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Upregulation of fibronectin but not of entactin, collagen IV and smooth muscle actin by anaphylatoxin C5a in rat hepatic stellate cells

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Summary. Rat Kupffer cells (KC), hepatic stellate cells (HSC) and sinusoidal endothelial cells (SEC) all express the C5a receptor (C5aR) constitutively in contrast to hepatocytes (HC). HSC showed an unexpectedly high level of expression of the C5aR. As these cells are known to play a key role in the induction of liver fibrosis we hypothesized that C5a may possibly induce fibrogenetic proteins in these cells. HSC are known to express the extracellular matrix (ECM) proteins collagen IV, fibronectin, entactin and the structure protein smooth muscle actin (SMA) which is regarded as a marker for the fibrotic conversion of HSC to myofibroblast-like cells. We investigated the effect of recombinant rat C5a (rrC5a) on the upregulation of these ECM-proteins and of SMA, all of which are known to be expressed by HSC. The profibrotic cytokine TGF-ß1 (2 ng/ml), which was used as a control, clearly upregulated the three matrix proteins but not SMA. In the absence of any stimulus HSC upregulated the three ECM-proteins as well as SMA during their conversion into myofibroblast-like cells. This resulted in a high stimulus-independent plateau of the mRNA expressions for all four proteins after four to five days of culture. Readouts were therefore taken at 72 h after the isolation of the HSC when the investigated mRNA levels had not yet reached their maxima due to the conversion of the cells. The first 24 h of culture were performed without stimulus and the following 48 h in the presence of 100 nM rrC5a (1 µg/ml) or TGF-ß1 (2 ng/ml). Only fibronectin-specific mRNA was clearly upregulated by C5a whereas entactin, collagen IV and SMA were not affected by C5a. By competitive-quantitative PCR the upregulation of fibronectin-specific mRNA was determined to be about five-fold. As TGF-ß1 upregulated all of the three investigated ECM-proteins but not SMA it was checked as to whether C5a might act indirectly by upregulating the expression of TGF-ß1 in KC and HSC, as both cell types are known to be sources of this profibrotic cytokine. However, using RT-PCR, such an effect was not detectable in either cell type after 3, 10 or 24 h.

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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 alpha-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 C5 anaphylatoxins are cationic glycoproteins that function as mediators of the inflammatory response. C5a increases the vascular permeability and mediates chemotactic migration of monocytes and neutrophilic granulocytes (Goldstein, 1988; Hugli and Morgan, 1996). Furthermore, it causes the production of reactive oxygen species in phagocytes, induces the release of histamin and of lysosomal contents from myeloid cells and the contraction of smooth muscle cells. All these effects are mediated by the binding of the C5a-ligand to its receptor (C5aR/CD88) which was first identified on the surface of myeloid cells like human neutrophils (Chenoweth and Hugli, 1978) 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 superfamily of G-protein-coupled rhodopsin-like receptors with seven transmembrane domains. The coding sequences of the receptors of man (Gerard and Gerard, 1991), 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 (Gerard and Gerard, 1991) first allowed the generation of specific antibodies against the human C5aR (Morgan et al., 1993; Oppermann et al.,

1993). These antibodies were used in immunohisto- and immunocytochemical investigations to demonstrate that the expression which had first been believed to be restricted to myeloid cells was also detectable on non-myeloid cells. The number of non-myeloid cells which have been reported to express the C5aR has increased from year to year (Haviland et al., 1995; McCoy et al., 1995; Floreani et al., 1998; Fayyazzi et al., 2000; Drouin et al., 2001). Investigations on the distribution of the C5aR on liver cells yielded controversial results. Haviland and coworkers reported a constitutive expression of C5aR in the human hepatoma cell line HepG2 and hepatocytes (HC) of human liver slices by in-situ hybridization and immunohistochemical analyses using polyclonal antibodies. These data on a constitutive expression on human HC are not in accord with later studies on rat liver cells (Schieferdecker et al., 1997; Schlaf et al., 1999, 2003) and those of human liver tissue slices (Zwirner et al., 1999; Fayyazzi et al., 2000) all of which were performed with specific monoclonal antibodies (mAb). These studies showed that primary HC of human and rat liver do not express the C5aR constitutively. Using primary HC isolated according to the enzyme-free method of Meredith (Meredith, 1988) Schieferdecker et al. (1997) first showed that HC lack C5aR-specific mRNA by RT-PCR analyses. Furthermore, they showed that Kupffer cells (KC) expressed about ten-fold more C5aR-specific mRNA than hepatic stellate cells (HSC), and that these cells showed nearly ten-fold more than sinusoidal endothelial cells (SEC), as demonstrated by quantitative-competitive RT-PCR. These mRNA data of Schieferdecker and coworkers were later confirmed immunochemically with respect to the receptor protein using the newly generated monoclonal antibody R63 against the rat C5aR and C5a-induced intracellular Ca^{2+}-release (Schlaf et al., 1999). Both studies showed an unexpectedly high level of expression of C5aR on HSC. The investigations on the C5aR-mediated intracellular Ca^{2+}-release in HSC resulted in signals which did not differ much from those obtained in KC in their intensities (Schlaf et al., 1999). A functional analysis demonstrated a C5a-induced time- and dose-dependent release of thromboxane A2 and of the prostaglandins D2, E2 and F2a in HSC (Schieferdecker et al., 1998). Even though HSC released far less prostanoids after stimulation with C5a than KC their levels were sufficient to activate glycogen phosphorylase in HC to a similar extent (Schieferdecker et al., 1998). As HSC are known to play an important role in the genesis of liver fibrosis by an increased and altered expression of components of the extracellular matrix (ECM) in the liver (Schwoegler et al., 1994; Gressner and Bachem, 1995; Pinzani, 1995) in the present study we have investigated whether the anaphylatoxin C5a may directly influence the development of liver fibrosis by the upregulation of fibrosis-relevant proteins or indirectly by the upregulation of TGF-ß1 in KC and/or HSC.

