Interleukin-6-dependent and -independent regulation of the human C-reactive protein gene

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We have investigated the function of different mediators of the regulation of the human C-reactive protein (hCRP) gene in transgenic mice. hCRP was induced by lipopolysaccharide and wounding in interleukin-6 (IL-6) +/+ mice, but not in IL-6 –/– mice. This finding suggested that IL-6 is necessary for the induction of hCRP. However, injection of IL-6 did not induce the hCRP gene. Thus, the induction of hCRP by IL-6 seems to require an additional cofactor. Therefore, we screened different

cytokines for their activity in IL-6 +/+ and IL-6 –/– mice. Surprisingly, interleukin-1 β , as well as oncostatin M or leukaemia inhibitory factor, led to an induction of hCRP in both genetic backgrounds. These results indicate an IL-6-dependent and -independent regulation of hCRP. These hCRP transgenic mice therefore represent a novel model system for defining the cytokine network involved in the regulation of acute-phase genes during the course of inflammation.

INTRODUCTION

The acute-phase response (APR) is the reaction of an organism to a local or systemic infection, to inflammation or to injury. This response leads to a characteristic change in the expression level of several serum proteins, produced mostly in the liver [1]. The change in the serum concentration is the result of a transcriptional and, for some cases, also translational regulation of the genes encoding acute-phase proteins [2]. Macrophages are a source of cytokines that are implicated in APR regulation [3] and interleukin 6 (IL-6) was identified as a potent regulator of a broad array of acute-phase genes in hepatocytes in mice and other mammals [4–9]. Besides IL-6, other cytokines, such as interleukin-1 (IL-1), leukaemia inhibitory factor (LIF), oncostatin M (OSTM) and interleukin-11 (IL-11) also contribute to the regulation of an APR [6,7,10–12].

In humans, the C-reactive protein (CRP) is the major acute-phase reactant; it is widely used as an indicator of an inflammatory state in patients. Its serum concentration in healthy individuals is below 1 mg/l and increases up to 300 mg/l during an APR [13,14]. This > 300-fold induction is the consequence of a transcriptional up-regulation of the hCRP gene in liver. Cell culture experiments have suggested that IL-6 is the mediator of this induction [15,16].

Using transgenic animals, we have shown that the regulation of hCRP in mice is similar to that in humans. Serum concentrations of up to 300 mg/l were only detectable upon injection of lipopolysaccharide (LPS) or croton oil [17,18]. However, injection of IL-6 never resulted in any transcriptional activation of the hCRP gene or in the detection of hCRP in the serum of mice [19]. In contrast, hCRP could be induced by IL-6 in primary hepatocyte cultures derived from the transgenic mice. Furthermore, the hCRP transgene was not inducible by LPS when tested in IL-6 negative knock-out mice [19]. These results clearly indicated that IL-6 is necessary but not sufficient for the induction of the hCRP gene. At least one additional factor, possibly present in cell culture, seems to be necessary to allow induction by IL-6.

To investigate this problem, we have used different cytokines such as IL-11, IL-1, LIF or OSTM to assay their activities in IL-6-negative and -positive mice. Surprisingly, several of these cytokines could induce hCRP even in the absence of IL-6. This result provides evidence for an IL-6-dependent and -independent regulation of the hCRP gene.

MATERIALS AND METHODS

Animals and treatment

hCRP transgenic mice have been described previously [17,18]. They carry a 31 kb genomic fragment of the hCRP locus containing the entire gene and a 17 kb 5'– as well as an 11 kb 3'–region. Mice with an inactivated IL-6 gene (IL-6 –/– mice) were generated and kindly provided by Dr. V. Poli, Instituto di Recherche di Biologia Molecolare P. Angeletti, Rome, Italy [20]. Female transgenic animals aged 8–10-weeks were used for experiments. They were maintained under standard conditions in a 12 h dark–light cycle.

