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Expression of a Functional C5a Receptor in Regenerating Hepatocytes and Its Involvement in a Proliferative Signaling Pathway in Rat

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Activation of the complement system generates the anaphylatoxin C5a whose activities are mediated through its binding to the widely expressed C5aR. C5aR mRNA and protein expressions are known to be induced in rat hepatocytes under inflammatory conditions. However, little is known about the role of the C5a/C5aR complex in liver and its involvement during a proliferative process. We have evaluated the expression of C5aR in regenerating rat hepatocytes following a partial hepatectomy and in hepatocyte cultures. C5aR induction was observed in hepatocytes from regenerating liver, as well as in normal hepatocytes under a culture-induced stress. The effect of a stimulation by a C5a agonist upon the synthesis of a growth factor/receptor pair (hepatocyte growth factor/c-Met) was also evaluated. Our data demonstrated an up-regulated expression of hepatocyte growth factor and c-Met mRNAs, but we failed to observe a direct mitogenic effect of C5a in culture. However, a significantly increased expression of cyclin E and D1 mRNA levels, as well as an increased BrdU incorporation, were observed in rats given an i.v. C5a agonist injection following an 80% partial hepatectomy. These studies demonstrate for the first time that: 1) C5aR is up-regulated during liver regeneration, 2) the binding of C5a to C5aR promotes a growth response, and 3) C5aR is involved in a cell cycle signaling pathway. Taken together, these findings point to a novel role for the hepatic C5aR implicating this complement system in the context of normal or abnormal proliferative pathways.

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Abbreviations used in this paper: APP, acute-phase proteins; AU, arbitrary units; HGF, hepatocyte growth factor; MAP-C5a, multiple-associated C5a peptide; PTX, pertussis toxin; qRT-PCR, quantitative RT-PCR; TK, thymidine kinase.
binding of C5a to its hepatic C5aR partner is involved in a proliferative process. Our data bring evidences that the C5a/C5aR complex enhances the proliferative process.

Materials and Methods

Animals

Adult male Sprague Dawley rats (Charles River Laboratories, Les Oncins, France) weighing ~250 g were kept under a 12-h light-dark cycle and fed with a standard laboratory chow and water ad libitum. They were kept at rest for at least 1 wk before any experiment. The animals were treated with human care and under the supervision of authorized investigators and the experiments were performed in compliance with the guidelines of a French law on animal protection.

Reagents

Multiple-associated C5a peptides (MAP-C5a) (a generous gift of Dr. A. Ishchenko, Research Institute of Highly Pure Biopreparations, St. Petersburg, Russia) correspond to the C-terminal part (aa 61–74) of the human anaphylatoxin C5a (eight peptide monomers) attached to a polylysine comb. A rabbit anti-rat C5aR antiserum (L-66) was obtained by immunization with a recombinant fusion protein consisting of GST fused to the 25 N-terminal amino acids of rat C5aR as described previously (15). Ab specificity was checked by cytometry on Chinese hamster ovary cells transiently transfected with a rat C5aR cDNA and by Western blotting on rat spleen and lung extracts. The IgGs were purified from rabbit serum by affinity chromatography using a Poros protein A column (PerSeptive Biosystems, Voisins le Bretonneux, France). An anti-CD26 mAb was purchased from Serotec (Argene Varilhes, France).

Other chemicals (Sigma-Aldrich, St. Louis, MO) were endotoxin (LPS) from Escherichia coli (LPS 011:B4 phenol extract) and pertussis toxin (PTX). They were diluted in a sterile isotonie solution before injection.

Surgical procedures

Under anesthesia, rats were injected i.p. with LPS (2.5 mg/kg) and then sacrificed at 12 h postinjection (this time was chosen from previous studies that exhibited an up- or down-regulated level for many mRNAs levels). Sham surgery (SHAM) was performed in three rats, consisting of median laparotomy and gentle manipulation of the liver lobes. Other rats underwent a 30, 60, or 80% hepatectomy as previously described (16). After a midline laparotomy, 30% hepatectomy consisted of resection of the median lobe, two-third hepatectomy consisted of the resection of the median and left lateral lobes, and the 80% hepatectomy consisted of resection of the median, left, and right lateral lobes of the liver. The animals were sacrificed at 6, 12, 24, or 48 h after hepatectomy (three animals per time point). The liver was removed and either perfused in situ with a collagenase solution to obtain pure hepatocytes (see below), or rapidly frozen in liquid nitrogen to obtain pure hepatocytes (see below), or rapidly frozen in liquid nitrogen.

