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Anaphylatoxin C5a receptor mRNA is strongly expressed in Kupffer and stellate cells and weakly in sinusoidal endothelial cells but not in hepatocytes of normal rat liver

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Abstract Anaphylatoxins (C5a and C3a), which are generated during complement activation, have recently been shown to increase glucose output from hepatocytes (HC) in perfused rat liver. They did not act directly on HC but indirectly by prostanoid release from non-parenchymal cells (NPC), probably Kupffer cells (KC). In order to corroborate this mechanism, the distribution of anaphylatoxin receptors in the different cell types of rat liver was determined by quantitative RT-PCR with primers specific for the rat C5a receptor (rC5aR) using RNA isolated from KC, sinusoidal endothelial cells (SEC), hepatic stellate cells (HSC) and HC. In line with functional data, C5aR mRNA was detected in freshly isolated NPC but not in HC of rat liver. Mainly KC but also HSC clearly expressed C5aR mRNA, while SEC did so only weakly. KC expressed up to 10-fold more C5aR mRNA than HSC and these in turn up to 10-fold more than SEC. These results support the proposed indirect action of anaphylatoxins on HC.

Key words: Anaphylatoxin C5a receptor; Rat liver; Non-parenchymal liver cell; Hepatocyte; Quantitative RT-PCR

1. Introduction

The anaphylatoxin C5a is generated by specific limited proteolysis of complement protein C5 during complement activation via the classical or the alternative pathway. It is a small diffusible glycopeptide derived from the first 74 (man) or 77 (rat) amino acid residues of the C5α-chain [1–4]. C5a acts as a potent inflammatory mediator by causing the degranulation of mast cells, the contraction of smooth muscle cells, an increase in vascular permeability and the chemotaxis and activation of neutrophils, resulting in the immigration of the cells into the inflamed tissue and the local release of reactive oxygen species, eicosanoids and cytokines [reviewed in [5–8]]. The effects of C5a are mediated via a high affinity C5a receptor (C5aR) which in man has been characterized as a 40–48 kDa membrane protein, which belongs to the 7-transmembrane domain receptor family and is coupled to the α-subunit of a pertussis toxin-sensitive G-protein [9,10]. Also sequences of the dog [11], mouse [12] and partial sequences of the bovine [11] and rat C5a receptors [11] have been cloned. The entire coding sequence of the rat C5aR has recently been communicated [Rothermel, E., Zwirner, J., Rabini, S. and Götze, O., in preparation, GenBank accession number Y09613].

In rat liver, anaphylatoxins have recently been shown to influence glucose metabolism and hemodynamics [13–15]. The enhanced glucose output and reduced flow elicited by recombinant rat C5a (rrC5a) in perfused rat liver were accompanied by an overflow of prostanoids [14,15] and largely prevented by the prostanoid synthesis inhibitor indomethacin [14]. Moreover, they were clearly reduced in livers in which the Kupffer cells (KC) — the resident macrophages of the liver — had been selectively eliminated by pretreatment with gadolinium chloride [15]. These results indicated that the rrC5a-induced metabolic and hemodynamic effects were at least in part mediated by prostanoids released from KC. In line with these observations rrC5a induced a time- and dose-dependent release of prostaglandins and thromboxane in KC cultures and led to the activation of glycogen phosphorylase in KC/hepatocyte (HC) cocultures but not in HC cultures alone [16]. Again, this effect was completely suppressed by inhibition of prostanoid synthesis [16]. Apparently, rrC5a did not act directly on HC but indirectly via prostanoids released from KC. This proposed mechanism might be at variance with the reported presence of C5aR on HC in human liver [17,18]. Therefore, the expression of mRNA for the C5aR was investigated in rat HC and KC and in other non-parenchymal cells (NPC), i.e. sinusoidal endothelial cells (SEC) and hepatic stellate cells (HSC) from rat liver.

2. Materials and methods

2.1. Animals

Male Wistar rats (Winkelmann, Borchen, Germany, 350–450 g for the isolation of KC, SEC and HSC, 200–250 g for HC) were kept on a 12 h day/night rhythm with free access to water and a standard rat diet (Ssniff, Soest, Germany).