LPS (L3254) and murine interferon- α/β (IFN- α/β ; I1258) were purchased from Sigma, and applied in a dose of 0.1 mg/mouse and 3×10^4 units (U)/mouse, respectively. Murine IL-6 was purchased from IC Chemikalien, Ismaning, Germany (ICC-06-5B) and injected at 2.5×10^4 U/mouse. Murine LIF and murine interferon- γ (IFN- γ) were purchased from Gibco-BRL (13275-029 and 13284-013 respectively) and injected at a dose of 1×10^5 U/mouse. Murine IL-1 β (1444590), human IL-11 (1581969) and human OSTM (1573675) were purchased from Boehringer Mannheim and injected at a dose of 1×10^4 U/mouse (IL-1 β), 2×10^3 U/mouse (IL-11) or 1.5 μ g/mouse (OSTM). LPS or cytokines were resuspended in sterile PBS solution and injected intraperitoneally in a total volume of 100 μ l/mouse. Blood was collected from the retro-orbital cavity before treatment, and 9 h and/or 24 h after treatment. The injection of 100 μ l of PBS and the retro-orbital puncture alone did not affect the hCRP serum level.

In addition, female mice were subjected to partial hepatectomies to induce an APR by wounding.

Abbreviations used: hCRP, human C-reactive protein; IL-6, interleukin-6; IL-1, interleukin-1; APR, acute-phase response; LIF, leukaemia inhibitory factor; OSTM, oncostatin M; IL-11, interleukin-11; LPS, lipopolysaccharide; IFN, interferon; CT-1, cardiotrophin-1; CNTF, ciliary neurotrophic factor; SAA, serum amyloid-A; JAK, janus kinase; PGC, primordial germ cell cultures; GAS, γ -interferon-activated sequence; IL-1 Ra, IL-1 receptor antagonist.

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Isolation and Northern blotting of total RNA

Mice were killed by cervical dislocation 9 h after treatment. RNA was isolated from livers by the guanidine isothiocyanate method [21], and subjected to Northern-blot analysis as previously described [18]. ³²P-Labelled cDNA fragments for hCRP, murine serum amyloid-A (SAA)-2, and rat glyceraldehyde-3-phosphate dehydrogenase were used as probes as previously described [18].

Protein analysis

Sera were prepared from blood and stored at -20°C until use. Titertek ELISA plates were coated with rabbit anti-hCRP antibodies (DAKO, Denmark A073) at 4°C overnight, washed with PBS/Tween and incubated with 0.2% gelatin solution at 37°C for 1 h to block unspecific binding sites. A 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 reactions, *O*-phenylenediamine dihydrochloride (P8287; Sigma) was used according to the supplier's instructions. Absorption coefficients were measured at 492 nm.

RESULTS

hCRP expression profile in transgenic mice after LPS injection or wounding

We have shown previously that IL-6 is unable to induce hCRP in transgenic mice. To further investigate the requirement for IL-6 in the induction of hCRP, we analysed the hCRP expression in transgenic mice with intact (IL-6 $+/+$) and with inactivated IL-6 gene (IL-6 $-/-$) upon different indirect stimuli such as LPS injection or sterile wounding by partial hepatectomy. LPS causes a systemic acute-phase reaction, whereas sterile wounding primarily results in a local reaction. Both reactions have been described to result in different APRs [22,23].

To ensure the absence of an acute-phase state in transgenic mice before the experimentally induced APR, blood was taken by retro-orbital puncture and the hCRP serum concentration was determined by a hCRP-specific ELISA method. The serum levels of uninduced animals did not exceed $0.1\text{ }\mu\text{g/ml}$. The intraperitoneal injection of $100\text{ }\mu\text{g}$ of LPS per mouse, as well as wounding caused by partial hepatectomy, led to 400- and 1000-fold increases in hCRP concentration respectively in IL-6 $+/+$ mice.