Hepatocyte culture

The animals were anesthetized by an i.p. injection of sodium pentobarbital (45 mg/kg). The liver was perfused via the portal vein, and the cells were isolated by an in situ collagenase perfusion as previously described (17). Hepatocytes were purified as in Ref. 18 by four successive low centrifugations at 50 × g for 2 min in ice-cold buffer. The viability of the final hepatocyte suspension was checked using trypan blue exclusion, and cell purity was assessed by morphological criteria under microscope. Moreover, OX-42-positive cells were not detected by flow cytometry. Contamination with nonparenchymal cells was <3%. The purified rat hepatocytes were then plated at a density of 4 × 10⁵ onto plastic culture dishes and allowed to attach for 4 h in RPMI 1640 culture medium supplemented with 2 mM glutamine, 10% decomplemented FCS (Boehringer Ingelheim, Meylan, France) and 1% antibiotic solution at 37°C under a 5% CO₂ atmosphere. The cells were next cultured overnight in a fresh medium containing 0.1 mM dexamethasone. After this attachment period, the cells were stimulated with a serum-free medium containing or not containing MAP-C5a (10⁻⁶ M) at different times (3, 6, or 18 h), and harvested for RNA extraction and quantification. For inhibition experiments, the cells were incubated or not with 200 ng/ml PTX for 4 h before stimulation with MAP-C5a.

qRT-PCR

Total cellular RNAs were extracted from crude liver or hepatocytes as in Ref. 19. Briefly, the cells were lysed with a denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 100 mM 2-ME), and the RNAs were obtained by sodium acetate-phenol-chloroform extraction, followed by an ethanol precipitation. The RNA integrity was assessed from the 18S and 28S RNA bands after agarose gel electrophoresis and the RNA concentration was measured at 260 nm. Two microliter of total RNA were reverse-transcribed in 60 μl of murine Moloney virus reverse transcriptase buffer (Promega, Madison, WI) containing 4 dNTPs (1 mM each), 120 U RNasin (Promega), 400 U murine Moloney virus reverse transcriptase (Promega), and 500 pmol random hexamer primers (Pharmacia, Peapack, NJ) at 37°C for 60 min and 95°C for 5 min. For the PCR step, 3 μl of the reverse transcriptase mixture were brought up to 20 μl with a PCR buffer (Promega) containing 4 dNTPs (200 μM each), 1.25 mM MgCl₂, 1.25 U Taq polymerase (Promega), 1 μCi [α³²P]dATP (Amersham Biosciences, Piscataway, NJ) and 100 pmol (each) of four primers (Eurogentec, Seraing, Belgium). The primers were chosen to have a 50–60% GC content. They covered separate exons so that RNA-associated PCR products could be readily distinguished from any PCR products resulting from contaminating genomic DNA. The following primer for rat genes were used: C5aR, 5′-TATTAGTCGGCCACGCTTCC-3′ and 5′-CGGATCAGGAGGAAGGTGTA-3′; hepatocyte growth factor (HGF), 5′-AATCTCTGAGGGAGAAGGG-3′ and 5′-TCTCGGAGGTTGTCATGC-3′; c-Met, 5′-GAAACCAATTTGGTGAGG-3′ and 5′-GAGTTATCTGATTCACAC-3′; and 18S, 5′-GTGGAGCGATTITGTAGTG-3′. Preliminary experiments (data not shown) were made to select the number of cycles that give a linear relationship between the number of cycles and the amount of PCR product, namely 30 cycles for C5aR, 28 cycles for HGF, 27 cycles for c-Met, and 8 cycles for 18S. After a first denaturation at 94°C (4 min), each PCR cycle consisted of (94°C/30 s) + (55–60°C/20 s) + (72°C/20 s). Aliquots of the PCR products were electrophoresed in a 6% polyacrylamide gel. The dried gels were exposed onto an x-ray film and the autoradiograms were analyzed by densitometric scanning. The resulting ampiclon amounts were normalized with the amounts of a 18S cDNA ampiclon.

