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Review

Expression and induction of anaphylatoxin C5a receptors in the rat liver

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Summary. The C5a-anaphylatoxin which is generated by limited proteolysis upon activation of the fifth component of complement may be induced by the classical, the alternative or the lectin pathway. C5a has been shown, under normal conditions, to induce the release of prostanoids from Kupffer cells (KC) and hepatic stellate cells (HSC) and thereby indirectly to increase glucose output from hepatocytes (HC). A direct action of C5a on HC would require the expression of the specific C5a receptor (C5aR). In studies using quantitative RT-PCR it was shown that non-stimulated HC lack C5aR, in contrast to KC, HSC and sinusoidal endothelial cells (SEC) all of which contained mRNA for the C5aR in decreasing amounts. FACS analyses, immunohisto- and immunocytochemistry as well as functional analyses confirmed the results of the RT-PCR assays. Under inflammatory situations the C5aR was found to be upregulated in various organs and tissues which included the liver. Interleukin-6 (IL-6) as a main inflammatory mediator in the liver induced a de novo expression of functional C5aR in HC in-vitro and in-vivo. In contrast, LPS failed to induce C5aR directly in cultured HC in-vitro but induced C5aR in HC in vivo and in co-cultures of HC and KC which release IL-6 upon stimulation with LPS. So far, the only known effector function of C5a on HSC was the induction of prostanoid release. In an approach to reveal new functions of C5aR in HSC, the cells responsible for liver fibrosis, it could be shown that C5a upregulated fibronectin-specific mRNA five-fold whereas entactin, collagen IV and the structure protein smooth muscle actin were not affected. In addition, C5a did not upregulate specific mRNA for the profibrotic cytokine TGF-ß1 in either isolated KC or HSC. Thus, C5a alone appears to have only a limited role in the induction of liver fibrosis.

Key words: C5a anaphylatoxin, C5a receptor, Hepatocyte, Kupffer cell, Rat liver

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1. Introduction

The anaphylatoxin C5a is cleaved from the fifth component of complement (C5) in the course of complement activation. It originates from the N-terminal region of the α-chain of C5 and consists of 74 (human) or 77 (rat) amino acid residues (Fernandez and Hugli, 1978; Cui et al., 1994; Rothermel et al., 1997a). The human and rat C5a molecules are cationic glycoproteins that function as mediators of the inflammatory response. C5a induces the release of histamin and of lysosomal contents from myeloid cells and the contraction of smooth muscle cells. Furthermore, it increases the vascular permeability and mediates chemotactic migration of neutrophilic granulocytes and monocytes by directing these cells into areas of inflammation (Hugli and Morgan, 1984; Goldstein, 1988). It also causes the production of reactive oxygen species in phagocytes. All these effects are mediated by the binding of the C5a-ligand to its receptor (C5aR/CD88) which has first been shown to be expressed on myeloid cells such as human neutrophils (Chenoweth and Hugli, 1988), eosinophils (Gerard et al., 1989), basophils (Kurimoto et al., 1989), monocytes (Marder et al., 1985; Werfel et al., 1992) and mouse macrophages (Chenoweth et al., 1982). The C5a receptor belongs to the large G-protein- coupled superfamily of rhodopsin-like receptors all of which possess an extracellular N-terminus, seven α-helical hydrophobic transmembrane regions and an intracellular C-terminus. For the binding of human C5a to its specific receptor, a model assuming two different binding sites has been proposed (Siciliano et al., 1994). The coding sequences of the receptors of man (Gerard and Gerard, 1991), mouse (Gerard et al., 1992), dog (Perret et al., 1992), rat (Rothermel et al., 1997b) and partial amino acid sequences of the bovine C5a receptor (Perret et al., 1992) have been reported. The cloning of the human C5aR cDNA (Gerard and Gerard, 1991) allowed the generation of specific antibodies against the human C5aR (Morgan et al., 1993; Oppermann et al., 1993; Haviland et al., 1995). These antibodies were used in immunohisto- and immunocytochemical investigations to demonstrate that the expression of the C5aR which