2.2. Cell preparation

HC were isolated by collagenase digestion or alternatively according to Meredith without the use of collagenase [19]. Briefly, the liver was perfused at 37°C in a non-recirculating manner via the portal vein with a Ca^{2+}-free Krebs-Henseleit buffer containing 15 mM glucose, 2 mM lactate, 0.2 mM pyruvate and 2 mM EDTA. The flow rate was 10 ml/min. After 45 min the liver was exised, the liver capsule was opened and hepatocytes were suspended in Krebs-Henseleit buffer containing 1 mM $CaCl_2$ and filtered through nylon gauze (mesh diameter 60 μm). Detritus was removed by two subsequent washing steps at 50 *g* and viable HC were further purified through a 58% Percoll gradient. Purity of hepatocytes as identified on the basis of their typical light microscopic appearance was greater than 95%.

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Abbreviations: r/hC5aR, rat/human anaphylatoxin C5a receptor; rrC5a, recombinant rat C5a; NPC, non-parenchymal cell(s); HC, hepatocyte(s); KC, Kupffer cell(s); HSC, hepatic stellate cell(s); SEC, sinusoidal endothelial cell(s); RT, reverse transcriptase

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KC and SEC 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 [20,21].

HSC were obtained by enzymatic digestion of the liver essentially as described in [22] with some modifications [N. Kawada, personally communicated]. The resulting cell suspension, subsequently handled at 4°C, was filtered through nylon gauze (mesh diameter 60 μm) and centrifuged for 10 min at 450 *g*. After two washing steps, cell sediments were resuspended in Hanks' Balanced Salt Solution (HBSS), mixed with Nycodenz to a final concentration of 8.13% and overlaid with HBSS. After centrifugation for 20 min at 1400×*g* HSC were obtained from the interphase. Purity of HSC as identified on the basis of their typical light microscopic appearance was about 95%.

2.3. RNA isolation and cDNA synthesis by reverse transcription

Total RNA from freshly isolated KC, HSC, SEC and HC was isolated either by homogenization in guanidine thiocyanate followed by cesium chloride gradient ultracentrifugation [23] or by an RNeasy Kit provided by Qiagen (Hilden, Germany). Total RNA (6 μg) was preincubated for 10 min at 68°C with 500 ng oligo-d$(T)_{12-18}$ and transcribed with reverse transcriptase (RT) (Superscript II, Gibco, Eggenstein, Germany) into cDNA. In some cases the efficiency of the cDNA synthesis was monitored by reverse transcription in the presence of [^{35}S]dATP (1000 Ci/mmol, ICN Pharmaceuticals, Eschwege, Germany) [24]. The cDNA thus generated was precipitated with ammonium acetate/ethanol in order to remove unincorporated radioactivity, washed 3 times with 70% ethanol and resuspended in deionized H_2O. The radioactivity incorporated into the cDNAs was assumed to be proportional to the amount of newly synthesized cDNAs [24].

2.4. Polymerase chain reaction

Equal amounts of cDNA from total RNA of KC, HSC, SEC and HC were amplified in a 50 μl reaction mix containing 1.5 mM $MgCl_2$, 0.6 μM of foreward and reverse oligonucleotide primers rC5aR-F1 and -R2, degC5aR-F3 and -R4, rβ-actin-F and -R, hβ-actin-F and -R (Table 1) or 0.1 μM primers for human C5aR (hC5aR, Stratagene, Heidelberg, Germany), 0.2 mM dNTPs, 6% DMSO and 0.5 U of thermostable DNA polymerase (Goldstar Red, Eurogentec, Seraing, Belgium). PCR reactions were prepared as master mixes before the addition of the respective cDNAs. The cDNA was denatured for 3 min at 94°C and then subjected to 35 cycles of 1 min at 94°C, 1 min at 58°C (rC5aR, degC5aR and hβ-actin), 61°C (rβ-actin) or 60°C (hC5aR), and 2 min at 72°C with a final elongation step of 10 min at 72°C. After amplification, polymerase chain reaction (PCR) products were separated in 2% agarose gels and visualized by ethidium bromide staining. The rC5aR product amplified from KC was cloned with SureClone ligation kit (Pharmacia, Freiburg, Germany) into pUC 18 and sequenced for identification using dyedeoxy terminator NTPs (Perkin Elmer, Weiterstadt, Germany).