Cycloheximide (CHX) and thymidine kinase (TK) mRNA levels were quantified using qRT-PCR that was done with the FastStart DNA Master SYBR Green I kit and 2–4 mM MgCl₂, in a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) exactly as recommended by the manufacturer. Primers were selected so as to span two separate exons, to have a tₚ of 58–62°C and to provide an ampiclon in the 200bp range.

Flow cytometry

The hepatocytes were incubated with the first Ab (10 μg whole IgGs) at 4°C for 30 min. Staining was performed by incubating the cells with an anti-rabbit IgG coupled to PE or FITC (Jackson Laboratories) during 30 min at 4°C. Then, the cells were analyzed on a FACSCalibur cytometer (BD Biosciences, Le pont de Claux, France) operating with the CellQuest software. Dead cells and debris were excluded from the analysis by gating live hepatocytes from size/structure dot plots. Data were displayed on a logarithmic scale of increasing fluorescence intensity and each histogram was recorded from at least 10⁶ events. The results are expressed as mean fluorescence intensity.

Immunohistochemistry

The animals (healthy or at 24 h after 80% hepatectomy) were anesthetized by an i.p. injection of sodium pentobarbital as previously described and the liver was perfused and fixed via the portal vein with a 4% paraformaldehyde solution. After sacrifice, the liver was collected, embedded in paraffin, and sliced at 5 μm. Sections were stained for BrdU incorporation.

BrdU incorporation

BrdU (Sigma-Aldrich) was administered to 80% hepatotomized rats, which received a C5a antagonist or a saline solution alone at 24 h after hepatectomy. BrdU was given by i.p. injection (50 mg/kg body
stress induced by a LPS challenge performed 12 h before a 66% hepatectomy (controls), SHAM rats, LPS-injected, or 66% hepatectomized animals (HP) or animals with an hepatectomy done at 12 h after LPS (HP 66% + LPS). Three animals per group were used and qRT-PCR was done in triplicate. Results are given in arbitrary units (AU) and normalized with 18S RNAs. *, p < 0.05; **, significant difference vs controls (p < 0.01).

Results

Inflammatory stress-induced expression of C5aR mRNA in rat hepatocytes

C5aR is not expressed in normal rat hepatocytes but is induced by IL-6 treatment in vivo (9), and recent data indicate that C5 is involved in liver regeneration (14). Therefore, we have compared the expression of C5aR mRNA in hepatocytes freshly isolated from rats subjected to a sham surgery, a LPS challenge, an hepatectomy, or a LPS challenge performed before the hepatectomy vs healthy animals used as controls. As shown in Fig. 1, the level of C5aR mRNA in hepatocytes from animals at 12 h after LPS challenge or after 66% hepatectomy was significantly increased (p < 0.05) as compared with controls. Following a major inflammatory stress induced by a LPS challenge performed 12 h before a 66% hepatectomy, the C5aR mRNA level was increased even further (p < 0.01). No significant modification was observed following a sham surgery.

C5aR expression in hepatocytes after an hepatectomy of variable extent

We next evaluated whether an hepatectomy of variable extent (30, 66, or 80%) caused a time- and dose-dependent increase in expression of C5aR mRNA in liver as compared with its level before resection. As shown in Fig. 2, a 30% resection caused a significant increase of this mRNA level (p < 0.01) that was maximally expressed at 24 h and returned to its basal value after 48 h. This kinetic of C5aR expression was not significantly modified by an extended resection (66%, data not shown). In contrast, an 80% resection induced a dramatic change in this kinetic, as this mRNA level strongly increased (p < 0.001) as early as 6 h after resection and remained at a high level until 48 h.