Materials and methods

Animals, recombinant rat C5a (rrC5a), recombinant TGF-ß1 and anti-fibronectin polyclonal antibody

Male Wistar rats weighing between 500g and 800g were used for the isolation of HSC. They were kept on a 12 hour day/night rhythm with free access to water and a standard rat diet from Ssniff (Soest, Germany). Treatment of the animals followed the German laws for the protection of animals.

Recombinant rat C5a (rrC5a) was cloned, expressed and purified using the Qiagen express system (Qiagen, Hilden, Germany) as described in detail by Rothermel et al. (1997a). For its purification a 6 histidine Ni^{2+}-chelate-binding domain as part of a 12 amino-acid tag [MRGSHHHHHHGS] was placed at the amino terminus by subcloning the C5a sequence into the pQE 30 vector. Purification of rrC5a from the bacterial lysate was performed using Ni^{2+} chelate chromatography followed by renaturation in 0.1 M Tris/HCl, pH 8.0; 0.005% Tween 80; 2 mM reduced gluthatione; 0.2 mM oxidyzed gluthatione. After removing the precipitated protein by centrifugation the yield was about 10 mg of protein per liter of bacterial culture. The recombinant protein was purified by FPLC cation exchange chromatography on a Mono-S column (Amersham-Pharmacia, Freiburg, Germany). The protein was homogeneous, as was demonstrated by SDS-PAGE (silver staining), and was found to be free of endotoxin, as tested by limulus assay (Sigma, Taufkirchen, Germany).

The recombinant human TGF-ß1, which was used in this study at a concentration of 2 ng/ml, was from R+D Systems (Wiesbaden, Germany). It was expressed in Chinese Hamster Ovary Cells (CHO cells) and is also active in rodents.

Sheep anti-human fibronectin polyclonal antibody, which shows species cross-reactivity with rat fibronectin, was from Serotec (Düsseldorf, Germany).

Preparation of HSC and KC

HSC were prepared using an enzymatic digestion procedure originally developed by Kawada et al. (1993) with modifications according to Schieferdecker et al. (1997). The resulting cell suspension was filtered through a nylon gauze (diameter 60 µm) and afterwards centrifuged at 450xg for 10 min (all steps were performed at 4 °C). The cell pellet was washed twice and then resuspended in Hank's Balanced Salt Solution (HBSS), which had been mixed with Lymphoprep (final concentration 8.13%) (Nycodenz, Oslo, Norway). The cell suspension was overlaid with HBSS. HSC were taken from the interphase after a centrifugation step at 1,400xg for 20 min. The purity of HSC was between 96% and 98%, as determined by the typical light microscopic appearance and by the immunocyto-chemical staining of the intermediary filament desmin with the mAb D33 (Serotec, Düsseldorf, Germany).

KC were prepared by a combined collagenase/ pronase perfusion and were purified by Nycodenz density gradient centrifugation and subsequent centrifugal elutriation using a Beckman JE-6 elutriation rotor in a J-21 Beckman centrifuge (Eyhorn et al., 1988).

Reverse PCR amplification (RT-PCR)

Messenger mRNA from HSC and KC was prepared with the RNeasy total kit (Qiagen, Hilden, Germany). The subsequent transcription into cDNA was performed using the SuperScript Preamplification system (Invitrogen, Karlsruhe, Germany). PCRs for the detection of the mRNA (cDNA-) samples of the ECM molecules were carried out with the following combination of primers: The primer "collagen IV sense" was (5'-CAC ACG TCA CAG TCA AAC AAC-3') and "collagen IV antisense" (5'-ATG CAT ACT TGA CAT CGG CTA-3') resulting in an amplificate of 653 nucleotides. The primer "entactin sense" was (5'-TTC CTG GCT GAC TTG GAC ACA-3') and "entactin antisense" was (5'-CCT AGG TAC ATT GTG TGG ATC-3') resulting in an amplificate of 715 nucleotides. The combination of primers for fibronectin was "fibronectin sense" (5'-GGA CAT CTG TGG TGT AGC ACA-3') and "fibronectin antisense" (5'-GTC TCC ACC TGA GAA TGT ACT-3') resulting in an amplificate of 756 nucleotides. For the structure protein smooth muscle actin (SMA) the primers were "SMA sense" (5'-TGT GCT GGA CTC TGG AGA TG-3') and "SMA antisense" (5'-GAT CAC CTG CCC ATC AGG-3') resulting in an amplificate of 292 nucleotides. The mRNA for TGF-ß1 was monitored using "TGF-ß1 sense" (5-CCT GCT GGC AAT AGC TTC CTA-3') and "TGF-ß1 anti-sense" (5'-TGC TTC CCG AAT GTC TGA CGT-3') which resulted in an amplificate of 711 nucleotides. As a control for each RT-PCR assay a primer pair was used to amplify rat ß-actin cDNA (769 nucleotides). The sense primer was 5'-GAT ATC GCT GCG CTC GTC GTC-3' and the antisense primer 5'-CCT CGG GGC ATC GGA ACC-3'. PCR assays were performed using Taq-polymerase (Red Taq, Sigma, Taufkirchen, Germany) with denaturing at 94 °C for 1 min, annealing at 56 °C for 1 min (59 °C for the collagen IV-specific primers), and extension at 72 °C for 1.5 min in 38 cycles (conventional semiquantitative PCR) or 35 cycles (quantitative-competitive PCR). For control purposes all amplified cDNA samples were partialy sequenced using the dideoxy chain termination method. All of the amplificates were identified to be part of their specific cDNA sequences.