2.5. Generation of the internal standard for the rC5aR

The rat C5aR cDNA was isolated using a hybridization probe produced by PCR with degenerate primers corresponding to conserved parts of the published C5aR sequences [Rothermel, E., Zwirner, J., Rabini, S. and Götze, O., in preparation, GenBank accession number Y09613]. A 678 bp *Hin*cII fragment of the cloned rC5aR sequence was cloned into the *Hin*cII restriction site of pBlueScript KS II. From this construct a 148 bp fragment was removed by restriction enzyme digestion with *Ava*II (Gibco, Eggenstein, Germany) at positions 568 and 716 (GenBank accession number Y09613), followed by gel electrophoresis, gel extraction (QIAquick gel extraction kit, Qiagen, Hilden, Germany) and religation with T4 Ligase (Gibco). The shortened insert ('internal standard') was isolated by restriction enzyme digestion of the flanking polylinker region with *Xho*I (MBI Fermentas, St. Leon-Rot, Germany) and *Eco*RI (Boehringer-Mannheim, Mannheim, Germany) and purified by agarose gel electrophoresis and extraction as described above.

2.6. Quantitative RT-PCR for C5aR cDNA

For quantitative PCR, a constant amount of KC-, HSC- or SEC-cDNA was co-amplified with the internal standard in 10-fold dilution steps under the conditions described above. After amplification, PCR products were separated in 2% agarose gels and visualized by ethidium bromide staining.

3. Results

3.1. Detection of rat C5a receptor mRNA in Kupffer, stellate and in sinusoidal endothelial cells but not in hepatocytes of rat liver

To investigate the expression of C5aR mRNA in different cell types of rat liver, equal amounts of RNA isolated from KC, HSC, SEC and HC were submitted to reverse transcription and PCR with primers specific for rC5aR and rβ-actin (rC5aR-F1 and rC5aR-R2; rβ-actin-F and rβ-actin-R, Table 1). PCR amplification yielded a single product corresponding to the expected 511 bp. With the cDNAs derived from KC and HSC product formation was strong and with the cDNA from SEC it was weak. No product was obtained with the cDNA from HC (Fig. 1). The identity of the amplified product was verified by cloning and sequencing the PCR product derived from the KC cDNA (data not shown).

The differences in C5aR mRNA expression observed in the different cell types were not due to differences in the efficiencies of cDNA synthesis, since all cell types expressed similar amounts of β-actin mRNA (Fig. 1). Moreover, the same results were obtained when reverse transcription was performed in the presence of [^{35}S]dATP to monitor the efficiency of cDNA synthesis and when equal amounts of radioactive cDNA were analysed (data not shown). The finding that hepatocytes did not express C5aR mRNA was independent from the kind of isolation procedure both for the cells and for RNA (see Section 2).

3.2. Detection of C5a receptor mRNA with degenerate primers in the human hepatoma cell line HepG2 and rat Kupffer cells but not rat hepatocytes

The human hepatocellular carcinoma cell line HepG2 has

Table 1
Oligonucleotide primers used for RT-PCR

Name	Sequence (5′→3′)	GenBank accession No	Position
rC5aR-F1	GGT **G**GC CGA C**C**T CCT CTC **G**TG	Y09613	267– 287
rC5aR-R2	**G**AC C̄AC **C**GC C**A**̄**T** CAC CAC T̄TT G		777– 756
degC5aR-F3	C̄TC AT$^{C}/_{T}$ C̄TG CT̄C AA$^{C}/_{T}$ ATḠ TA	X57250	343– 362
degC5aR-R4	CAC CAC $^{C}/_{T}$TT GAG $^{C}/_{T}$GT $^{C}/_{T}$TT GG		732– 713
		Y09613	373– 392
			765– 746
rβ-actin-F	GAT ATC GCT GCG CTC GTC GTC	J00691	1251–1271
rβ-actin-R	CCT CGG GGC ATC GGA ACC		2570–2553
hβ-actin-F	CCC AGC CAT GTA CGT TGC TAT	HSAC07	428– 448
hβ-actin-R	GGG TGG CTT TTA GGA TGG CAA		1474–1454

In the rat C5a receptor primers (rC5aR-F1, -R2) deviations from the human sequence are printed in **bold face**.

previously been shown to express C5aR mRNA [25]. Since it was not possible to detect C5aR mRNA in HepG2 cells with the rat primers used (rC5aR-F1, rC5aR-R2; Table 1; Fig. 2) control PCR with degenerate primers for C5aR (degC5aR-F3, degC5aR-R4; Table 1) binding in an area of high homology between the human and the rat sequence was performed. Also with this primer pair C5aR was detected in rat KC but not in rat HC. As expected these primers revealed C5aR mRNA expression also in HepG2 cells (Fig. 2). Moreover, the human primers originally employed for the detection of C5aR mRNA in HepG2 cells [25] cross-reacted with the rat sequence, thus yielding a product again in HepG2 cells and rat KC but not rat HC (data not shown).