We next checked whether the induction of C5aR mRNA by partial resection of the liver resulted in an expression of the protein at the hepatocyte membrane. Hepatocytes from healthy controls or animals that underwent an 80% resection were purified and analyzed by flow cytometry using a specific anti-C5aR antiserum (L-66, see Materials and Methods). As shown in Fig. 3, the hepatocytes from healthy rats were not stained by this antiserum, whereas a clear-cut shift of the whole histogram was observed with cells obtained at 24 h after resection from animals with an 80% liver resection. The control histogram (open) was obtained with the preimmune IgG of the rabbit. These data clearly showed an induction of C5aR at the membrane of all regenerating hepatocytes. Furthermore, these findings were supported by data obtained by immunohistochemistry (Fig. 4), where a homogeneous staining of hepatocyte membrane by L-66 Ab was seen on liver sections from

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**Figure 1.** C5aR mRNA expression in rat hepatocytes after an inflammatory stress. mRNAs from purified hepatocytes were from healthy rats (controls), SHAM rats, LPS-injected, or 66% hepatectomized animals (HP) or animals with an hepatectomy done at 12 h after LPS (HP 66% + LPS). Three animals per group were used and qRT-PCR was done in triplicate. Results are given in arbitrary units (AU) and normalized with 18S RNAs. *, p < 0.05; **, significant difference vs controls (p < 0.01).

**Figure 2.** C5aR mRNA expression after a partial hepatectomy. C5aR mRNA level in liver from healthy rats (H0) or in a regenerating liver after hepatectomy (30% HP and 80% HP) at various times was measured by a qRT-PCR method. Three animals per group were used and qRT-PCR was done in triplicate. A significant difference vs controls is noted. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Figure 3.** Analysis of C5aR expression on hepatocyte membrane by flow cytometry. A, Hepatocytes from healthy rats; B, hepatocytes taken at 24 h after resection in animals with an 80% hepatectomy. Open histograms, Incubation of hepatocytes with preimmune rabbit IgGs used as control. Filled histograms, Incubation of hepatocytes with specific anti-C5aR Abs (antiserum L-66).

**Figure 4.** C5aR protein expression on hepatocyte membrane by immunohistochemistry. Photomicrographs of peroxidase-stained liver sections with an anti-C5aR Ab (L-66, cf Materials and Methods). A, Healthy rats; B, 80% hepatectomized rats 24 h after resection.
hepatectomized animals only. Moreover, a staining was also observed on nonparenchymal cells most probably on Kupffer cells.

**Induction of C5aR mRNA expression in cultured hepatocytes**

To evaluate the stimulatory effects of C5a on cultivated hepatocytes, we first confirmed that C5aR was expressed under our culture conditions. We measured the level of C5aR mRNA in freshly isolated hepatocytes before cell attachment on dish or at 24 h after culture onset. The data in Fig. 5 show that C5aR mRNA level was significantly up-regulated ($p < 0.001$) at 24 h after culture onset as compared with its basal level before cell attachment. Flow cytometry further allowed us to observe an induction of the C5aR protein at the membrane of cultivated hepatocytes (Fig. 6A). This induction paralleled the expression of the hepatocyte-specific Ag CD26, an adhesion marker (Fig. 6B).

Moreover, to assimilate this cell attachment to an inflammatory stimulus, we have compared the expression of two acknowledged rat APPs (α1-acid glycoprotein and α2-macroglobulin) in freshly isolated hepatocytes vs cells at 24 h after culture onset. As shown in Fig. 5, the mRNA levels for both APPs were strongly expressed in hepatocytes at 24 h of culture as compared with an undetectable expression in freshly isolated hepatocytes.

**Induction of hgf and c-met gene expression by MAP-C5a**

The most potent mitogenic factor for the liver, namely the HGF, promotes hepatocyte proliferation by its binding to a specific c-Met protooncogene receptor. We have quantified the mRNA levels for HGF and c-Met in rat hepatocytes stimulated by the C5a agonist. Hepatocytes from healthy rats were purified and cultivated for 24 h. At 24 h after culture onset, the anaphylatoxin analog MAP-C5a at 10^{-8} M final concentration was added or not to the cell cultures, and the cells were harvested at 3, 6, or 24 h after stimulation for RNA extraction and measure of mRNA levels of HGF and c-Met. This concentration was chosen from a pilot study using 10^{-7}, 10^{-8}, or 10^{-9} M MAP-C5a concentrations. It fitted well with a previous study (20) where a maximum effect of the peptide was observed at 10^{-8} M (plateau phase) on different cell types. As shown in Fig. 7, stimulation by the C5a agonist caused a transient up-regulation of the HGF mRNA level at 6 h after stimulation as compared with its level in unstimulated cells. No modifications were observed at 3 and 18 h after stimulation. By

