C5a Receptor and Interleukin-6 Are Expressed in Tissue Macrophages and Stimulated Keratinocytes but not in Pulmonary and Intestinal Epithelial Cells

Afshin Fayyazi,* Raoul Sandau,[†]
Le Quynh Duong,* Otto Götze,[†]
Heinz Joachim Radzun,* Stefan Schweyer,*
Afsaneh Soruri,[†] and Jörg Zwirner[†]

From the Departments of Pathology* and Immunology,[†]
University of Göttingen, Göttingen, Germany

The anaphylatoxin derived from the fifth component of the human complement system (C5a) mediates its effects by binding to a single high-affinity receptor (C5aR/CD88), the expression of which has been traditionally thought to be restricted to granulocytes, monocytes, macrophages (M ϕ), and cell lines of myeloid origin. Recent immunohistochemical data suggested that human bronchial and alveolar cells express C5aR as well. To reexamine the tissue distribution of human C5aR expression, transcription of the C5aR gene was investigated in normal and pathologically affected human lung (bronchopneumonia, tuberculosis), large intestine (acute appendicitis, Crohn's disease), and skin (pyogenic granuloma, lichen planus) using *in situ* hybridization. In contrast to previous evidence, C5aR mRNA could not be detected in pulmonary or intestinal epithelial cells, whereas keratinocytes in inflamed but not in normal skin revealed detectable levels of C5aR transcripts. Additionally, it could be documented that only migrating M ϕ express C5aR mRNA, whereas sessile M ϕ in normal tissues and epithelioid/multinucleated M ϕ found in granulomatous lesions do not. Because C5a has been demonstrated to upregulate the expression of interleukin (IL)-6 in human monocytes, we also studied IL-6 gene transcription in parallel to the C5aR. IL-6 mRNA was detectable in many tissue M ϕ . Surprisingly, a tight co-expression of C5aR and IL-6 mRNA was observed in keratinocytes from lesions of pyogenic granuloma and lichen planus. These results point to an as yet unknown role for C5a in the pathogenesis of skin disorders beyond its well-defined function as a chemoattractant and activator of leukocytes. (Am J Pathol 1999, 154:495–501)

C5a, a 74-amino acid peptide cleaved from the complement protein C5, represents the most potent anaphylatoxin. At nanomolar levels, C5a leads to a complex patho-

physiological response including cellular migration into inflammatory sites, changes in blood flow, and impairment of vascular integrity associated with edema.^{1,2} In addition, C5a possesses immunoregulatory activities through the induction of cytokines (tumor necrosis factor, IL-1, IL-6, and IL-8) in human monocytes.^{3–6}

C5a mediates its effects by binding to a specific high-affinity receptor, C5aR/CD88, a member of the G-protein-coupled rhodopsin receptor family with seven transmembrane segments.^{7–9} A wealth of functional and binding data suggested that expression of the C5aR was limited to leukocytes and leukocyte-derived cell lines.¹⁰ By flow cytometry, immunohistochemistry, and Western blotting, Gasque et al¹¹ showed recently that human astrocytes also express the C5aR. At the same time, Haviland et al¹² reported that several non-myeloid-derived tissue cells including liver parenchymal cells, bronchial and alveolar epithelial cells, lung vascular smooth muscle, and endothelial cells also express the C5aR. In that study the cellular localization of C5aR production within the liver was demonstrated by immunohistochemistry and *in situ* hybridization, whereas C5aR expression in the lung was determined by immunohistochemistry.¹² Although antibodies against C5aR/CD88 are valuable tools for investigating the C5aR on hemopoietic cells, results with non-myeloid cells should be considered with caution. All antibodies presently available have been raised against synthetic peptides of the aminoterminal receptor portion and may bind to other antigens such as desmosomal antigens localized on human keratinocytes.¹³ To exclude false positive immunohistochemical signals within the human lung or other tissues, it is therefore important to apply *in situ* hybridization for C5aR mRNA detection.