3.3. *Quantification of mRNA for the rat C5a receptor in Kupffer, hepatic stellate and sinusoidal endothelial cells by quantitative RT-PCR*

Rat KC and HSC strongly expressed C5aR mRNA whereas SEC did so only weakly (Fig. 1). In order to quantitate C5aR mRNA expression in the different cell types, PCR was performed with cDNAs derived from KC, HSC and SEC in the presence of an internal C5aR cDNA standard shortened by 148 bp. When equal amounts of cDNA were amplified in the presence of decreasing amounts of the internal standard (6000–0.006 amol), both competed for the oligonucleotide primers according to their molar ratio in the reaction mixture (Fig. 3). At high molar excesses of either internal standard or cell-derived cDNA, essentially only standard or cDNA were amplified by PCR, while both products were formed in equal intensity when present in equimolar amounts (Fig. 3). By determining the amount of internal standard that resulted in the same quantity of PCR product as cell-derived cDNA, the amount of C5aR cDNA per μg RNA could be determined. For example, when 6 μg RNA were reverse transcribed in a volume of 20 μl and subsequently 1 μl of a 1:10 dilution of this cDNA was coamplified with different dilutions of the internal standard, in case of equimolarity of cDNA and internal standard at 6 amol standard this corresponded to 200 amol C5aR cDNA/μg total RNA. For KC the amount of cDNA thus was evaluated as 200 amol C5aR cDNA/μg total RNA in three different experiments. For HSC up to 10-fold and for SEC 10- to 100-fold lower expression levels were detected, whereas for HC the amount of cDNA was always

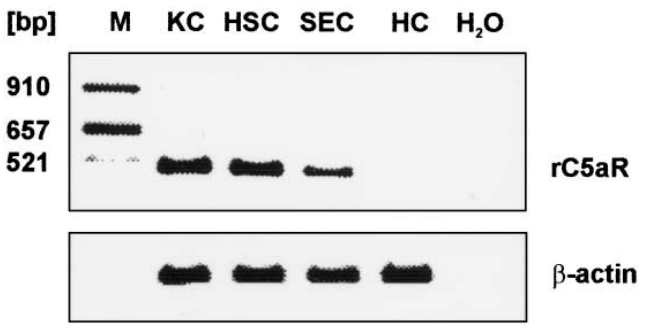

Fig. 1. Detection of mRNA for the C5a receptor in Kupffer (KC), stellate (HSC) and sinusoidal endothelial cells (SEC) but not hepatocytes (HC) of rat liver by RT-PCR (rat primers). cDNA was generated by reverse transcription of equal amounts of total RNA from the different cell types and subjected to 35 cycles of PCR in the presence of sequence specific primers for the rat C5a receptor (primers rC5aR-F1 and rC5aR-R2, 511 bp) and rβ-actin (primers rβ-actin-F and rβ-actin-R, 769 bp).

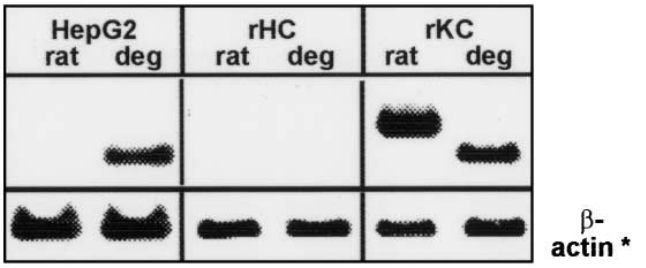

Fig. 2. Detection of mRNA for the C5a receptor in HepG2 cells and rat Kupffer cells (KC) but not rat hepatocytes (HC) by RT-PCR with degenerate primers. cDNA was generated by reverse transcription of equal amounts of total RNA from the different cell types and subjected to 35 cycles of PCR in the presence of degenerate primers for the human and rat C5a receptor (primers degC5aR-F3 and degC5aR-R4, 390 bp and 393 bp, respectively) and with sequence specific primers for the rat C5a receptor (see Fig. 1). *β-actin-mRNA was detected in rat KC and HC with primers rβ-actin-F and -R (see Fig. 1) and in HepG2 cells with primers hβ-actin-F and -R (1047 bp).

below the detection limit of 0.2 amol C5aR cDNA/μg total RNA (three experiments, each) (Table 2).