In transgenic mice, hCRP is regulated on the transcriptional level [17]. To ensure that the protein data reflected the situation on the RNA level, we performed Northern-blot analysis with RNA from livers of IL-6 $+/+$ and IL-6 $-/-$ mice after both LPS treatment and partial hepatectomy. Blots were hybridized with probes specific for hCRP and for the endogenous acute-phase gene SAA. In uninduced animals, signals for hCRP or SAA were not detectable. In IL-6 $+/+$ mice, hCRP and SAA were highly induced both by LPS injection and by wounding. In contrast with these results, hCRP was not inducible in IL-6 $-/-$ mice. SAA was expressed in IL-6 $-/-$ mice only after LPS administration, and not after wounding (Figure 1), indicating that LPS can still induce an APR in the absence of IL-6, whereas wounding cannot. Nevertheless, IL-6 is required to induce hCRP in both types of experimentally induced inflammation.

Figure 1 Transgenic hCRP mRNA expression in IL-6 $+/+$ (lanes 1–3) and IL-6 $-/-$ (lanes 4 and 5) mice upon addition of unspecific inducers

The Figure shows a Northern-blot analysis of liver RNA from transgenic mice. Livers were isolated after PBS injection as an untreated control (lane 1), after injection of $100\text{ }\mu\text{g}$ LPS (lanes 2 and 4) or after partial hepatectomy (lanes 3 and 5). The filters were sequentially hybridized with probes for hCRP, murine SAA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Figure 2 Transgenic hCRP mRNA expression in IL-6 $+/+$ mice after injection of specific inducers.

The Figure shows a Northern-blot analysis of liver RNA from hCRP transgenic IL-6 $+/+$ mice after injection of PBS (lane 1), $100\text{ }\mu\text{g}$ LPS (lane 2), $2.5 \times 10^4\text{ U}$ IL-6 (lane 3), $1 \times 10^4\text{ U}$ IL- 1β (lane 4), $1 \times 10^5\text{ U}$ LIF (lane 5), $1.5\text{ }\mu\text{g}$ OSTM (lane 6), $1 \times 10^5\text{ U}$ IFN- γ (lane 7), $2 \times 10^3\text{ U}$ IL-11 (lane 8) and $3 \times 10^4\text{ U}$ IFN- α/β (lane 9). The filters were hybridized with probes for hCRP, murine SAA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Induction of hCRP by specific cytokines

The fact that IL-6 cannot in itself induce hCRP, but is nevertheless required for hCRP gene induction to occur, indicates that, besides IL-6, at least one additional factor is necessary to induce hCRP.

In our screen for a co-activator of hCRP expression we first tested the cytokines IL- 1β , IL-11, LIF, OSTM, IFN- γ and IFN- α/β for their ability to induce hCRP in IL-6 $+/+$ mice. These cytokines were chosen because they can mediate an APR in a variety of different species, either alone or in combination with IL-6 [6,7,10–12].

Each cytokine was injected intraperitoneally into transgenic mice. The animals were killed 9 h after administration of the cytokine and analysed for hCRP and SAA RNA expression. In agreement with previous results, IL-6 did not induce hCRP but

Table 1 hCRP serum levels in IL-6 +/+ mice

Mean values $\pm$ S.D. for $n = 3$ animals (LPS and IL-11), $n = 5$ animals (IL-6) or $n = 2$ animals (all others)

Treatment	hCRP concn. (μ g/ml)
Without	0.1
LPS	40 $\pm$ 30
IL-6	0.1
IFN- γ	0.1
IFN- α/β	0.1
LIF	44 $\pm$ 7
OStM	50 $\pm$ 26
IL-11	5 $\pm$ 3
IL-1 β	78 $\pm$ 16

Table 2 Human CRP serum levels in IL-6 -/- mice

Mean values $\pm$ S.D. for $n = 7$ animals (LPS) and $n = 2$ animals (all others).