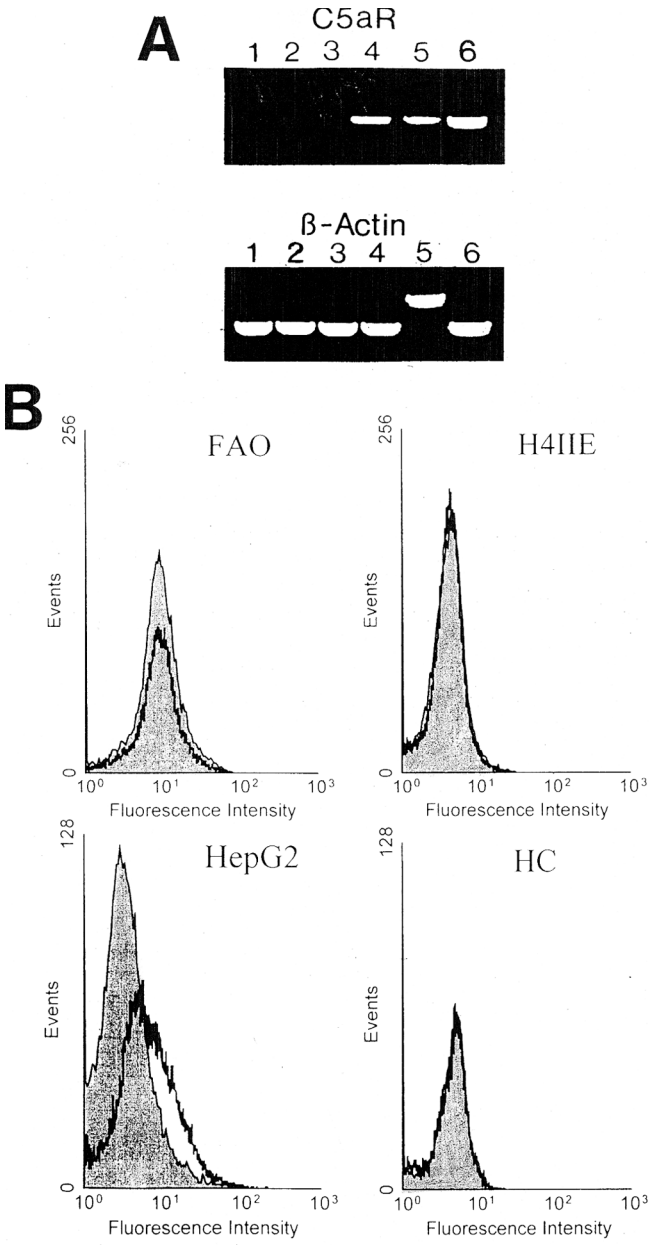

Fig. 1. A. Identification of the C5aR-specific mRNA by RT-PCR analysis. No mRNA specific for C5aR is present in FAO hepatoma cells (1), H4IIE hepatoma cells (2) and isolated rat hepatocytes (3). C5aR-specifc mRNA (amplificate of 393 bp) is detectable in rat Kupffer cells (4), HepG2 hepatoma cells (5) and RBL cells which have been transfected with the rat C5aR gene (6) using degenerated primers which amplify the human and the rat C5aR. For control purposes the corresponding ß-actin -specific amplificates are shown which comprise 749 bp in cells derived or isolated from rat (1, 2, 3, 4, 6) and 1027 bp for the HepG2 hepatoma cell line (5) which is of human origin. **B.** Flow cytometric analyses of the hepatoma cell lines FAO, H4IIE, HepG2 and hepatocytes isolated from rat liver. FAO cells, H4IIE cells and isolated rat HC are stained with the mAb R63 for the rat C5aR (broad lines), HepG2 cells are stained with the anti-human C5aR mAb S5/1 (broad line). As controls all cells are stained with the IgG1-isotype-specific control antibody MOPC-21 followed by a FITC-conjugated secondary antibody (thin lines and shaded areas). C5aR expression is only detectable on HepG2 hepatoma cells.

had first been believed to be restricted to myeloid cells as mentioned above is also detectable on non-myeloid cells. The number of non-myeloid cell types which have been reported to express the C5aR increased from year to year (Haviland et al., 1995; McCoy et al., 1995; Floreani et al., 1998; Zwirner et al., 1999; Fayyazzi et al., 2000; Drouin et al., 2001). The recently described expression of the C5aR on human T-lymphocytes (Nataf et al., 1999) was not observed in earlier investigations by Werfel et al. (1992) and our own unpublished observations (Schlaf and Götze, 2000), possibly due to a special stimulus which may be required to express C5aR on these cells. Investigations of the C5aR-distribution on liver cells resulted in controversial results. Here we review experimental evidence concerning the expression of the C5aR on rat and to a minor part on human liver cells. We review our results obtained with molecular, immunocytochemical as well as functional assays which clearly show that HC do not express C5aR under normal conditions. We discuss data on the expression of C5aR on hepatocytes (HC) of rat and man which have been reported previously but are not in accord with our findings. The observation that C5aR are inducible by treatment of rats with LPS or IL-6 in-vivo or by the in-vitro treatment of HC with IL-6 may be an explanation for the controversially reported identification of C5aR on HC. In a second part of this review we present experimental evidence concerning the expression of C5aR on hepatic stellate cells (HSC). We discuss our most recent investigations concerning a possible C5a-mediated effect on HSC with respect to the induction of liver fibrosis which suggest a limited role in the induction of liver fibrosis.

2. Identification of the C5aR on the surface of KC, HSC and SEC but not on HC in rat liver under normal conditions

There are four main cell types in the liver. The parenchymal cells (HC) contribute more than 60% of all liver cells. The resident macrophages of the liver, the Kupffer cells (KC) amount to about 8% of the liver cells but they make up 80% of the total number of macrophages in the body. They are located in the blood sinusoids with processes of their cell bodies reaching through the layer of sinusoidal endothelial cells (SEC) into the "Space of Disse". The SEC, which amount to about 28% of the liver cells, separate the sinusoids and the "Space of Disse" but allow, via their "fenestrae", the passage of soluble substances between these two compartments. Thus, they are a cellular filter between the sinusoidal blood stream and the "Space of Disse" (Wisse et al., 1985). The fourth main liver cell type, the hepatic stellate cells (HSC), which account for about 4% of all liver cells and which are also called fat-storing-cells or Ito cells, are located within the "Space of Disse". They store vitamin A within conspicuous cytoplasmic vacuoles of 1-2 μm diameter. These cells are the main producers of extracellular matrix proteins and, thus, have

a decisive role in the genesis of liver fibrosis.