Quantitative-competitive PCR

For quantitative-competitive PCR a constant amount of cDNA from HSC, without or after stimulation, was co-amplified with an external lambda-phage DNA standard of 500 nucleotides (TaKaRa Biochemicals, Tokyo, Japan) flanked by chimeric segments. Co-amplification using combined primers was performed at five-fold dilution steps of the standard under the conditions given above. Afterwards amplification PCR products were separated in 1.5% agarose gels. Bands were visualized by ethidium bromide staining and amplificates of standard lambda-DNA and fibronectin were checked for equal staining intensity.

Immunoblot analysis

Supernatants of HSC, which contained 70 µg of protein, were precipitated with TCA from supernatants of stimulated and unstimulated HSC. As a standard 250 ng bovine fibronectin was chosen. The precipitated samples and the standard protein fibronectin were boiled in SDS sample buffer under reducing conditions and applied to an SDS-minigel system (Biometra, Göttingen, Germany). The separated samples were transferred onto a nitrocellulose sheet under a current of 150 mA for 2 hours using a semi-dry transfer chamber (Multiphor-Novablot, Amersham-Pharmacia, Freiburg, Germany). The nitrocellulose sheet was blocked with 2% BSA (30 min) and incubated with a sheep anti-human fibronectin pAb which cross- reacts with rat fibronectin (Serotec, Düsseldorf, Germany) for 2 h. The secondary antibody, peroxidase-conjugated donkey anti-sheep IgG (Dianova, Hamburg, Germany) was used at a concentration of 1:4000 for 2 h. The color reaction was performed with diaminobenzidine (Sigma, Aidenbach, Germany) and H_2O_2.

Results

Immunochemical detection of the C5aR and C5a-induced Ca^{2+}-release in HSC

With the exception of HC of normal rat liver the other three main cell types, i.e. KC, HSC and SEC express the C5aR in decreasing amounts, as shown by mRNA determinations (Schieferdecker et al., 1997) and by immunochemical methods (Schlaf et al., 1999). The data were supported by the demonstration of an intracellular Ca^{2+}-release induced by rrC5a. However, in this previous study (Schlaf et al., 1999) recombinant rat C5a was used at a concentration of 250 nM which, most probably, was unphysiologically high and therefore resulted in untypical Ca^{2+}-release-kinetics. The maximum was lower than expected and the fall of the signal after having reached its maximum was about 3 min, i.e. quite slow. We therefore repeated the Ca^{2+}-release experiments with ten-fold lower concentrations of the ligand i.e. with 25 nM rrC5a. As shown in figure 1A a stimulus with 25 nM rrC5a resulted in a signal with a higher maximum than that obtained with a stimulus of 250 nM rrC5a. A complete reduction of the signal was monitored in no more than 45 seconds. As C5a is known to stick to tissue culture plates and our aim was to provide this stimulus at a sufficient but not supraphysiological concentration we used it at

concentrations of 100 nM (1 µg/ml) in all the following experiments. The use of this concentration for the stimulation experiments of 24 h or 48h was supported by the study of Solomkin et al. (1981) who had reported increased C5a serum levels of > 100 ng/ml during sepsis. The dose-response experiments were accompanied by studies which confirmed the activity of the rrC5a-preparation used in this study by an assay which resulted in the ligand-mediated release of the lysosomal enzyme N-acetyl-glucosaminidase (NAGA) (Rothermel et al., 2000) in C5aR-transfected RBL cells (not shown). A plateau value of the NAGA-release was reached at a C5a concentration of 40 nM and higher. These experiments additionally suggested 100 nM rrC5a to be an adequate concentration for stimulation periods of 48h and longer. In accordance with Schlaf et al. (1999) the anti-C5aR mAb R63 clearly identified the receptors on the surface of HSC (Fig. 1B). To demonstrate the homogeneity of the population the cells were labeled with the anti-desmin mAb D33. Among the liver cells only HSC