Treatment	hCRP concn. (μ g/ml)
PBS	0.1
LPS	0.1
IL-6	0.1
LIF	66 $\pm$ 20
OStM	53 $\pm$ 27
IL-1 β	24 $\pm$ 17

IL-6 alone does not affect hCRP expression, several other cytokines, such as IL-1 β , LIF and OStM, can strongly induce hCRP.

Figure 3 hCRP mRNA expression in IL-6 -/- mice after injection of specific cytokines

Liver RNA from transgenic IL-6 +/+ mice after injection of 100 μ g LPS as a positive control (lane 1) and from IL-6 -/- mice after injection of PBS as a negative control (lane 2) or 1×10^5 U LIF (lane 3) or 1×10^4 U IL-1 β (lane 4) was subjected to Northern-blot analysis; the filters were hybridized with probes for hCRP, murine SAA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

led to strong SAA expression, showing that the administered IL-6 was biologically active. IL-1 β , LIF, OStM, IFN- γ and IL-11 were strong inducers of SAA, whereas IFN- α/β led to a weaker induction of the SAA gene (Figure 2). Interestingly, only IL-1 β , LIF and OStM induced the hCRP gene, although all tested cytokines were mediators of an APR. Additionally, the hCRP serum concentrations were measured in IL-6 +/+ mice before and after injection of the cytokines. The serum levels of the animals did not exceed 0.1 μ g/ml before induction, showing that the mice were not in an acute-phase state. Again, IL-6 did not increase hCRP protein expression whereas LIF, OStM and IL-1 β led to an increase in hCRP serum concentration which was comparable with serum levels after LPS administration. IL-11 caused only a minor increase in hCRP, which might reflect the high sensitivity of the ELISA assay (Table 1). IFN- γ and IFN- α/β had no effect on hCRP serum levels.

To exclude the possibility that induction of hCRP by OStM, LIF and IL-1 β was caused by an endotoxin contamination of the appropriate cytokine, each cytokine was heated for 5 min at 95 $^{\circ}$ C. This procedure destroys the cytokine activity but does not influence the endotoxin. None of the boiled cytokines led to an hCRP response in transgenic mice, demonstrating that the APR was not caused by endotoxin (results not shown). Thus, although

Induction of hCRP in IL-6 -/- mice

We then investigated whether IL-1 β , LIF and OStM need to cooperate with IL-6 to induce hCRP expression. These cytokines were therefore injected into IL-6 -/- mice. The animals were killed 9 h later and analysed for hCRP and murine SAA RNA expression by Northern-blot analysis. As shown in Figure 3, IL-1 and LIF could induce hCRP and SAA expression in the livers of these animals even in the absence of IL-6. Furthermore, the hCRP serum level was determined in IL-6 -/- mice both before and 24 h after injection of LIF, OStM and IL-1, respectively. LIF and OStM led to a > 500-fold increase (Table 2), which resembled the hCRP response to these cytokines in IL-6 +/+ mice. IL-1 β led to a 200-fold increase in hCRP serum concentration in IL-6 -/- mice which was moderately lower than that found in IL-6 +/+ mice. Thus IL-1, OStM and LIF can induce hCRP, even in the absence of IL-6.

DISCUSSION

Our results indicate that the hCRP gene is differentially regulated according to the stimulus; in response to LPS and wounding, the induction of hCRP requires IL-6, whereas LIF, OStM and IL-1 β cause an IL-6-independent hCRP expression.