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**FIGURE 5.** C5aR mRNA expression on hepatocytes before and after seeding in culture dishes. C5aR mRNA level in hepatocytes from healthy rat obtained after dissociation by a collagenase perfusion before cell attachment or at 24 h after culture was measured by a qRT-PCR method. Three animals per group were used and qRT-PCR was done in triplicate. A significant difference between the two conditions was noted; $p < 0.001$.

**FIGURE 6.** Analysis of C5aR expression on cultured hepatocytes by flow cytometry. The control histogram was obtained by staining of freshly isolated hepatocytes with the rabbit L-66 (A, open histogram). The filled histogram was obtained with rat hepatocytes after 56-h culture stained with the L-66 Ab. B, In parallel, the expression of the specific hepatocyte marker CD26 was investigated. The open histogram was obtained with freshly isolated hepatocytes and the filled histogram with cultivated hepatocytes after staining with anti-CD26 mAb.

**FIGURE 7.** MAP-C5a-induced variations of HGF and c-Met mRNA expressions in cultivated hepatocytes. mRNA levels for these factors were measured at different times after stimulation by a qRT-PCR method done in triplicate for each sample. The results (mean ± SEM) are expressed as a ratio of the mRNA levels in C5a-treated vs unstimulated cells. Differences between the various time points are noted by horizontal brackets and a statistically significant difference (unpaired $t$ test) is noted.

**FIGURE 8.** PTX induced blockade of MAP-C5a-induced HGF mRNA expression. The hepatocytes were incubated with PTX for 4 h and MAP-C5a was then added. The HGF mRNA level was measured by a qRT-PCR method at 6 h after stimulation in PTX-treated vs untreated cells. Significant differences between samples (unpaired $t$ test) were noted by horizontal brackets.
Contrast, c-Met mRNA expression was slightly up-regulated over the 6- to 18-h stimulation period. The values provided by our qRT-PCR were confirmed by real-time RT-PCR (data not shown) that indicated an excellent agreement between both techniques (HGF, r = 0.89, p < 0.01; c-Met, r = 0.81, p < 0.01). To determine whether the above effects were mediated by a Gi protein-coupled receptor, cells were preincubated with PTX (200 ng/ml) for 4 h, then stimulated by MAP-C5a for 6 h, and the level of HGF mRNA was determined. As shown in Fig. 8, a PTX pretreatment prevented the up-regulation of HGF mRNA level by MAP-C5a, suggesting the involvement of a Gi-coupled receptor.

Effect of MAP-C5a on hepatic regeneration in vivo
As C5aR expression in hepatocytes was induced during liver regeneration and a C5a agonist enhanced the expression of factors involved in hepatocyte proliferation, we next attempted to observe a direct mitogenic effect of C5a on hepatocytes. In vitro experiments with a measure of BrdU incorporation or an ATP bioluminescence test as a measure of cell proliferation failed to demonstrate a possible involvement of MAP-C5a in a proliferative process (data not shown). We then postulated that our in vitro conditions were inappropriate and we performed in vivo experiments.

Cell cycle–associated genes such as those for cyclins or TK, a key regulatory enzyme of DNA synthesis, are assumed to regulate cell cycle progression in the regenerating liver. We previously demonstrated that after an 80% liver resection, cyclin E, cyclin D1, and TK gene expressions are enhanced and delayed up to 48 h after resection, as compared with their expression that is up-regulated at 24 h after a 66% resection (21). Given the timing of membranous expression of C5aR after a major hepatectomy (see above), an i.v. injection of MAP-C5a was performed at 24 h after an 80% liver resection and the livers were removed at various times (15, 24, or 48 h) postinjection. We then evaluated whether the regenerative process as assessed from the cyclin E, D1, and TK mRNA levels was modified by this MAP-C5a input as compared with a mere 80% hepatectomy used as control. No significant modification of the TK mRNA level could be observed at any time. On the contrary, and as shown in Fig. 9, the cyclin E mRNA level was significantly increased in the MAP-C5a-treated animals at 15 h postinjection (39 h after hepatectomy), but without changes at 24 and 48 h postinjection (48 and 72 h after surgery). For the cyclin D1, as shown in Fig. 10, we observed a strong and significant increase of mRNA level at 24 h postinjection (48 h after hepatectomy).