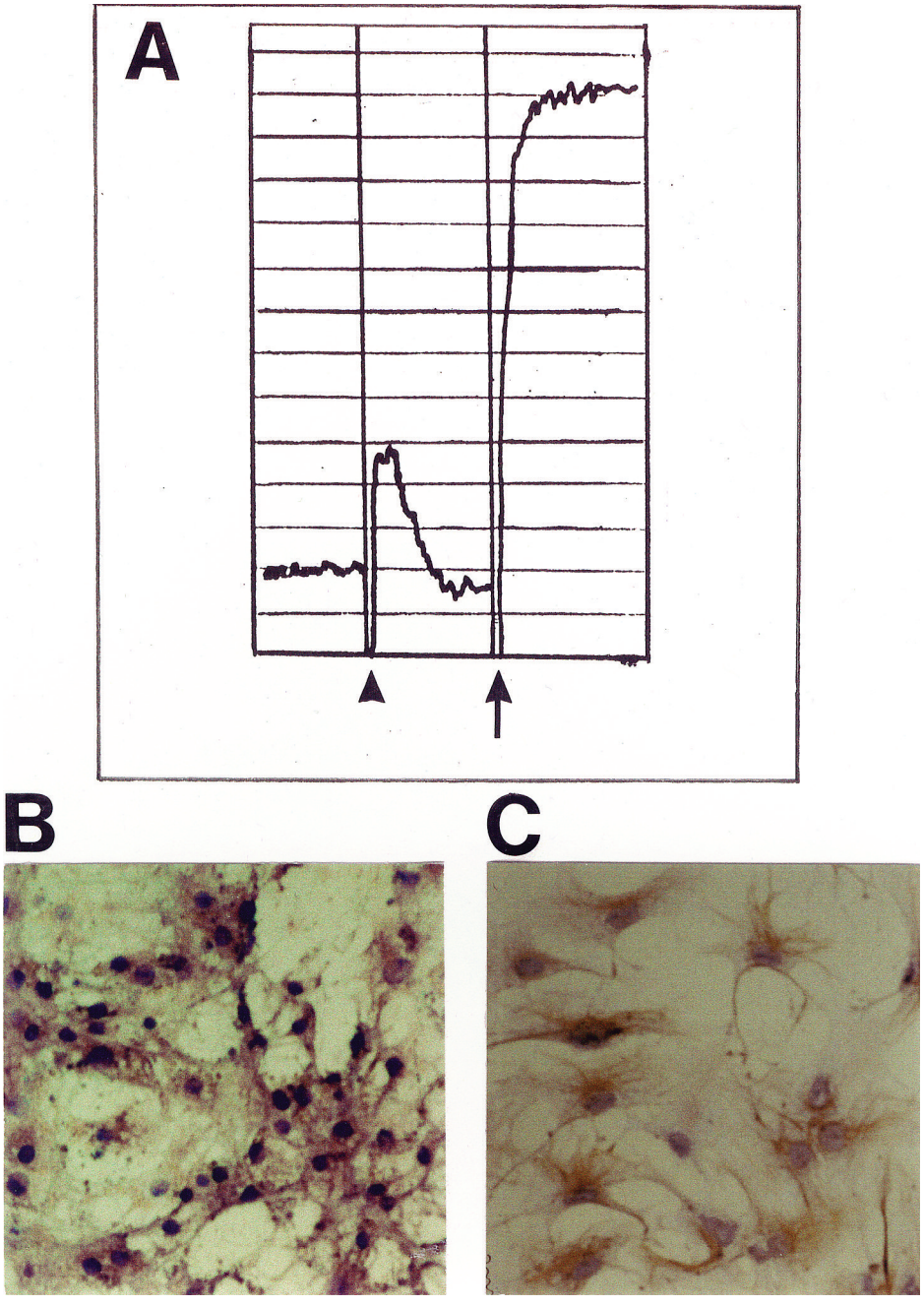

Fig. 1. A. Intracellular release of Ca^{2+} by rrC5a in HSC.Recombinant rat C5a was used at a final concentration of 25 nM. The arrowhead indicates the application of the C5a ligand and the arrow the response to Triton X-100-mediated lysis of the cells. **B, C.** Immunocytochemical staining of isolated HSC. HSC are stained extracellularly with the anti-C5aR mAb R63 **(B)** or intracellularly with the anti-desmin mAb D33 **(C)** after permeabilization of the cells with acetone /methanol. B, x 300; C, x 400

expressed this intermediary filament (Fig. 1C). By microscopic analyses using the intracellular desmin staining the population was determined to consist of at least 96% of HSC.

Transforming growth factor beta 1 (TGF-ß1) upregulates the extracellular matrix proteins collagen IV, entactin, fibronectin but not the structure protein smooth muscle actin

FACS analyses of HSC had shown that after the enzymatic isolation procedure the C5aR was completely reexpressed after a cultivation period of 24 h. There was no recognizable increase in the expression of the receptor by culturing the HSC for a further 24 h (data not shown). In agreement with previous studies (Knittel et al., 1996) the conversion of HSC into myofibroblast-like cells, which results from the isolation of these cells, was accompanied by an enhanced expression of the structure protein smooth muscle actin (SMA) and of the extracellular matrix proteins collagen IV, entactin and fibronectin. Therefore, stimulation experiments were performed to determine the times of cultivation and stimulation which are required to demonstrate the maximal difference in the mRNA expression of the investigated extracellular matrix proteins collagen IV, entactin, fibronectin and the structure protein smooth muscle actin in unstimulated and stimulated cells. The profibrotic cytokine TGF-ß, which is known to upregulate the expression of ECM-proteins, was used as a positive control at a concentration of 2 ng/ml. As shown in figure 2 the conversion into the myofibroblast-