The first data dealing with the expression of the C5aR on liver cells were published by Buchner et al. (1995). In their study Buchner and coworkers described a C5a-mediated increased expression of the acute phase proteins α1-antitrypsin and α1-antichymotrypsin in the hepatoma-derived cell line HepG2 as assayed by mRNA-determinations using RT-PCR. An increased α1-antitrypsin concentration in the supernatants of HepG2 cells after stimulating these cells with C5a was, in addition, measured by a solid-phase ELISA. The C5a receptor was identified on HepG2 cells in FACS analyses using polyclonal anti-peptide antibodies (aa-position 9-29 of the human primary structure). These data showed that the human HepG2 hepatoma-derived cell line constitutively expresses the C5a receptor and that binding of its ligand results in the upregulation of acute phase proteins i.e. that it is functionally coupled. Haviland et al. (1995) supported the data on the expression of the C5aR on HepG2 cells but, in addition, identified the C5aR in/on HC in human liver slices by in-situ hybridization and immunohistochemical analyses. Their data on a constitutive expression on human HC are not in accord with studies on rat liver cells (Schieferdecker et al., 1997; Schlaf et al., 1999) and those of human liver tissue slices (Zwirner et al., 1999; Fayyazzi et al., 2000). The data of Haviland and coworkers may have to be reevaluated: the faint signal of the Northern Blot analyses of C5aR-specific mRNA from liver tissue is in sharp contrast to the abundant mRNA signal obtained from HepG2 hepatoma-derived cells. As HC make up 60% of the liver cells the signal in whole liver would be expected to be much larger. However, the signal's low intensity could be explained if it were due to KC which amount to only about 8% of the liver cells. KC, which are known to bear the highest number of C5aR and, thus, show the most prominent signal of all liver cells in any assay (Schieferdecker et al., 1997; Schlaf et al., 1999, Zwirner et al., 1999) did not display a signal in the immunohistochemical studies of Haviland and coworkers. This disagrees with all other immunohistochemical studies of liver tissue using monoclonal antibodies (Fig. 2) (Schlaf et al., 1999; Zwirner et al., 1999; Fayyazzi et al., 2000). The *in-situ* hybridization data of Haviland et al. (1995) show distinct signals but their location is difficult to determine. They seem to be located lateral to the sinusoids and may thus rather represent KC. The differences in the studies of liver tissue performed by Haviland and coworkers as compared with the other groups may, in addition, depend on background signals which may have been generated by the endogenous biotin of HC. In our own studies isolated HC as well as HC from rat liver sections showed a high background which was completely absent after the endogenous biotin had been blocked. Furthermore, Haviland and

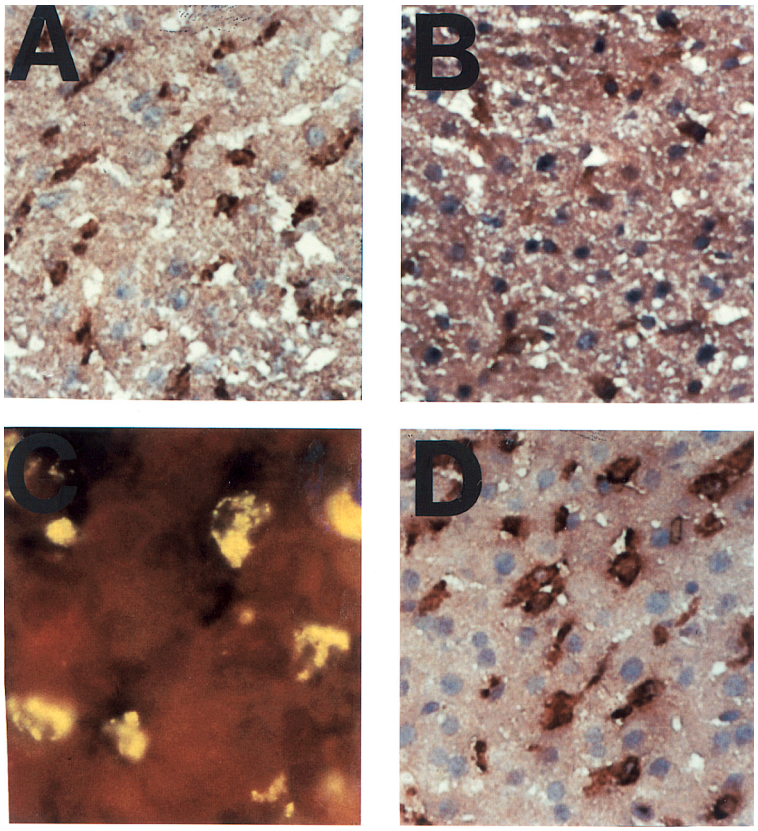

Fig. 2. Immunohistochemical staining of rC5aR in normal liver tissue **(A, C, D)** and liver tissue of a rat intraperitoneally treated with LPS 12 hours before the removal of the liver **(B)**. **A and B.** Staining with the mAb R63 (anti rC5aR) followed by a biotin-conjugated goat-anti-mouse IgG secondary antibody and final AEC/peroxidase staining after incubation with peroxidase-conjugated streptavidin. **C.** Parallel staining with the FLUOS-conjugated mAb R63 in combination with the HC-specific mAb MRC OX-61 (anti-rat dipeptidyl-peptidase IV), followed by a Texas-Red -conjugated anti-mouse secondary antibody. In **D** the Kupffer cell-specific mAb ED2 is used. The Kupffer cells, as the only detectable cell type in this procedure, appear as brown spots interspersed among the parenchyma **(A, D)** or as yellow spots among the dark red-stained HC **(C)**. In tissue slices of livers after LPS treatment the KC are hardly visible due to the prominent expression and staining of C5aR on HC **(B)** (for details, see Ref. Schlaf et al., 1999). A, B, D, x 250; C, x 350