C5a enhanced proliferative process in regenerating hepatocytes
To evidence a direct effect of C5a on hepatocyte proliferation in vivo, we evaluated the percentage of positive hepatocytes after BrdU incorporation and stained cells were identified by the presence of round dark nuclei (Fig. 11). Whereas control rats given a saline solution at 24 h after 80% hepatectomy presented a barely detectable staining in the liver (1.50% ± 1.99), the C5a-challenged rats exhibited a strong and significantly increased staining (45.24% ± 9.9) in the liver parenchyma. These observations are consistent with our results of cyclin expression, which was increased in C5a-treated animals.

Discussion
Numerous recent studies have shown that C5aR is expressed not only by myeloid cells but also by nonmyeloid cells in different organs, and particularly the liver. In humans, C5aR expression occurs in liver cells and in the HepG2 hepatoma cell line (7, 8, 22). In rats, C5aR is mainly expressed by the nonparenchymal liver cells and to a much lower extent by hepatocytes (5, 23) where it is up-regulated by an inflammatory stress (9, 22, 24). However, the actions of the C5a/C5aR complex in the liver are still poorly characterized. Therefore, our present work is a first step to determine to what extent and how the anaphylatoxin C5a and its C5aR receptor are regulatory components of hepatic homeostasis. We have found that C5aR expression in hepatocytes is strongly enhanced by an inflammatory condition. Our results are in agreement with former studies in which an up-regulated expression of C5aR on hepatocytes was seen during an inflammatory process, and more particularly in LPS-challenged rats (25). However, in this latter work, LPS failed to induce C5aR in cultured hepatocytes in vitro.
Our data are also in keeping with an enhanced binding of C5a, along with an increased level of C5aR mRNA as observed in rat alveolar epithelial cells stimulated by LPS, IL-6, or TNF-α (26).

Most importantly, we now report for the first time that C5aR expression in hepatocytes is strongly enhanced by a regenerative process induced by an 80% partial hepatectomy and the level of C5aR mRNA expression is related to the extent of resection. Moreover, the C5aR protein induction in regenerating hepatocytes is also demonstrated by FACS analysis as well as immunohistochemistry. We have then investigated whether C5a binding to C5aR can regulate the mRNA level for some proliferation-related genes. We have specifically studied cell proliferation-inducers such as HGF and its c-Met receptor that are essential actors of liver regeneration (27, 28) and have been associated with proliferation of normal and neoplastic hepatocytes. In a first step, under our experimental culture conditions, we observed an increased expression of the C5aR mRNA by hepatocytes that are able to synthesize typical well-known hepatic proteins (α2-macroglobulin and α1-acid glycoprotein) whose expression is strongly enhanced during an inflammatory stress. We next demonstrated and confirmed by flow cytometry studies the induction of C5aR protein expression at the membrane of cultivated hepatocytes. In a second step, by an in vitro treatment of the hepatocytes with the MAP-C5a peptide, we investigated at different times after stimulation the involvement of this anaphylatoxin concerning the mRNA expression of the genes under study as detailed above. Under these experimental conditions, our results demonstrate for the first time that a binding of the anaphylatoxin C5a to the hepatocyte C5aR promotes an increased expression of both HGF and c-Met mRNAs. This cascade is quite specific for C5a and its receptor, as a cell pretreatment with PTX completely abrogated the ability of hepatocytes to respond to C5a and blocked the up-regulation of the HGF mRNA. These data now argue for a new role for hepatic C5a receptor, particularly in a proliferative pathway. But we failed (data not shown) to demonstrate by in vitro experiments a possible involvement of MAP-C5a in a proliferative process. So, we postulated that our in vitro system was unable to produce or did not contain all the factors necessarily implicated in this process, and then to address this question, in vivo experiments were performed. Moreover, our data obtained in vivo strengthen those obtained in vitro. Indeed, after a MAP-C5a injection following an 80% hepatectomy, an up-regulated expression of the cyclin E and cyclin D1 mRNA levels was observed at 39 and 48 h, respectively, after surgery. In control rats, no increased cyclin D1 mRNA levels was observed at 39 and 48 h after surgery because, as previously described (21) the peak of response for this cyclin following an 80% hepatectomy was observed at 24 h after surgery and then strongly declined. For the cyclin E, as compared with control, we observed a significant increased mRNA level in MAP-C5a-treated animals at 39 h after hepatectomy because, as previously described (21), the peak of response for this cyclin was different and observed between 24 and 48 h after surgery alone before return to basal value. These up-regulated expressions observed at 39 and 48 h after the C5a agonist injection allow us to provide evidence for the effect of C5a on cell cycle regulation and proliferation. Furthermore, by BrdU incorporation, we observed an increase in the number of proliferating cells, thus establishing that regenerating hepatocytes have a proliferative response after C5a agonist injection. Therefore, C5a/C5aR binding now appears to play an important role in liver regeneration by triggering signal transduction events that are involved in cell cycling. Furthermore, we have recently described that in rat, when a LPS challenge is given before partial hepatectomy, the major source of hepatic HGF appears to be the hepatocyte itself, rather than nonparenchymal cells. An autocrine HGF/c-Met loop that involves the hepatic parenchymal cells, promotes their proliferative potential, and participates in liver regeneration had been postulated (29). According to our present data, we can now consider that following a LPS challenge, binding of C5a to its up-regulated receptor C5aR might be one of the mechanisms that promotes the increased HGF expression that eventually results in a proliferative response of the liver as proven by the BrdU incorporation assay.