4. Discussion

C5aR mRNA expression was investigated in hepatocytes (HC), Kupffer cells (KC), hepatic stellate cells (HSC) and sinusoidal endothelial cells (SEC) of rat liver by means of quantitative RT-PCR. This technique was chosen, because a low abundance of mRNA for C5aR was expected at least in some cell types of the liver: in a recent study investigating the expression of the C5aR in human liver and lung, only a weak expression of C5aR mRNA could be detected in RNA extracted from whole liver by Northern blot analysis [17]. As expected, cDNAs derived from equal amounts of total RNA from KC, HSC, SEC and HC of rat liver showed clear differences in the expression of C5aR mRNA (Fig. 1, Table 2).

4.1. *Expression of C5aR mRNA in Kupffer cells but not in hepatocytes of rat liver*

The liver might be confronted with anaphylatoxins generated by complement activation both systemically reaching the liver via the portal vein and locally in the liver tissue itself. Alternatively, active proteases formed during blood clotting or other serine hydrolases of tissue or leukocyte origin can result in the localized production of C5a at sites of local injury [26]. C5aR mRNA was strongly expressed in KC but not at all in HC (Fig. 1). These results are consistent with the recent findings that anaphylatoxins increased glucose output in perfused rat liver and in KC/HC cocultures via prostanoids released from KC [13–16]. They are also in accord with the observation that C5aR so far have been found predominantly on cells of myeloid origin such as neutrophils, eosinophils, monocytes, macrophages and the myeloid cell lines U937 and HL-60. The lack of C5aR mRNA in HC of rat liver — although in correspondence with functional data — is in contrast to recent studies in the human system in which the detection of C5aR mRNA by in situ hybridisation of human liver tissue was interpreted as expression of C5aR mRNA in HC [17,18]. This divergence might be due either to differences in the methodologic approach including interpretation of results or to genuine species differences between man and rat.

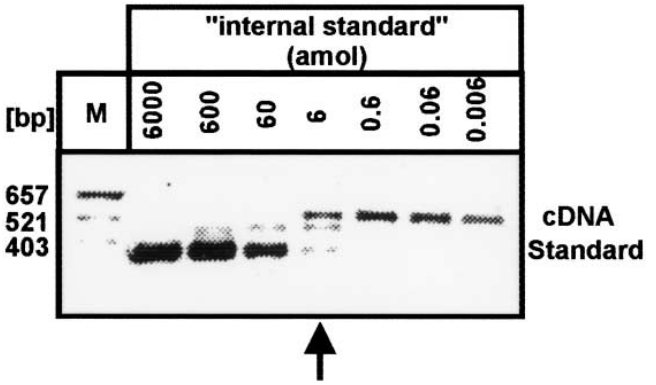

Fig. 3. Quantification of cDNA for the C5a receptor in rat KC by competitive PCR. Equal amounts of KC-cDNA derived from 6 μg total RNA were coamplified with 10-fold dilution steps of the internal standard ranging from 6000 to 0.006 amol (PCR products: 511 bp (cDNA) and 363 bp (internal standard)). The arrow denotes the point of equimolarity between cell-derived cDNA and internal standard.

The presumed expression of C5aR mRNA in human HC was supported by the detection of C5aR mRNA in the human hepatoma cell line HepG2 [25,17]. Using degenerate primers (Table 1) and the same human primer pair as in the original study [25] C5aR mRNA could be detected in HepG2 cells and in rat KC but not in rat HC (Fig. 2 and data not shown). Again, this difference between human hepatoma and rat hepatocytes might be due to species differences or to malignant transformation. Therefore, the possibility has to be considered that acute and/or chronic inflammation could induce C5aR mRNA expression also in rat HC.