coworkers used polyclonal IgG-antibodies which may have cross-reacted with another structure of unknown origin. A third and probably important reason may be that the bioptic human material, the source of which has not been disclosed, does not represent normal tissue. In some studies an enhanced expression of C5aR mRNA in whole liver after intraperitoneal injection of LPS was shown (Haviland et al., 1995; Akatsu et al., 1997; Fukuoka et al., 1998). This demonstrates that the C5aR is induceable in HC under inflammatory conditions, as discussed in the following sections of this review. The expression of C5aR in the human hepatoma-derived cell line HepG2 (Buchner et al., 1995; Haviland et al., 1995; McCoy et al., 1995), should not be taken to represent C5aR expression in wildtype HC or in other hepatoma-derived cell lines as we could not identify the C5aR in the cell lines FAO and H4IIE nor in primary rat HC (Fig. 1). This suggests that the expression of the C5aR on HepG2 hepatoma cells may be a result of their malignant transformation which, however, appears not to represent a general mechanism, as the rat hepatoma cells FAO and H4IIE do not express the C5aR. In this context we have to correct former data of our group (Schlaf et al., 1999) which showed C5aR-specific mRNA expression in the two rat hepatoma cells FAO and H4IIE. These signals were most probably due to an incomplete digest of genomic DNA which had been amplified instead of C5aR-specific cDNA derived from mRNA as there is no intron between the chosen primer positions in the C5aR gene of man (Gerard et al., 1993) and mouse (Gerard et al., 1992). Therefore, it is most probably also lacking in the rat. By analogy with the human gene the mouse homologue contains two exons with the 5' untranslated region and the initiating methionine codon in exon 1 and the remainder of the nucleotide sequence in exon 2 (Gerard et al., 1992, 1993). The data, therefore, suggested that the activation of the C5aR gene might be a general consequence of the malignant transformation of the cell lines. This is, however, not the case, as shown by figure 1. In recent investigations using RT-PCR assays and FACS analyses with the anti-human C5aR mAb S5/1 (Oppermann et al., 1993) and the anti-rat C5aR mAb R63 (Schlaf et al., 1999; Rothermel et al., 2000) we could demonstrate that only HepG2 cells but not FAO and H4IIE hepatoma cells express the C5aR (Fig. 1) i.e. HepG2 cells are apparently an exception among HC and other hepatoma cells.

There are studies which clearly show that primary HC of human and rat liver do not express the C5aR constitutively. Using HC of rats isolated according to the enzyme-free method of Meredith (1988), Schieferdecker et al. (1997) first showed, by RT-PCR analyses, the lack of C5aR-specific mRNA in HC. In contrast, Kupffer cells (KC) expressed about ten-fold more C5aR-mRNA than hepatic stellate cells (HSC) and these cells nearly ten-fold more than sinusoidal endothelial cells (SEC), as was demonstrated by quantitative-competitive RT-PCR. In contrast to the lack of a signal from primary rat HC, Schieferdecker and coworkers obtained an HepG2-derived amplificate using degenerated primers which amplified C5aR-specific cDNA from human as well as from rat cells. These data suggest once more that malignantly transformed cells are not good models of primary cells as they do not necessarily display the properties of wild-type cells. The mRNA data obtained by Schieferdecker et al. (1997) were later confirmed on the protein level using the newly generated monoclonal antibody R63 against the rat C5aR (Schlaf et al., 1999; Rothermel et al., 2000). This mAb had been generated by immunizing mice with rat RBL2H3 cells which had been transfected with the coding sequence for the rat C5a receptor. In FACS analyses and by immunocytochemistry the highest levels of expression were demonstrated on the surface of KC (Fig. 2A,C,D) followed by an unexpectedly high level on HSC and a rather weak level on SEC. HC did not bind the mAb R63. In immunohistochemical analyses only KC were clearly identified as C5aR-positive, as shown by parallel staining with the KC-specific mAb ED2 (Fig. 2D). The immunochemical analyses were strengthened by the demonstration of an intracellular Ca^{2+}-release induced by recombinant rat C5a (rrC5a) (Rothermel et al., 1997a; Schlaf et al., 1999). Unequivocal data on the functional coupling of the C5aR were obtained for KC, HSC and SEC by this assay. The lack of a signal in these assays in HC confirmed the lack of C5aR on this cell type.

The argument that the differences between the data of Haviland and coworkers and our studies may be due to species differences of man versus rat has been cleared up (Zwirner et al., 1999; Fayyazzi et al., 2000). Using monoclonal antibodies against the aminoterminal peptide (Ex1) they showed that in slices of normal human liver no C5aR-protein was found on HC whereas KC distinctly expressed it. Thus, the more recent data of our group (Schieferdecker et al., 1997; Schlaf et al., 1999; Rothermel et al., 2000) and of Fayyazzi et al. (2000) suggest the necessity for a reevaluation of the formerly published data.