Regulation of hCRP by cytokines of the IL-6 family

The IL-6 cytokine family members IL-6, IL-11, LIF, OStM, cardiotrophin-1 (CT-1) and ciliary neurotropic factor (CNTF) show remarkable redundancy in their biological activities. These activities include the regulation of haematopoiesis, the acute phase and immune responses, as well as regulatory functions in the endocrine and nervous system [24]. This redundancy is at least in part explained by the fact that all these cytokines share a common cytokine receptor subunit, the glycoprotein gp130 [24]. In our experimental system, the induction of the endogenous mouse acute-phase gene SAA served as a 'read-out' system for the induction of an acute-phase reaction and thereby the biological activity of the administered substance. Each tested cytokine induced the SAA gene, which is in agreement with previous results [25]. In contrast, the hCRP gene was regulated by a less broad array of cytokines in transgenic mice (Figure 2), demonstrating both redundancy as well as specificity of IL-6 cytokine family members in the regulation of acute-phase genes. In particular, our investigations revealed considerable differences

between IL-6/IL-11 and OStM/LIF with respect to hCRP gene regulation. hCRP gene induction by IL-6 and IL-11 was not detectable at the RNA level. In contrast, OStM and LIF strongly induced the hCRP gene, which closely correlated the induction seen at the protein level. Differences in the functional properties between OStM/LIF and IL-6/IL-11 have also been reported on mouse primordial germ cell cultures (PGC): OStM and LIF enhance the viability of post-migratory (colonizing) PGC, whereas IL-11 and IL-6 have no effect on this process [26].

These differences might be explained by the different compositions of the appropriate receptor complexes of IL-6/IL-11 and OStM/LIF.

Besides gp130, a second receptor subunit, the LIF-receptor β (LIFR β) is shared by the receptors of CNTF, LIF, OStM and CT-1, but not by those of IL-6 and IL-11 [24,27,28]. These differences might enable the cytokines to trigger different signals to the target gene. In addition, it has been reported that different monoclonal antibodies against gp130 can specifically block the action of distinct cytokines of the IL-6 family [28,29]. This indicates that different specific functional sites of gp130 are recruited by different cytokines, creating functional diversity in signal transduction via gp130 among the cytokines of the IL-6 family. This diversity might involve the activation of a different set of janus kinases (JAK) as signal transducers to regulate hCRP gene expression.

IL-6-dependent regulation of the hCRP gene

Wounding-mediated gene expression of hCRP and SAA caused by partial hepatectomy required IL-6 (Figure 1). Furthermore, it was reported that IL-6 is also required to induce murine SAA upon crotonoil or turpentine treatment of mice [22,30]. Thus IL-6 seems to be necessary to mediate a sterile local inflammation. In terms of LPS-caused induction, the hCRP and murine SAA genes were differentially regulated: the murine SAA gene was induced by LPS in IL-6 $+/+$ as well as in IL-6 $-/-$ mice (Figure 1) showing that IL-6 is not necessary for this response, which is in agreement with previous reports [22,30]. In contrast, the induction of hCRP by LPS required IL-6 in transgenic mice (Figure 1). Nevertheless, IL-6 alone cannot induce hCRP in these animals (Figure 2). This indicates that the induction of hCRP by LPS is mediated by IL-6 in co-operation with a second, so far unidentified, factor.

In the human hepatoma cell line Hep3B a hCRP reporter gene was synergistically induced by IL-6 and IL-1 β . IL-1 β was assumed to act as a post-transcriptional regulator of hCRP in these cells because it could not induce hCRP alone [31]. Another group reported the requirement of IL-1 for hCRP induction in the human hepatoma cell line Hep3B [15]. Nevertheless, co-injection of IL-1 β and IL-6 did not show any synergistic or additional effect on hCRP serum levels in transgenic mice, even when suboptimal IL-1 dosages that did not induce the full hCRP response were used (results not shown). Furthermore, IL-1 β could induce hCRP even in the absence of IL-6 in transgenic mice (Figure 3 and Table 2). Thus IL-1 β does not require IL-6 to induce hCRP and does not serve as a co-activator of hCRP expression in transgenic mice.