In the present study, we have sought to map the cellular expression of C5aR mRNA, investigate the expression of C5aR mRNA in migrating as compared to sessile M ϕ , and find *in situ* evidence for the effects of C5a on IL-6 induction. The investigations were performed on specimens of normal human lung, large intestine, and skin, and on samples of these tissues with granulocyte/monocyte- or lymphocyte-mediated inflammatory reactions.

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Address reprint requests to Dr. Jörg Zwirner, Abteilung Immunologie, Universität Göttingen, Kreuzberggring 57, D-37075 Göttingen, Germany.

Materials and Methods

Tissue Samples

Studies were performed on surgically removed lung, large intestine, and skin biopsy specimens (i) without any pathological disorder, (ii) with granulocyte/monocyte-mediated inflammatory reactions (bronchopneumonia, acute appendicitis, and pyogenic granuloma), and (iii) with lymphocyte-mediated reactions (pulmonary tuberculosis, Crohn's disease of the vermiform appendix, and lichen planus of the skin). The tissue samples ($n = 3$ for each category of specimens) were fixed in 4% formaldehyde and embedded in paraffin. Five- to 10- μ m-thick sections were mounted on silanized slides (Perkin Elmer, Langen, Germany). After deparaffinization, the sections were stained histochemically with hematoxylin and eosin. In addition, the tissues were analyzed by *in situ* hybridization (ISH), *in situ* reverse transcription-polymerase chain reaction (*in situ* RT-PCR), and indirect immunofluorescence (IF).

Detection of C5aR mRNA by Nonradioactive in Situ Hybridization

cRNA probes for the human C5aR were synthesized from PCR-generated cDNA as described.¹⁴ In brief, total RNA was isolated from peripheral blood mononuclear cells and subjected to a reverse transcriptase reaction as described previously.¹⁵ Amplification of a 409-bp cDNA fragment of the human C5aR (position 373-782 of the coding cDNA) was performed in an OmniGene Thermocycler (Hybaid, Middlesex, UK). Reaction mixtures of 50 μ l contained cDNA corresponding to 4 ng RNA, 100 pmol of each C5aR specific primer including an additional sequence at the 5'-end complementary to promoters of T3-polymerase (antisense primer: 5' cagagatgcaattaaccctcactaaa gggaga-GTCACCTGGTAGGGCAACCAGAAG 3'), and T7-polymerase (sense primer: 5' ccaagcttctaatacgcactactagggaga-CTGCTCCTGGCCACCATCA 3') (MWG Biotech, Ebersberg, Germany), 10 nmol of desoxynucleotides (dATP, dCTP, dGTP, dTTP), 5 μ l Taq PCR buffer $\times 10$, and 1 U Taq polymerase (Pharmacia, Freiburg, Germany). After an initial 95°C denaturation step of 2 minutes, 35 PCR cycles were carried out at 95°C (60 seconds), 55°C (60 seconds) and 72°C (60 seconds), followed by a final extension cycle of 10 minutes at 72°C. Amplification products were separated on a 1% agarose gel and visualized by ethidium bromide staining. After purification, PCR-generated cDNA fragments were subjected to *in vitro* transcription. In this process, Digoxigenin-11-dUTP-labeled antisense and sense probes were generated using T3- and T7-polymerases, respectively, according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). The amount of transcripts was monitored by 1% agarose gel electrophoresis. Labeling efficiency was controlled by dot blot analysis of serial dilutions of the probes.

ISH was performed according to a modified method published by Breitschopf et al.¹⁶ In brief, tissue sections