3. C5a functions in liver under normal conditions

The investigations which were undertaken to provide direct evidence for the expression of the C5aR were supported by functional data of the C5a-induced release of prostanoids from KC and HSC. Effector functions of HC which are dependent of C5a were, in addition, shown to be strictly dependent on the release of soluble mediators from non-parenchymal cells (KC, HSC) which express the C5a receptor. In this context, KC are the most important cell type. Besides their classical immunological tasks (phagocytosis, antigen processing and presentation) this cell type, upon activation by inflammatory stimuli, e.g. LPS (Grewe et al., 1992), zymosan (Birmelin and Decker, 1984) and platelet-activating factor (Gandhi et al., 1992), releases prostanoids and other mediators such as cytokines and nitric oxide (Scholz et al., 1990; Montz et al., 1991). Cytokines are released by C5a-stimulation alone or in

combination with other stimulatory substances such as LPS (Cavaillon et al., 1990; Mäck et al., 2001). After stimulation with recombinant rat C5a (rrC5a) (Rothermel et al., 1997a), KC released predominantly the prostanoids PGD2 and TXA2 but also PGF2α and PGE2. Prostanoids which are generated enzymatically from arachidonic acid can be synthesized rapidly and, for this reason, are tools of short term effector functions. After stimulating KC with rrC5a the release of prostanoids was maximal within the first two minutes and then decreased. The kinetics observed after the exposure of KC to zymosan or LPS were different. The prostanoid release caused by zymosan was initiated as fast as that induced by rrC5a. However, its maximal peak was reached after 15-30 min (Püschel et al., 1993; Pestel et al., 2001). Prostanoid release, which had been induced by LPS, was demonstrable only after 3.5 hours (Peters et al., 1990; Pestel et al., 2001). The different kinetics show that different stimuli induce the release of the same prostanoids via different pathways, i.e. that different stimuli regulate the response time of the hepatic defense reactions differently. Although HC do not express C5aR under normal conditions it was found that C5a induced a rapid increase in glucose output from this cell type in perfused rat livers (Püschel et al., 1996). These effects of C5a on HC were mediated by prostanoids the main source of which were KC. When KC were depleted in rat livers by pretreating the animals with gadolinium chloride the C5a-mediated glucose output and the prostanoid release into the hepatic vein were substantially diminished (Püschel et al., 1996). The remaining effect of C5a on the output of glucose, i.e. on the activation of glycogen phosphorylase was mediated by prostanoids which had been released by HSC (Schieferdecker et al., 1998). The activation of glycogen phosphorylase in HC via prostanoids as a response to rrC5a was similar if they were released from HSC or from KC although the prostanoids were released in different ratios (Schieferdecker et al., 1999). Additionally, a reduced flow rate was observed (Püschel et al., 1989; Schieferdecker et al., 1999). As both effects could be blocked completely by inhibitors of prostanoid function and synthesis, i.e. by daltroban as an antagonist of the thromboxane receptor and indomethacin as an inhibitor of the cyclooxygenase, they were mediated only by prostanoids.

The data reviewed in sections 2 and 3, indicate that the hepatocyte is the only one of the four main cell types of the normal rat liver and, most probably, of human liver as well in which no C5aR-specific mRNA and no surface C5aR-protein is detectable. This was shown by direct approaches to identify the receptor in/on isolated HC and on tissue slices through the use of antibodies and the recombinant ligand. These data were supported by functional data which showed that the C5a-induced effects on HC are mediated by prostanoids, i.e. that C5a under normal circumstances acts only indirectly on HC. It was the aim of nearly all of our studies to provide convincing evidence for this hypothesis. In this context it is puzzling that the data of Schlaf et al. (1999) which show that HC do not express the C5aR were interpreted by Drouin et al. (2001) to contain the message that HC express the C5aR when the main message was indeed that they do not, unless stimulated by IL-6 (Schieferdecker et al., 2000). Drouin et al. (2001) used the same methodologies for the investigations of lung tissue which had formerly been established by Haviland and coworkers for liver cells. For critical comments concerning those methodologies see Zwirner et al. (1999).

