of this gene by IL-6 is not influenced by species-specific effects. The mouse CRP gene, although not considered to be a major acute-phase gene because of its weak inducibility, can be induced by IL-1, but not by IL-6, in primary mouse hepatocytes [44]. Therefore the expression of hCRP in mouse hepatocytes from transgenic mice is not governed by a mechanism that regulates the mouse CRP gene.

In principle, two explanations could be proposed for differences between cell culture and transgenic animals. First, the medium used for the cell cultures may contain factors that synergize with IL-6 in the induction of hCRP, although these factors are unable to induce the gene on their own. These co-activators might be components of the fetal calf serum. Serum contains an undefined mixture of factors that might not be present or available at comparable concentrations and composition in an adult animal. The culture of hepatocytes in chemically defined medium without fetal calf serum should allow the identification of any such factors that are involved in the regulation of hCRP expression.

One supplement that we used in our hepatocyte cultures was dexamethasone. This hormone can synergize with IL-6 in the induction of an APR in two different ways: it can increase the number of IL-6 receptors at the cell surface, or it can exert its action via an IL-6 responsive element containing a CTGGGA motif, which is present in the α_2-macroglobulin promotor in rats [45–48]. Although the hCRP promotor contains this motif, the injection of dexamethasone alone or in combination with IL-6 did not influence hCRP expression in transgenic mice (results not shown). Thus it seems unlikely that this hormone alone is responsible for the described differences in the regulation of the hCRP gene.

Secondly, factors may be present in mice but not in cell culture that counteract the regulation of hCRP expression by IL-6. In an organism, the action of a cytokine is often counter-regulated to guarantee fine tuning that is necessary to re-establish homoeostasis and to avoid an over-reaction. For instance, restrictin-P/activin-A is a protein that acts antagonistically to IL-6 and IL-11. This action is most probably achieved by interference with cytokine signal transduction pathways [49]. As activin-A is produced by stromal cells, its availability in hepatocyte cultures is unlikely. Thus it would be interesting to study the regulation of hCRP in activin-A negative mice and in co-cultures of hepatocytes with stromal cells.

Differences in gene regulation between cell cultures and organisms are not restricted to the hCRP gene, but have also been described for other genes encoding APPs, such as human α_1-acid glycoprotein and nuclear factor-IL-6. The α_1-acid glycoprotein gene has been shown to lose its tissue-specific expression in cell culture, but not in transgenic mice [50], whereas nuclear factor-IL-6 was constitutively expressed and was not inducible in hepatoma cells [51], but was expressed at low levels in liver, where it could be induced upon inflamatory stimulation [52]. This demonstrates that results derived from cell culture may not always reflect true physiological conditions, a fact of particular importance for medicine. In this respect, the transgenic mouse represents a useful alternative to the cell culture system.

IL-6 is necessary for regulation of the hCRP gene

In transgenic mice the hCRP gene was highly inducible upon LPS administration, and in this respect showed a similar pattern of expression to that in humans. Although LPS induces a variety of APR mediators, it failed to induce hCRP in mice lacking IL-6. This, together with the observation that hCRP did not respond to IL-6 alone in transgenic mice, strongly indicates that IL-6 is necessary, but not sufficient, for the induction of hCRP in organisms.

It has become apparent that changes in the expression of many APPs are mediated not by IL-6 alone but by a combination of cytokines [53]. This conclusion is based on the observation that the endogenous APR to LPS was similar in IL-6$^{-/-}$ and IL-6$^{+/+}$ mice. These findings indicate that, for most of the APPs, the activity of IL-6 can be substituted by other cytokines *in vivo*. So far the only gene apart from hCRP that could not be induced by LPS in IL-6$^{-/-}$ mice is the endogenous α_2-macroglobulin gene

[54]. Interestingly, in rats α_2-macroglobulin gene regulation with respect to IL-6 is sex-specific: IL-6 induced α_2-macroglobulin in male but not in female rats; however it could be strongly induced in both sexes by turpentine, an indirect mediator of an inflammatory reaction. The reason for this sex specificity remains unclear [22]. Nevertheless, in our studies IL-6 did not induce hCRP expression in either male or female mice. However, it will be of interest to investigate whether the regulation of these two major APP genes relies on the same mechanism.

Up to now, no results are available that define a role for IL-6 in the regulation of hCRP expression in healthy humans. In patients suffering from cancer, the injection of IL-6 is correlated with an increase in hCRP serum levels [55,56]. These findings do not contradict our results in transgenic mice, as it can been proposed that the investigated subjects expressed a variety of growth factors and cytokines as a result of their disease or medical treatment. These factors could then interact with administered IL-6 in the regulation of hCRP expression.

Other cytokines, such as IL-1, IL-11, leukaemia inhibitory factor, oncostatin M and ciliary neurotrophic factor, have also been shown to induce several acute-phase genes in hepatic cells [1,5,57–60]. We aim to investigate the involvement of these factors and their co-operation with IL-6 in the regulation of hCRP expression in transgenic mice. Additionally, knockout mice are available that lack leukaemia inhibitory factor, IL-1 and ciliary neurotrophic factor [61–63]. These mice can be bred to hCRP transgenic mice and will provide a unique model in which to investigate the roles of the various cytokines in the regulation of an APR *in vivo*. These studies may result in new perspectives for the medical treatment of inflammatory diseases.

We thank Karin Bork for help in the establishment of human hepatocyte cultures, and Christoph Ahlers for critical reading of the manuscript. This work was supported by a grant to U.R. (DFG; SFB 244).

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Received 17 January 1997/14 March 1997; accepted 27 March 1997