were deparaffinized and rehydrated in serial dilutions of ethanol. Samples were permeabilized using proteinase K (10 μ g/ml) for 10–30 minutes at 37°C. Digestion was stopped by washing the samples in Tris-buffered saline (TBS). Tissue specimens were then incubated in 0.25% acetic anhydride and dehydrated in serial dilutions of ethanol. Digoxigenin-labeled riboprobes were diluted in hybridization solution containing 50% deionized formamide, $5 \times$ SSC, 1% sodium dodecyl sulfate (SDS), 50 μ g/ml t-RNA, and 50 μ g/ml heparin. After application of antisense or sense probes the samples were covered with sterile coverslips and placed on a hot plate at 85°C for 5 minutes for probe and target denaturation. Hybridization was performed overnight at 55°C in a sealed humid chamber containing 50% formamide. After hybridization the sections were submerged in $2 \times$ SSC to remove the coverslips. Nonspecifically bound and unbound probes were removed by the following post-hybridization washes: $1 \times$ SSC/1% SDS at room temperature (3×5 minutes), $0.2 \times$ SSC/1% SDS at 55°C (2×10 minutes). Finally, the sections were washed in TBS (50 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.5) containing 0.1% (v/v) Tween-20 (Boehringer Mannheim) (3×5 minutes).

Signals were detected using a sheep polyclonal antibody F(ab)₂ fragment against digoxigenin conjugated with alkaline phosphatase (1:500) (Boehringer Mannheim). Alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate as substrate and nitro blue tetrazolium chloride as coupler (Boehringer Mannheim). Specimens were either counterstained with Mayer's hematoxylin or nuclear fast red and mounted in Aquamount (BDH Laboratories, Poole, UK) or subjected to indirect immunofluorescence.

A light microscopic examination followed. Positive cells showed strong cytoplasmic staining around the clearly demarcated nuclei.

For each tissue sample sense riboprobes were used as controls and proved to be negative.

Detection of IL-6 mRNA by in Situ RT-PCR

Tissue sections were incubated three times for 5 minutes in 0.01 mol/L citrate buffer (pH 6.0) in a microwave oven set to high power (600–700 W). Thereafter, *in situ* cycling and labeling of the PCR products were performed as described.¹⁷ Finally, signals were detected using a sheep polyclonal antibody F(ab)₂ fragment against digoxigenin conjugated with alkaline phosphatase (1:500) (Boehringer Mannheim). Alkaline phosphatase activity was visualized by applying 5-bromo-4-chloro-3-indolyl phosphate as substrate and nitro blue tetrazolium chloride as coupler (Boehringer Mannheim). All specimens were mounted in Aquamount (BDH Laboratories).

Light microscopic examination followed. Positive cells showed strong cytoplasmic staining around the clearly demarcated nuclei.

Two slides were run as controls for each *in situ* RT-PCR experiment. Lymph node sections served as positive controls run under cycling conditions as described

above. They exhibited positive signals in the compartments described by Peters et al.¹⁷ Sections from the materials subjected to *in situ* RT-PCR were run without primers serving as controls and proved to be negative.

Indirect Immunofluorescence

The primary monoclonal antibody Ki-M1P (anti-CD68) recognizing all populations of monocytes and M ϕ ¹⁸ was obtained from the Department of Pathology, University of Kiel, Germany.

Indirect immunofluorescence with the antibody Ki-M1P was performed on the same sections immediately after ISH or, in the case of IL-6 mRNA detection, on serial sections from the materials subjected to *in situ* RT-PCR. Sections were incubated for 2 hours with the monoclonal antibody Ki-M1P (hybridoma culture supernatant diluted 1:2000). Thereafter, samples were incubated with FITC-labeled goat anti-mouse IgG for 1 hour (working dilution 1:50) (Dako, Hamburg, Germany). An examination by fluorescence microscopy followed.

Results

Investigation of C5aR and IL-6 mRNA Expression in Human Lung

In normal human lung specimens, C5aR mRNA was detected in all small Ki-M1P-positive M ϕ localized in the subepithelial connective tissue of the bronchial mucous membrane and within the alveolar walls. IL-6 mRNA was detectable in many Ki-M1P-positive M ϕ and in lymphocytes infiltrating the alveolar walls. In contrast, C5aR or IL-6 mRNA expression could not be observed in bronchial and alveolar epithelial cells, in large Ki-M1P-positive M ϕ within the alveolar airspace, or in vascular smooth muscle or endothelial cells (Figures 1, a and b, and 2, a–d).