4. The expression of the C5aR is inducible in HC under inflammatory conditions

It has previously been shown that the expression of C5aR is upregulated in some tissues under pathological conditions. C5aR were inducible on the surface of bronchial epithelial cells of rats which had been infected with mycoplasms (Rothermel et al., 2000). An enhanced responsiveness to C5a was also demonstrable in human bronchial epithelial cells upon exposure to cigarette smoke (Floreani et al., 1998). The expression of C5aR was upregulated in the human central nerve system under inflammatory conditions (Gasque et al., 1997). Moreover, the receptor was strongly upregulated in neurons of mice in experimental meningoencephalitis caused by listeria (Stahel et al., 1997a) and in neurons of rats with experimental diffuse axonal injury (Stahel et al., 1997b). In addition, intraperitoneal injection of lipopolysaccharide (LPS) strongly upregulated the C5aR-specific mRNA expression in organs and tissues of rat (Haviland et al., 1995) and mouse (Fukuoka et al., 1998). Also, in the liver C5aR-specific mRNA was significantly upregulated by LPS in both species. We demonstrated that this LPS-dependent expression of C5aR was due to an induction of C5aR in HC (Fig. 3) (Koleva/Schlaf et al., 2002). Furthermore, we investigated whether this effect was mediated by inflammatory cytokines, as LPS does not act directly on HC (Koleva/Schlaf et al., 2002; Schlaf et al., 2002) and the effect of LPS could therefore only have been mediated indirectly. A possible candidate for this function was the proinflammatory cytokine interleukin-6 (IL-6), formerly called hepatocyte-stimulating factor (HSF), which is known to be pivotal for hepatocellular defense reactions (Castell et al., 1989; Heinrich et al., 1990; Baumann and Gauldie, 1994; Ramadori and Christ, 1999). Furthermore, IL-6 is released from KC upon stimulation with LPS (Decker, 1990; Grewe et al., 1992). Indeed, treatment of rats with IL-6 caused a time-dependent expression of C5aR mRNA in HC with a maximum at about 4h (Schieferdecker et al., 2000). The C5aR protein was maximally expressed between 8 and 10h after this treatment. The newly expressed C5aR was functional as C5a could directly activate glycogen phosphorylase in isolated HC. The direct effect of rrC5a on HC was also demonstrated in the perfused rat liver. Indomethacin and daltroban did not influence this

metabolic effect of C5a-dependent glucose output, in contrast to rats treated with saline/BSA in which these inhibitors blocked the activation of glycogen phosphorylase and, thus, glucose output completely as had been shown previously (Püschel et al., 1996; Schieferdecker et al., 1999). The time dependence of the enhancement of glycogen phosphorylase activity was in good accord with the kinetics of the immunochemically-detected appearance of the receptor on the surface of HC (Schieferdecker et al., 2000). The effects of noradrenaline, which acts directly on HC, were not modulated by these prostanoid inhibitors either in LPS-treated or in saline/BSA treated rats. The data on the prominent role of IL-6 as the inducer of C5aR on HC was supported by a recent study (Koleva/Schlaf et al., 2002) which was performed to elucidate the formerly described mechanism of LPS as an upregulator of C5aR-specific mRNA in tissues of mice (Fukuoka et al., 1998) and rats (Haviland et al., 1995). The treatment of rats with LPS induced C5aR-mRNA in HC with a maximum at 10h (Fig. 3) (Koleva/Schlaf et al., 2002). As in IL-6 - treated animals the maximal expression of C5aR-specific