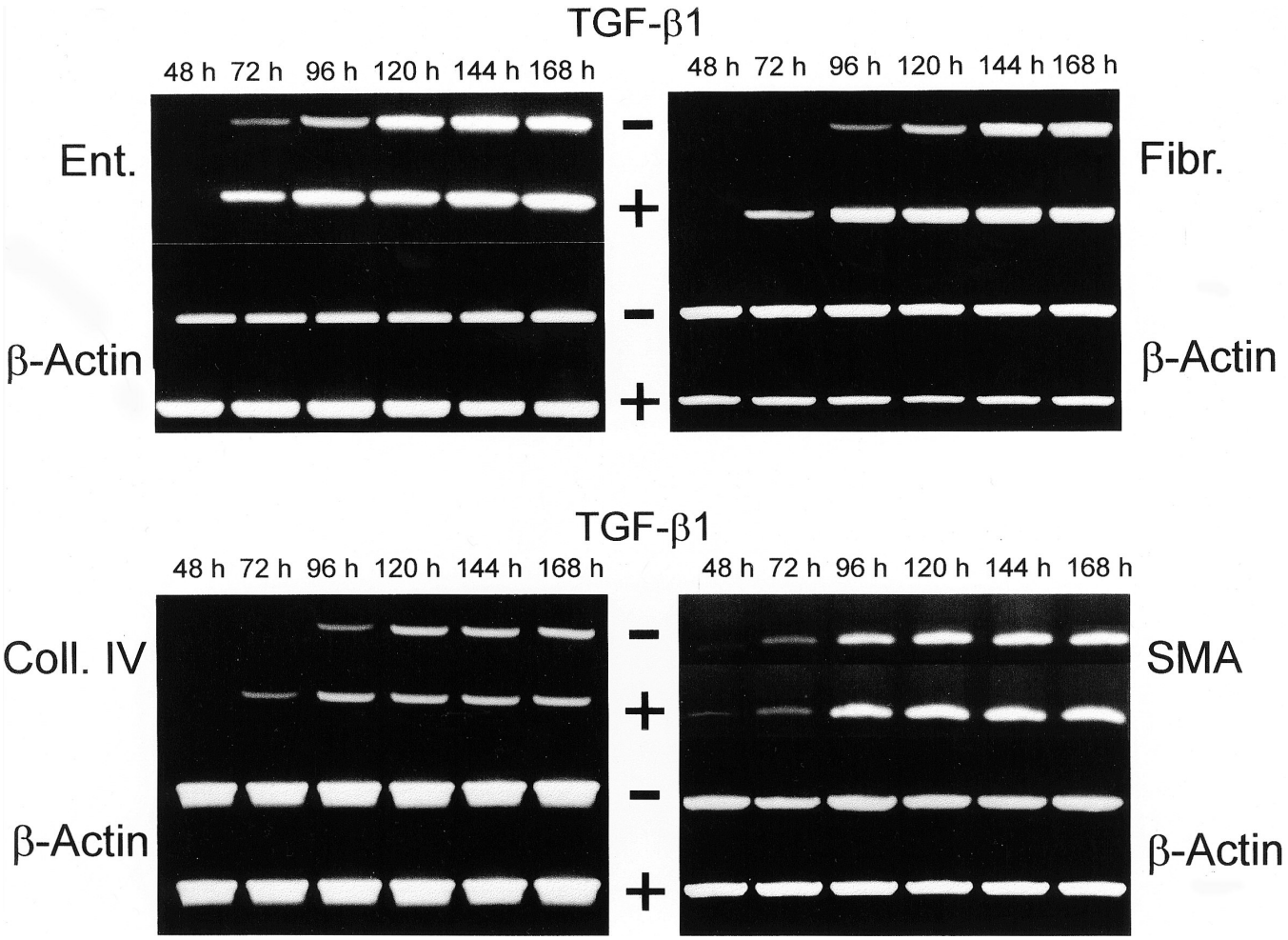

Fig. 2. Upregulation of entactin-, fibronectin-, collagen IV- and smooth muscle actin-specific mRNA with and without stimulation of HSC by TGF-ß1 as demonstrated by RT-PCR analysis. HSC are cultured for 24h to allow the reexpression of the C5aR and then for various time periods without (-) and with (+) stimulation of the cells by TGF-ß1 (2 ng/ml). The time spans given above include the initial 24 h without any stimulation which allowed for the reexpression of cell surface molecules. The specific amplificates of entactin, fibronectin and collagen IV are augmented by TGF-ß1 as is visible after 72 h or 96 h of cultivation (i.e. 48 h or 72 h of stimulation with TGF-ß1) whereas the specific amplificate of smooth muscle actin is not affected. The corresponding control amplificates of ß-actin -specific cDNA show equal stainings.

like phenotype resulted in the upregulation of the mRNA specimens of all three ECM-components and of SMA. The intensities of the specific amplificates reached a maximum after a cultivation period of 120 h. There was no further increase during an additional 48 h cultivation period (Fig. 2). HSC which were cultured in the presence of TGF-ß1 (2 ng/ml) after an initial 24 h of culture to allow for the reexpression of the cell surface molecules showed a stronger expression of the mRNA specimens of the ECM molecules at 72 h of cultivation (48 h of stimulation). The level of maximal entactin-specific mRNA expression was already reached after 96 h of cultivation (72 h of stimulation) i.e. 24 h earlier than in the unstimulated cells.

ß-actin-specific mRNA control amplificates did not change, whether they were taken from unstimulated or stimulated cells during cultivation periods between 48 h and 168 h. The specific amplificates of the cDNA specimens of fibronectin and of collagen IV showed similar developments in their intensities. Both amplificates became visible after 96 h of cultivation of HSC whereas after stimulation with TGF-ß1 the specific mRNA specimens were already detectable after 72 h of cultivation (48 h of stimulation). The plateau of both the amplificates of collagen IV and entactin were reached 24 h earlier in stimulated (after 96 h) than in unstimulated HSC (after 120 h). The SMA-specific amplificate became detectable after 48 h of cultivation (24 h of stimulation) in unstimulated and TGF-ß1-stimulated cells. In both stimulated and unstimulated cells a cultivation time of 96 h was necessary to reach plateau levels. In contrast to its accelerating effect on the mRNA-expression of the three ECM-molecules entactin, fibronectin and collagen IV, TGF-ß1 did not affect the SMA-specific mRNA expression. No control amplificates of ß-actin cDNA differed in their intensities