In human lung biopsy specimens from bronchopneumonia with abundant scarring, C5aR mRNA was noted in all small, infiltrating Ki-M1P-positive M ϕ localized within the bronchial walls and fibrous septa. IL-6 mRNA was detected in many small macrophages, in lymphocytes infiltrating the alveolar walls, and also in the endothelium of many capillaries. In contrast, C5aR or IL-6 mRNA expression could not be detected in large, Ki-M1P-positive M ϕ found within the alveolar airspace (data not shown).

In tissue samples from pulmonary tuberculosis, C5aR mRNA was detected in all small infiltrating Ki-M1P-positive M ϕ localized within the fibrous septa. IL-6 transcripts were detected in many small infiltrating M ϕ and in lymphocytes infiltrating alveolar walls and those surrounding epithelioid granulomas, as well as within endothelium of some capillaries. In contrast, C5aR or IL-6 mRNA expression could not be noted in epithelioid or multinucleated, Ki-M1P-positive M ϕ present in granulomas (Figure 3, a–d).

Figure 1. Bronchial epithelial cells express neither C5aR nor IL-6 mRNA. C5aR mRNA was detected by *in situ* hybridization followed by counterstaining with Mayer's hematoxylin (a), whereas IL-6 mRNA was detected by *in situ* RT-PCR without nuclear counterstaining (b). C5aR and IL-6 mRNA could be noted in leucocytes localized in the subepithelial connective tissue of the bronchial mucous membrane (filled arrows), but not within bronchial epithelial cells (open arrows). Original magnification, $\times 400$.

Investigation of C5aR and IL-6 mRNA Expression in Human Large Intestine

In the normal human large intestine, C5aR mRNA was detected in all small Ki-M1P-positive M ϕ localized in the subepithelial connective tissue of the intestinal mucous membrane. Positive cytoplasmic staining for IL-6 mRNA was seen in many small M ϕ and interfollicular lymphocytes, some germinal center cells, many interfollicular lymphocytes, and several fibroblasts localized within the subserosa. In contrast, C5aR or IL-6 mRNA expression could not be observed in epithelial cells, vascular smooth muscle, or endothelial cells (Figure 4, a–b).

In vermiform appendices from patients with acute appendicitis, C5aR mRNA was noted in all small infiltrating Ki-M1P-positive M ϕ scattered through the appendix wall. IL-6 mRNA was detected in many small infiltrating M ϕ and interfollicular lymphocytes, some germinal center cells, and in the endothelium of many capillaries (data not shown).

In vermiform appendices from patients with Crohn's disease, C5aR mRNA was detected in all small infiltrating Ki-M1P-positive M ϕ scattered through the appendix wall. IL-6 transcripts were detected in many small M ϕ and

Figure 2. C5aR and IL-6 mRNA expression in the normal human lung parenchyma. C5aR mRNA was detected by *in situ* hybridization (a) combined with indirect immunofluorescent labeling (Ki-M1P) of Mφ on the same section (b). IL-6 mRNA was detected by *in situ* RT-PCR (c) followed by indirect immunofluorescent labeling (Ki-M1P) of Mφ on the serial section (d). In lung parenchyma C5aR and IL-6 mRNA expression could be noted in leucocytes localized within alveolar walls (filled arrows) (a and c). Whereas C5aR expression was restricted to small Mφ (filled arrows) (a and b), IL-6 mRNA was noted not only in Mφ but also in lymphocytes. Filled arrows denote some IL-6 mRNA-expressing Mφ (c and d). Note that alveolar epithelial cells (open arrows) and large Mφ within the alveolar airspace (arrowheads) fail to express C5aR or IL-6 mRNA (a-d). Original magnification, $\times 400$ (a, b) and $\times 250$ (c, d).

interfollicular lymphocytes, some germinal center cells, and in the endothelium of many capillaries. In contrast, neither C5aR nor IL-6 mRNA expression was detected in epithelioid and multinucleated Ki-M1P-positive Mφ present in granulomas (data not shown).