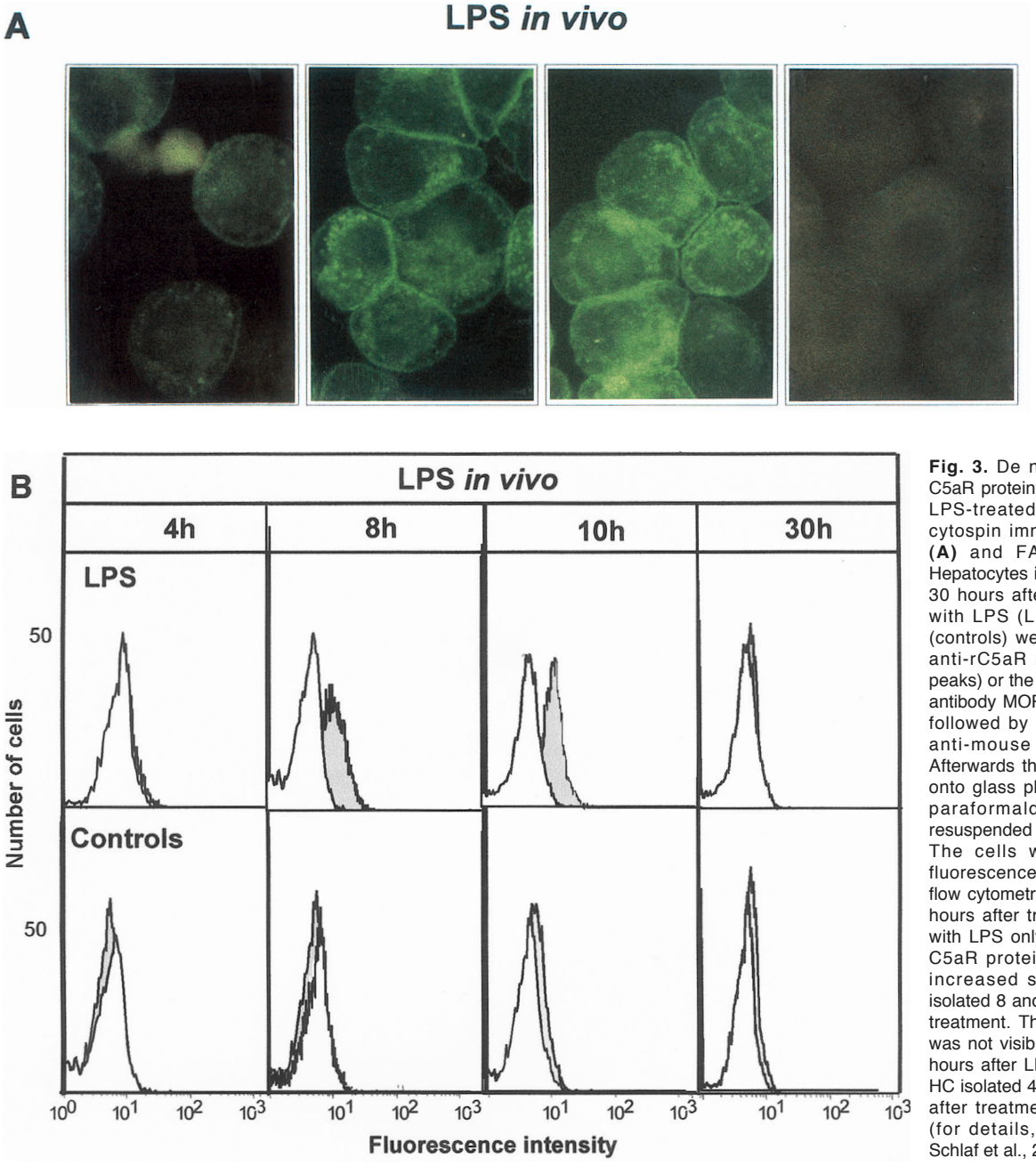

Fig. 3. De novo expression of C5aR protein in HC isolated from LPS-treated rats as shown by cytospin immunocytochemistry **(A)** and FACS-analysis **(B)**. Hepatocytes isolated 4, 8, 10 and 30 hours after injections of rats with LPS (LPS) or saline/BSA (controls) were stained with the anti-rC5aR mAb R63 (shaded peaks) or the IgG1-isotype control antibody MOPC-21 (open peaks), followed by a FITC-conjugated anti-mouse IgG antibody. Afterwards they were centrifuged onto glass plates and fixed with paraformaldehyde **(A)** or resuspended in FACS buffer **(B)**. The cells were analyzed by fluorescence microscopy **(A)** or flow cytometry **(B)**. HC isolated 4 hours after treatment of the rats with LPS only weakly expressed C5aR protein. The expression increased significantly in HC isolated 8 and 10 hours after LPS treatment. The C5aR expression was not visible in HC isolated 30 hours after LPS treatment and in HC isolated 4, 8, 10 and 30 hours after treatment with saline/BSA (for details, see Ref. Koleva/Schlaf et al., 2002). x 300

mRNA in HC is already visible at about 4h (Schieferdecker et al., 2000); this shift in the C5aR-specific mRNA expression suggests an indirect effect of LPS on HC. This conclusion is strengthened by the observation that LPS does not act directly on this cell type (Fig. 4) (Koleva/Schlaf et al., 2002; Schlaf et al., 2002). C5a activated glycogen phosphorylase in isolated HC of LPS-treated rats directly and enhanced glucose output in their perfused livers without an involvement of prostanoids. LPS did not induce C5aR in-vitro in cultured HC in contrast to IL-6 which is known to be released from KC upon treatment with LPS. Co-culture experiments of KC and HC showed an LPS-dependent induction of C5aR protein in HC (Fig. 4) (Koleva/Schlaf et al., 2002). This induction could be suppressed by an inhibitory polyclonal anti-IL-6 IgG (Fig. 4). The conclusion was thus strengthened that IL-6 released by KC is the main mediator of an LPS-induced de novo expression of C5a receptors in HC. It is supported by recent unpublished results by our group which demonstrate, by FACS-analyses, that HC isolated from rats which had been treated with gadoliniumchloride to destroy all KC do not express C5aR after LPS-treatment of the animals. These in-vivo experiments strongly support the results of the HC/KC co-culture experiments (Fig. 4) which demonstrated that IL-6 which is released from KC upon stimulation with LPS functions as the main mediator of the LPS-induced C5aR expression in HC.

5. An approach to novel functions of C5a

After we observed the expression of C5aR in HSC at a high level with respect to both mRNA (Schieferdecker et al., 1997) and protein (Schlaf et al., 1999) we checked whether C5a, besides inducing the release of prostanoids (Schieferdecker et al., 1998), may also induce additional functions in these cells. Investigations on the C5aR-mediated intracellular Ca^{2+}-release resulted in signals which in their intensities did not differ much from those obtained in KC (Schlaf et al., 1999). A functional analysis (Schieferdecker et al., 1998) demonstrated a C5a-induced time- and dose-dependent release of thromboxane A2 and of the prostaglandins D2, E2 and F2α. The activation of glycogen phosphorylase in HC was in the same range regardless of whether it depended on prostanoids released by KC or by HSC (see section 2) (Schieferdecker et al., 1998). As HSC are known to play an important role in the genesis of liver fibrosis by expressing components of the extracellular matrix (ECM) in the liver (Schwoegler et al., 1994; Gressner and Bachem, 1995; Pinzani, 1995) we investigated whether the activation of HSC via the anaphylatoxin C5a may influence the development of liver fibrosis (unpublished results). The investigations turned out to be difficult, as isolated HSC in culture upregulated all monitored ECM-molecules (entactin, collagen IV, fibronectin) as well as the structure protein smooth muscle actin (SMA) during their conversion in vitro from HSC to myofibroblast-like cells as a consequence of their isolation. The profibrotic cytokine TGF-ß1 increased the mRNA expression of all four investigated proteins and was, therefore, used as a universal positive control. For the stimulation of HSC by TGF-ß1 an adequate incubation period was chosen to demonstrate stimulus-mediated effects which could clearly be