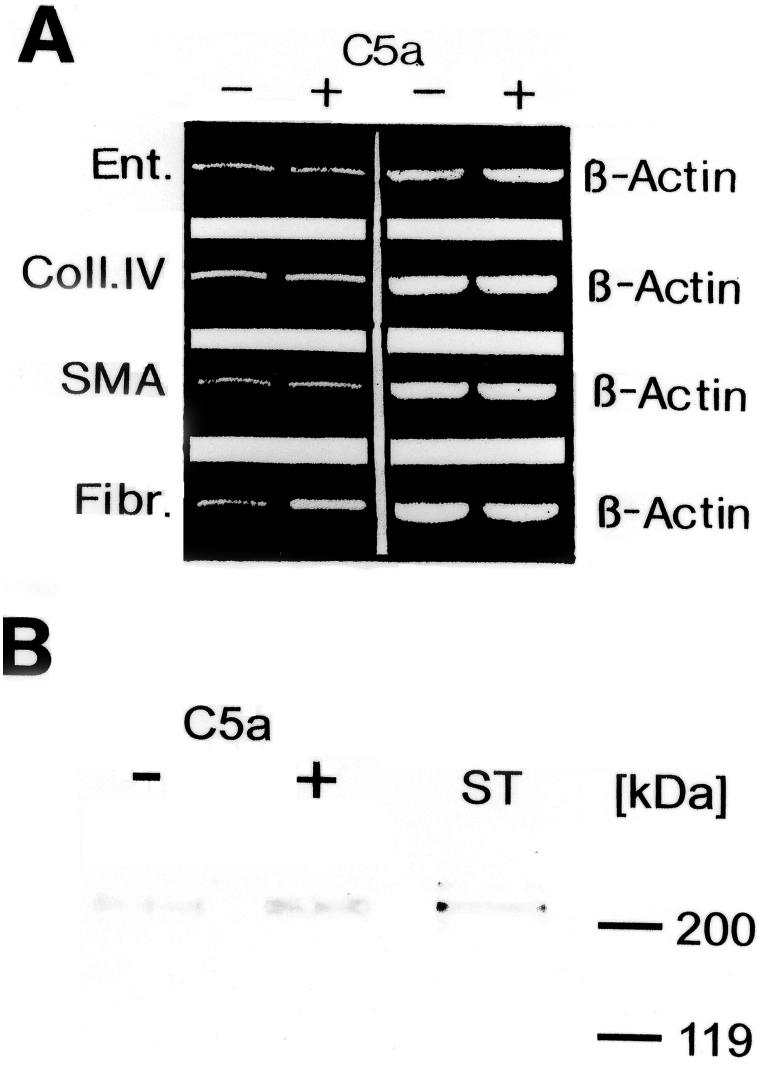

Fig. 3. A. Upregulation of fibronectin-specific mRNA but not of the mRNA for entactin, collagen IV or for smooth muscle actin by stimulation of the cells with rrC5a as demonstrated by RT-PCR analysis. HSC are initially cultured for 24 h to allow the reexpression of the receptor and afterwards stimulated for 48 h with 100 nM rrC5a. Only the fibronectin-specific amplificate (Fibr) is upregulated whereas the other amplificates including the ß-actin control do not show changes in their intensities. **B.** Upregulation of fibronectin protein in the supernatants of HSC without (-) and after (+) stimulation of the cells with 100 nM C5a for 48 h as demonstrated by semi-quantitative immunoblot analysis. The fibronectin-specific band in the range of 210 kDa (under reducing conditions) deriving from 70 µg of precipitated protein is clearly increased after stimulation with C5a. As a standard protein (ST) purified bovine fibronectin (250 ng) is applied to the gel.

after cultivation periods from 48 h to 168 h, or between stimulated and unstimulated cells. Thus, the stimulation of HSC with TGF-ß1 clearly upregulated the specific mRNA specimens of the three ECM-molecules collagen IV, entactin and fibronectin. However, the mRNA of the three ECM-proteins as well as of SMA were already upregulated without any stimulation and reached plateau levels after 96 to 120 h of cultivation. A potential regulatory effect of C5a thus had to be investigated during the first 72 h of cultivation (48 h of stimulation) as it should not completely be covered by the time-dependent conversion of the HSC into myofibroblast-like cells. In the following experiments HSC were therefore stimulated for 48 h using 100 nM (1µg/ml) rrC5a after an initial cultivation period of 24 h.

Fibronectin, but not collagen IV, entactin or smooth muscle actin is upregulated by C5a

As shown in figure 3 only fibronectin-specific mRNA was increased by the stimulation of HSC with 100 nM rrC5a. The amplificates of collagen IV, entactin and of the structure protein smooth muscle actin were not changed by the stimulation of the HSC for 48 h, as the intensities of the specific amplificates derived from unstimulated and stimulated cells were not different (Fig. 3A). The C5a-mediated upregulation of fibronectin-specific mRNA was also demonstrable on the protein level as the signal of about 210 kDa derived from precipitated supernatants of HSC was stronger after a stimulation of these cells for 48 h with 100 nM C5a (Fig. 3B). Furthermore, stimulations using various concentrations of rrC5a (100 nM, 1 µM and 10 µM) showed that concentrations of 1 µM or 10 µM C5a did not further enhance the effect of 100 nM C5a (Fig. 4). The specifity of the C5a-mediated effect on the fibronectin-specific mRNA expression was demonstrated by a functional blockade of the C5a molecules using a 15-fold molar excess of the inhibitory mAb 6-9F for 1 h. A 16-fold molar excess of this mAb had formerly been shown to completely block the C5a-dependent Ca^{2+}-release and a twenty-fold molar excess to completely block a release of NAGA (Rothermel et al., 2000). The ß-actin-specific control amplificates did not differ (Fig. 4). These control experiments confirmed that the upregulation of fibronectin-specific mRNA was indeed specific for C5a.