4.2. Expression of C5aR mRNA in hepatic stellate cells

C5aR mRNA was also expressed in HSC although less strongly than in KC (Fig. 1, Table 2). HSC can clearly be differentiated from SEC and KC on the basis of their typical light microscopic appearance due to intracytoplasmatic vitamin A droplets. The cell preparations were microscopically pure; thus, minor contaminations with other non-parenchymal cells (i.e. a mixture of KC and SEC) can hardly explain the strong expression of C5aR mRNA in these cells. HSC (Ito-, fat-storing- or perisinusoidal cells) have great similarities with smooth muscle-like pericytes in other organs concerning morphology, physiology and pathophysiology [27]. Activation of HSC results in proliferation and transformation into 'myofibroblast-like' cells, a process which is accompanied by the expression of α-smooth muscle actin [28] which plays a key role in contraction processes. Several studies have demonstrated that isolated cultured HSC contract in response to various vasoconstricting stimuli such as prostaglandin $F_{2\alpha}$, thromboxane, thrombin, angiotensin-II, endothelin, and substance P [22,29–32]. Also C5a has vasoactive capacities and causes smooth muscle contraction [33,34]. Thus, the most probable function of C5aR expression on HSC might be the mediation of cell contraction resulting in a reduced blood flow and thereby facilitating the immigration of inflammatory cells into the liver tissue. Consequently, the portion of flow reduction observed after stimulation with rrC5a in perfused rat liver which was not inhibitable by the prostanoid synthesis inhibitor indomethacin or the thromboxane receptor antagonist daltroban (37% and 19%, respectively, [14]) might have been due to HSC contraction induced directly by rrC5a. However, until now contraction of HSC has only been demonstrated for cultured cells resembling 'activated' myofibroblast-like cells rather than quiescent HSC. Therefore it has to be investigated, whether cell contraction can be induced by rrC5a in short-term cultures of HSC and whether prolonged culture of these cells leads to alterations in C5aR expression and possibly in their capacity to contract in response to C5a.

4.3. Weak expression of C5aR mRNA in sinusoidal endothelial cells

Besides KC and HSC also SEC weakly expressed C5aR mRNA (Fig. 1, Table 2). While cultured KC and SEC can be differentiated based on their capacity to phagocytose latex particles of different diameters or by expression of cell specific surface antigens, these parameters cannot be tested with freshly isolated KC and SEC. Due to the pronase/collagenase digestion necessary for the isolation of non-parenchymal cells surface structures required for phagocytosis and some surface markers have been lost. SEC cultured for 48 h contain about 5% KC (own data, not shown). Thus, the weak C5aR mRNA expression in SEC (Fig. 1), which by quantitative RT-PCR has been shown to represent only 1 to 10% of C5aR cDNA found in KC (Table 2) might be due to contamination of SEC with KC. Nevertheless, it cannot be excluded from these ex-

Table 2
Quantification of C5a receptor cDNA in Kupffer-, hepatic stellate-, sinusoidal endothelial cells and hepatocytes of rat liver by means of competitive RT-PCR

Cell type	C5aR cDNA (amol/μg total RNA)	C5aR
Kupffer cells	200	++++
Hepatic stellate cells	20–200	+++
Sinusoidal endothelial cells	2–20	+?
Hepatocytes	<0.2	−

Values are from three experiments each: ?, low level expression or contamination.

periments that also SEC themselves weakly express C5aR mRNA. In human umbilical vein and rat pulmonary artery endothelial cells, C5a has been shown to activate protein kinase C and generate inositol-1,4,5-trisphosphate, to produce O^{2-} radical and to convert xanthine dehydrogenase to xanthine oxidase [35,36]. Moreover, in a recent study demonstrating the induction of P-selectin expression in human umbilical vein endothelial cells by C5a [37], C5aR expression was also shown by RT-PCR and by the demonstration of specific binding of [^{125}I]C5a to these cells. Also in this case only a rather low number of binding sites — i.e. 10% of the number bound to human neutrophils — could be detected. Another indirect hint that also SEC might express C5aR mRNA could be that SEC together with KC belong to the reticulohistiocytotic system representing the totality of mesenchymal cells with phagocytotic capacity in the body. Binding studies and functional data have to show whether SEC from rat liver express C5a receptors.

5. Conclusions

In the present study C5a receptor mRNA expression was investigated for the first time in freshly isolated parenchymal and non-parenchymal liver cells rather than in whole liver or in the malignant cell line HepG2. Kupffer and stellate cells strongly expressed C5a receptor mRNA, whereas sinusoidal endothelial cells contained 10–100-fold less C5a receptor mRNA than Kupffer cells. Hepatocytes were devoid of C5a receptor mRNA. This receptor distribution is in line with the proposed indirect mechanism of action of the anaphylatoxin C5a on liver metabolism via Kupffer cell derived prostanoids.

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