Moreover, our results now account for the impaired liver regeneration documented in C5-deficient mice and the ability of murine C5 or C5a to restore hepatocyte regeneration in these animals (14). Recently, data (30) indicated that C5a contribute to the early priming stages of hepatocyte regeneration. So, we can hypothesize that C5a might have two major functions: the first involved in the early priming of the regenerative process via its interaction with a constitutive C5aR expressed on non parenchymatous cells, and the second involved in the amplification of the proliferative response via the interaction with its inducible hepatocyte C5aR. Taken together, our observations and former data argue for a C5a/C5aR-controlled transduction pathway, which may result in normal or abnormal proliferative processes in liver.

As yet, few studies have dealt with an involvement of C5aR in a proliferative pathway in humans. C5aR expression in the skin lesions of patients with systemic sclerosis suggests an activation of pathways that end up with a growth factor-dependent matrix synthesis (31). In mesangial proliferative glomerulonephritis, the glomerular expression of C5aR mRNA and protein correlate positively with the extent of mesangial hypercellularity and mesangial matrix expansion (32). In rheumatoid arthritis, i.e., another condition with inflammatory and proliferative stages, some chondrocytes express C5aR (33, 34) that argues for a contribution of this receptor in arthritis pathogenesis. Contrasting with an involvement of C5aR in cell proliferation, some studies have pointed to the role of C5aR in the apoptotic pathway. For instance, at an early stage of sepsis onset, an increased expression of C5aR occurs in thymocytes that increases their susceptibility to the C5a-induced apoptosis (35). In neuroblastoma cells, an abnormal activation of the C5aR-associated transduction pathway could result in apoptosis and neurodegeneration (36, 37). However, the involvement of the C5a/C5aR complex in these important processes requires further investigations. In fact, accumulating data suggest that complement proteins might exert important and novel functions in diverse processes and pathways, might interact with other networks, and eventually play a role in complex biologic programs such as those described above (38).

Taken together, our results demonstrate for the first time that anaphylatoxin C5a through the specific interaction with its receptor expressed in rat hepatocytes is involved in the proliferative process and may elicit a growth factor response stimulating this process. These findings provide a novel role for the hepatic C5aR, implicating this complement system in the context of normal or abnormal proliferative pathway. This new regulatory role of the hepatic homeostasis might be important in the induction of such pathologic processes as carcinogenesis. Further clinical investigations, particularly during the development of hepatocarcinoma, are now essential to improve these data.

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