Signalling by IL-6 and other cytokine members of the IL-6 family (OStM, LIF, IL-11, CT-1 and CNTF) is mediated by the APR factor APRF, now also called 'signal transducer' and 'activator of transcription 3' (Stat3) [32,33]. This factor binds to an IL-6-responsive element (IL-6 RE) containing a TT(N)₄AA or TT(N)₅AA motif, and is involved in the regulation of the hCRP gene in human hepatoma cells [34]. The binding motif for Stat3 also represents a γ -IFN-activated sequence (GAS), which

is bound by the transcription factor Stat1/p91. Knock-out experiments revealed that this factor is required for signal transduction mediated by IFNs [35,36]. Stat1 *in vitro* could also be activated by members of the IL-6 family [32]. Nevertheless, we never observed an induction of hCRP by IFNs in our transgenic mice (Figure 2 and Table 1), indicating that the signalling by the IL-6 family members LIF or OStM is different from the IFN-signalling pathway.

Regulation of the hCRP gene by IL-1

We have demonstrated an IL-6-independent regulation of the hCRP gene not only by OStM or LIF, but also by IL-1. It has been shown that IL-1 β induces acute-phase genes by triggering transcription factors of the CCAAT enhancer binding protein (C/EBP) family, which bind to a T[T/G]NNGNAA[T/G] DNA consensus sequence [nuclear factor (NF)-IL-6 binding site] [37,38]. These DNA elements are present in the hCRP promoter in our transgenic mice [18,39], but further investigation is required to evaluate the role of this signalling pathway in the regulation of the hCRP gene. In addition, it will be of interest to see whether IL-1 β can induce OStM/LIF or whether these cytokines share a common signalling pathway, which has not yet been reported.

As LPS is a potent inducer of IL-1 β , the failure of hCRP induction by LPS in IL-6 $-/-$ mice was surprising and indicated that either the gene expression of IL-1 β or the biological activity of the IL-1 β protein must be impaired in the absence of IL-6. Reports that IL-6 $-/-$ mice fail to combat infections of *Listeria monocytogenes*, a reaction which is IL-1-dependent [30,40], strengthen this assumption.

Endogenous modulation of IL-1 activity can be achieved at various levels, including regulation of IL-1 gene activity, protein synthesis or processing. IL-1 protein processing results in the production of a biologically active protein via modification of the cytoplasmic inactive form with the help of the IL-1 converting enzyme ICE; active IL-1 is then released by the cell [41,42]. In addition, expression of IL-1 receptors and IL-1 receptor antagonist (IL-1 Ra) can modulate IL-1 effects [42]. An influence of IL-6 on these processes has not been reported to date.

We first investigated the IL-1 β expression in peritoneal macrophages of IL-6 $-/-$ mice and found a strong induction of the IL-1 β RNA upon LPS injection (results not shown). Thus IL-1 β RNA expression is not impaired in the absence of IL-6 in these cells. We are currently investigating the requirement of IL-6 for the formation of a biologically active IL-1 β protein. It has been shown that transgenic mice overexpressing IL-1 Ra are more susceptible to infection by *Listeria monocytogenes*, whereas the susceptibility to this infection is decreased in mice with an inactivated IL-1 Ra gene (IL-1 Ra $-/-$). In addition, elevated IL-6 levels were observed in IL-1 Ra $-/-$ mice [43]. Thus it will be interesting to investigate whether IL-1 Ra is upregulated in IL-6 $-/-$ mice and thereby counteracts IL-1. In this context, it is worth noting that we observed a 4-fold higher increase in hCRP serum level in IL-6 $+/+$ mice compared with IL-6 $-/-$ mice after injection of IL-1 β (Tables 1 and 2), which would correlate with this assumption. However, further analysis is necessary to define the regulatory cytokine network regulating the major human acute-phase gene hCRP. In this respect, the hCRP transgenic mouse seems to be a suitable system of analysis for this cytokine network in the context of an organism. Such investigation might result in the development of new therapeutic approaches for the treatment of inflammatory diseases.

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