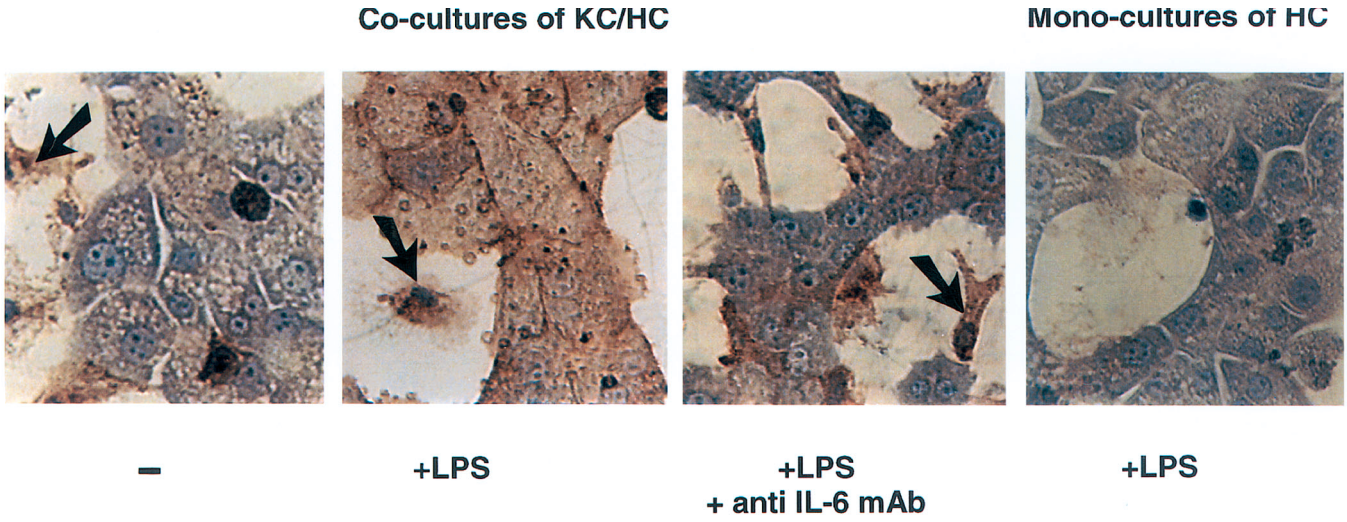

Fig. 4. IL-6 -mediated induction of C5aR expression in rat co-cultures of hepatocytes and Kupffer cells. Cells were co-cultured for at least 48 hours and then stimulated for 24 hours with 1 μg/ml LPS in the absence or presence of an inhibitory anti-rat IL-6 polyclonal antibody. Cells were fixed with paraformaldehyde, incubated with the anti-rC5aR mAb R63, followed by a biotinylated goat-anti-mouse IgG secondary antibody and labeled by incubation with peroxidase-conjugated streptavidin. In non-stimulated co-cultures (-) only Kupffer cells (arrows) appear as stained brown spots whereas in co-cultures treated with LPS (+LPS) both KC and HC are stained. The LPS-dependent induction of the C5aR on HC is reduced in the presence of an inhibitory anti-rat IL-6 polyclonal antibody (+LPS/+anti IL-6 mAb), i.e. only Kupffer cells are stained. No staining is visible in mono-cultures of HC after their in-vitro stimulation with LPS (for details, see Ref. Koleva/Schlaf et al., 2002). x 300

distinguished from the lesser conversion-mediated upregulation of the ECM proteins. This was best achieved after the reexpression of the C5aR after 24h of culture. The cells were then stimulated for 24h or 48h, a time at which the upregulation of the proteins due to the isolation and cultivation had not yet reached a high level. Using rrC5a at a concentration of 1 μM (10 μg/ml) or 100 nM (1 μg/ml), respectively, fibronectin-specific mRNA was clearly upregulated, whereas the specific amplificates of entactin, collagen IV and SMA were hardly affected when compared to the conversion-mediated upregulation. As the C5a-mediated effect on the expression of fibronectin could be blocked by the anti-C5a mAb 6-9F (Rothermel et al., 2000) the effect was specific for C5a. The upregulation of fibronectin-specific mRNA was about five-fold as shown by quantitative-competitive PCR using five-fold dilution steps of the competitor.

Because of the finding that only one of the investigated ECM proteins was upregulated by C5a we investigated its possible indirect effect via the upregulation of TGF-ß1 in KC and HSC both of which constitutively express this profibrotic cytokine. However, a C5a-mediated effect on the amount of mRNA specific for TGF-ß1 was neither observed in HSC nor in KC. KC constitutively express nearly ten times as much TGF-ß1 as do HSC (Armendariz-Borunda et al., 1993; De Bleser et al., 1997). This proves that TGF-ß1 is neither upregulated by C5a in HSC, which could result in an autocrine upregulation of ECM-molecules, nor in KC, which would result in a paracrine upregulation. Taken together, C5a seems to have only a limited role in the induction of liver fibrosis as only one of the four investigated fibrosis-relevant molecules was directly upregulated by it and C5a had no effect on the expression of TGF-ß1 in KC and HSC. Further investigations will have to be performed to define novel functions of C5a-stimulated HSC and to analyze possible co-factor functions of C5a in profibrotic processes.

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