Quantification of the upregulation of fibronectin-specific mRNA

In order to quantify the upregulation of fibronectin-specific mRNA more precisely a competitive RT-PCR assay was established with cDNA derived from HSC before and after stimulation with 100 nM rrC5a. The PCR assay was performed in the presence of a lambda-DNA standard of 500 nucleotides which was 256 nucleotides shorter than the fibronectin-specific amplificate. When equal amounts of cDNA were co-amplified with decreasing amounts of the lambda-DNA they both competed for the oligonucleotide primers in the reaction mixture. Both products were amplified to equal intensities when the templates were present in equal amounts. As shown in figure 5 the equilibrium between fibronectin-specific cDNA (upper lanes) and competitor DNA (lower lanes), which in unstimulated HSC is in lane 3, shifted after treatment with 100 nM rrC5a to lane 2, i.e., by a factor of close to five as the competitor was used at five-fold dilutions. As a control ß-actin-specific amplificates of stimulated and unstimulated HSC were also analysed. They showed equal intensities (data not shown). As the aim of this assay was only to determine the degree by which fibronectin-specific mRNA was upregulated and not to

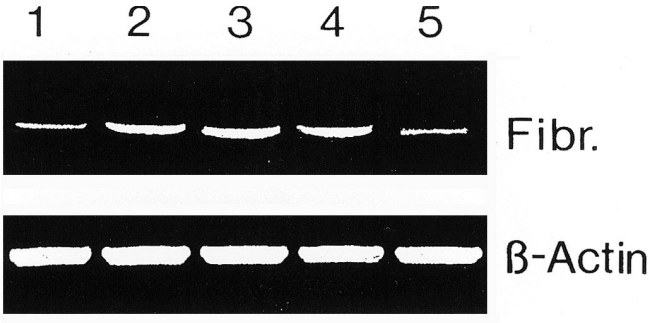

Fig. 4. Upregulation of fibronectin-specific mRNA after stimulation of HSC with various concentrations of rrC5a and with C5a after treatment with the blocking anti-C5a mAb 6-9F, as demonstrated by RT-PCR analysis. HSC are not stimulated (lane 1) or are stimulated with 100 nM rrC5a (lane 2), 1 µM rrC5a (lane 3) or 10 µM rrC5a (lane 4) for 48 h after initial cultivation of the cells for 24h for the reexpression of the receptor. Recombinant rat C5a pretreated with a 15-fold molar excess of the anti-rat C5a mAb 6-9F for 1 h reduces the intensity of the amplificate to that of unstimulated cells (lane 5). The corresponding ß-actin-specific control amplificates show equal staining intensities.

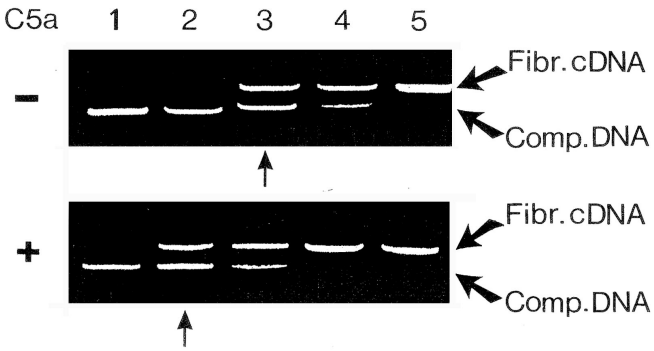

Fig. 5. Quantification of the upregulation of fibronectin-specific mRNA by quantitative-competitve PCR. Equal amounts of fibronectin-specific cDNA are co-amplified with five-fold dilutions of a λ-standard DNA. The arrows denote the points of near equimolarity and indicate that the fibronectin-specific mRNA expression between unstimulated and C5a-stimulated HSC is upregulated by a factor of close to five.

compare absolute molarities of standard lambda DNA and fibronectin-specific cDNA with and without stimulation only the lanes with equal intensities of both amplificates were compared.

C5a does not upregulate the profibrotic cytokine TGF-ß1 in KC or HSC

As C5a upregulated only one of the three ECM-proteins under investigation the question arose as to whether this anaphylatoxin might upregulate the synthesis of the profibrotic cytokine TGF-ß1 which is known to upregulate ECM-proteins and, therefore, served as a universal positive control in these investigations (Fig. 2). An upregulation of TGF-ß1 upon stimulation with C5a would result in the indirect upregulation of the ECM-components of the HSC. It is known that both KC and HSC are sources of this profibrotic cytokine (Armendariz-Borunda et al., 1993). For this reason both cell types were investigated for a possible C5a-induced upregulation of TGF-ß1. Both cell types, i.e. isolated HSC, which, according to FACS-analyses maximally reexpress the receptors for C5a after an initial cultivation period of 24 h and KC which completely reexpress the receptors after an initial cultivation of 48 h (data not shown), were subsequently stimulated for 3 h, 6 h, 10 h and 24 h, respectively. TGF-ß1-specific cDNA was amplified and specific amplificates from unstimulated and stimulated cells were compared. There were no differences in the intensities of the TGF-ß1 -specific amplificates in unstimulated and stimulated HSC or KC at any of the chosen time points after stimulation, as shown with the example of TGF-ß1 -specific amplificates of HSC and KC after 10 h of stimulation (Fig. 6). However, in accord with published data (Armendariz-Borunda et al., 1993), the level of TGF-ß1 -specific mRNA expression was at least five-fold higher in KC than in HSC when equal amounts of isolated RNA were reversely transcribed. Thus, TGF-ß1-specific mRNA is not upregulated by C5a in either cell type.