Investigation of C5aR and IL-6 mRNA in Human Skin

In normal human skin, C5aR and IL-6 mRNA could not be detected in any tissue compartment. In skin from pyogenic granuloma, both C5aR and IL-6 mRNA expression were detectable in the basal and suprabasal cell layer of the epidermis. In addition, all small infiltrating Ki-M1P-positive Mφ expressed C5aR mRNA and many of them expressed IL-6 mRNA. IL-6 mRNA was also expressed in many infiltrating small lymphocytes and some endothelial cells. In skin from lichen planus, C5aR mRNA (Figure 5, a-c) and IL-6 mRNA (Figure 6) were detected within the basal and suprabasal cell layers of the epidermis and also within the infiltrating Ki-M1P-positive Mφ scattered through the subepidermal connective tissue. Infiltrating small lymphocytes and some vascular endothelial cells revealed IL-6 mRNA expression as well.

Discussion

Cellular Expression of C5aR mRNA

Originally it was postulated that C5aR expression is restricted to peripheral blood leukocytes and related cell lines.¹⁰ However, by applying immunohistochemistry, Haviland et al¹² demonstrated that bronchial and alveolar epithelial cells, as well as lung vascular smooth muscle and endothelial cells, may express the C5aR. In the present study, we investigated the cellular expression of C5aR mRNA by applying *in situ* hybridization. In the normal human lung and large intestine and in specimens from those tissues with granulocyte/monocyte or lymphocyte infiltrations, C5aR mRNA could not be detected in the epithelium (bronchial epithelium, alveolar lining cells, and enterocytes), in vascular muscle, or in endothelial cells. The immunohistochemical findings reported by Haviland et al¹² could therefore possibly be a consequence of antibody binding to antigens other than CD88. This interpretation is in accordance with evidence reported by Werfel et al¹³ that specific binding of anti-C5aR monoclonal antibodies to desmosomal antigens is the cause of false positive immunohistochemical signals

Figure 3. C5aR and IL-6 mRNA expression in human lung from pulmonary tuberculosis. C5aR mRNA was detected by *in situ* hybridization (a) combined with immunofluorescent labeling (Ki-M1P) of M ϕ on the same section (b). IL-6 mRNA was detected by *in situ* RT-PCR (c) followed by indirect immunofluorescent labeling (Ki-M1P) of M ϕ on the serial section (d). Whereas C5aR expression (black) (a) was restricted to small M ϕ (green) (b), IL-6 mRNA was noted not only in M ϕ but also in lymphocytes (black) (c). Filled arrows denote some IL-6 mRNA-expressing M ϕ (c and d), whereas the open arrow denotes two lymphocytes expressing IL-6 mRNA (c). Note that M ϕ arranged in epithelioid granulomas (G) do not express C5aR (a) or IL-6 (c), but are Ki-M1P positive (green) (b and d). Arrowheads denote multinucleated M ϕ , which fail to express C5aR or IL-6. Original magnification, $\times 400$.

within the human epidermis. We next asked whether human keratinocytes more closely resemble C5aR-negative pulmonary/intestinal epithelial cells or epithelial liver cells shown to express the C5aR constitutively. To our surprise, we could not observe any C5aR mRNA in the normal epidermis, whereas in those specimens from inflammatory lesions (pyogenic granuloma and lichen planus) keratinocytes as well as tissue-infiltrating M ϕ revealed strong C5aR mRNA expression. This finding demonstrates that in skin disorders, keratinocyte stimulation may not only modulate the functions of migrating inflammatory cells via the production of cytokines,^{19–20} but may also lead to the expression of receptors, eg, for IL-8²¹ and C5a, thereby rendering these cells responsive to signals from the cutaneous microenvironment.