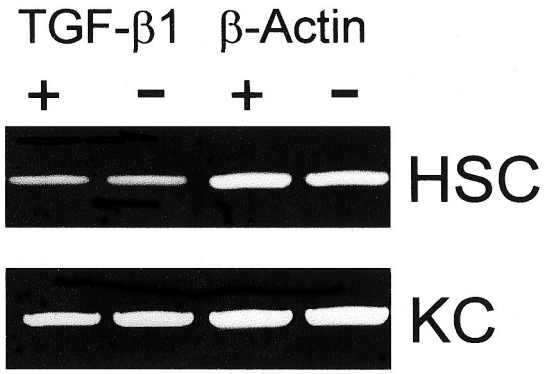

Fig. 6. Monitoring of the TGF-ß1 -specific mRNA expression after (+) and without (-) stimulation of HSC and KC with 100 nM rrC5a for 10h. HSC are cultivated for an initial 24 h and KC for an initial 48 h for the complete reexpression of their C5a receptors. The TGF-ß1 -specific amplificates do not show increases in their intensities in HSC and KC after stimulation with rrC5a (TGF-ß1, +). The corresponding ß-actin-specific control amplificates show equal intensities.

Discussion

The expression of C5aR in rat HSC was first shown by RT-PCR assays (Schieferdecker et al., 1997). These data were later confirmed by immunochemical analyses using a newly generated anti-rat C5aR mAb (Schlaf et al., 1999; Rothermel et al., 2000) as well as functional analyses using C5a-mediated Ca^{2+}-release assays. These functional assays suggested an unexpectedly high level of expression of C5aR in HSC. Furthermore, it was shown that HSC were involved in the C5a-dependent increase in glucose output from HC in perfused rat livers (Püschel et al., 1996) although HC do not express C5aR under normal conditions (Schieferdecker et al., 1997; Schlaf et al., 1999, 2003). This effect of C5a on HC was mediated by prostanoids, the main source of which in the liver are KC (Püschel et al., 1996). 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 but not completely interrupted (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). In experiments with co-cultures of isolated cells the activation of glycogen phosphorylase in HC via prostanoids as a response to C5a was in the same range regardless of whether they were released from KC or from HSC although the prostanoids (thromboxane A_2 and prostaglandins D_2, E_2 and $F_{2\alpha}$) were released in different amounts and ratios (Schieferdecker et al., 1999). Thus, it seemed unlikely that the C5a-mediated prostanoid release and the resulting activation of glycogen phosphorylase in HC are the only C5a-mediated effects in HSC. As HSC are known to play an important role in the genesis of liver fibrosis by expressing components of the ECM (Schwoegler et al., 1994; Gressner and Bachem, 1995; Pinzani, 1995) we investigated whether the activation of HSC by the anaphylatoxin C5a might influence the development of liver fibrosis. These investigations turned out to be difficult as isolated HSC in culture upregulated all of the monitored ECM-molecules (entactin, collagen IV, fibronectin) as well as the structure protein SMA during their in-vitro conversion from HSC into myofibroblast-like cells. In this study the profibrotic cytokine TGF-ß1 was used as a universal positive control as it upregulated the specific mRNA specimens of the three ECM-molecules in cultivated HSC. In these experiments an adequate incubation period was chosen which allowed the differentiation of stimulus-mediated effects from

those induced by conversion processes, i.e. development of the myofibroblast-like cell type. This was best achieved by a stimulation of 48 h after the reexpression of the C5aR, i.e. after 24 h of culture.

To the best of our knowledge this is the first study dealing with a correlation between complement activation and liver fibrosis which is based on an unexpectedly high level of expression of the C5aR on hepatic stellate cells. In this context, in a recent study Hillebrandt et al. (2002) used a completely different approach, the genetic quantitative trait loci (QTL) analysis, to identify "fibrogenic genes" that confer genetic susceptibility to hepatic fibrosis in mice. One of the two gene regions which were significantly correlated with an increased susceptibility to liver fibrosis was identified to contain the gene encoding complement component C5. In the fibrosis-resistant murine strains A and AKR this complement gene is known to have a two-base pair deletion in exon 6 which leads to a deficiency of this component and promotes an anti-inflammatory phenotype. The strains BALB/c, C57BL/6 and C3H/He which do not bear the mutated gene and are not deficient in functional C5/C5a display higher levels of hydroxyproline (collagen) (Hillebrand et al., 2002). However, in our study C5a did not display an effect on the level of expression of collagen IV. Most probably, the effect of C5a on the upregulation of fibronectin-specific mRNA which is shown in this study is only one of the complex pathophysiological effects of a C5 deficiency on liver fibrosis. This will have to be analysed in detail in future studies. Our results indicate that C5a may have a role in the induction of liver fibrosis, as one of the four investigated fibrosis-relevant molecules was directly upregulated by it. C5a had no effect on the level of expression of TGF-ß1 in isolated KC or HSC. Whether C5a may be a co-factor for the induction of other fibrogenic proteins will have to be determined.

As there are differences in the expression patterns of some proteins which are expressed in myofibroblast-like cells (protease P 100, α2-macroglobulin, desmin) or in "real" myofibroblasts (fibulin 2, interleukin-6), and only myofibroblasts can be passaged, both cell types cannot be regarded as equal (Knittel et al., 1999) although after five to six days of cultivation their morphologies are very similar (Knittel et al., 1996, 1999). Further investigations will have to be performed to define novel functions of C5a-stimulated HSC (myofibroblast-like cells) or C5a-stimulated "real" myofibroblasts. These cells have so far not been clearly characterized and their isolation will have yet to be established. Whether they contribute to liver fibrosis through their activation of C5a receptors will then have to be analysed.

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