Heterogeneous Expression of C5aR mRNA in Tissue Macrophages

In the normal lung all small Ki-M1P-positive M ϕ localized in subepithelial connective tissue of the bronchial mucous membrane and within the alveolar walls revealed C5aR mRNA expression, whereas receptor transcripts could not be detected in large Ki-M1P-positive M ϕ local-

ized within the alveolar airspace. This evidence suggests that newly arrived M ϕ still express the C5aR, whereas older M ϕ , which have transversed alveolar walls and entered into the alveolar airspace, down-regulate their receptor expression. The same phenomenon was observed in tissue samples of bronchopneumonia and pulmonary tuberculosis, in which freshly immigrated small M ϕ found within the bronchial wall or fibrous septa expressed C5aR transcripts, whereas M ϕ localized within the airspace or present in granulomas did not reveal any detectable C5aR expression. In line with these observations, we did not observe C5aR expression in epithelioid/multinucleated M ϕ found within granulomas in Crohn's disease. Considering the role of C5aR as a chemoattractant receptor, these findings suggest that tissue M ϕ cease to express C5aR when they stop migrating.

Co-expression of C5aR and IL-6 mRNA

Using a double staining method based on a combination of ISH and indirect immunofluorescence on the same section, it could be demonstrated that in biopsy specimens of normal tissues and also in those of pathological disorders, all migrating but not resident M ϕ express

Figure 4. Epithelial cells of the large intestine express neither C5aR nor IL-6 mRNA. C5aR mRNA was detected by *in situ* hybridization followed by counterstaining with Mayer's hematoxylin (a) whereas IL-6 mRNA was detected by *in situ* RT-PCR without nuclear counterstaining (b). In the human large intestine C5aR or IL-6 mRNA could be noted in leucocytes localized in the subepithelial connective tissue (filled arrows), but not within epithelial cells (open arrows). Original magnification, $\times 400$.

C5aR mRNA. However, technical reasons such as the denaturation of CD68 after 60 cycles of PCR excluded the detection of CD68 and IL-6 mRNA on the same section. Because serial sections 5–10 μm apart from each other were used, we found it difficult to locate unequivocally the Ki-M1P- and the IL-6 mRNA-derived signals to the same cells in the tissues examined. We estimate that 10 to 40% of the CD68 and C5aR mRNA-positive infiltrating M ϕ coexpress IL-6 mRNA. This is in line with recently published data showing that about 20% of Ki-M1P-positive M ϕ in lymph nodes express IL-6 mRNA.¹⁷ Surprisingly, a close correlation of C5aR and IL-6 mRNA expression was found in almost all basal and suprabasal keratinocytes from pyogenic granuloma and lichen planus, but not in keratinocytes from normal skin.

IL-6 is a cytokine with multiple growth and differentiation activities,²² the expression of which has been demonstrated in cultured monocytes and keratinocytes following stimulation.^{23,24} Previously, Scholz et al²⁵ showed that recombinant human C5a induces IL-6 production in monocytes. Subsequently, Höpken et al²⁶ reported that anti-C5a monoclonal antibodies significantly lower peripheral blood IL-6 concentrations in pigs infused with *E. coli*. They concluded that C5a plays a key role for the

Figure 5. C5aR mRNA expression in human skin from lichen planus. C5aR mRNA was detected by *in situ* hybridization followed by counterstaining with nuclear fast red. Small infiltrating M ϕ within the subepidermal connective tissue (a) (arrowheads) and basal/suprabasal keratinocytes (a and b) (filled arrows) show strong cytoplasmic staining, whereas superficially localized keratinocytes (b) (open arrow) remain negative for C5aR mRNA. By applying sense riboprobes, no staining could be noted in basal/suprabasal keratinocytes (c) (filled arrow), or superficially localized keratinocytes (c) (open arrow). Original magnification, $\times 125$ (a), $\times 1000$ (b), and $\times 500$ (c).

expression of IL-6 during the early phase of acute inflammatory reactions. Our observation that *in situ*, both C5aR and IL-6 are coexpressed in 10–40% of migrating M ϕ and in almost all stimulated keratinocytes may be in line with the regulatory role of C5a on IL-6 expression in cell types that express the C5aR constitutively or upon stimulation.

Further studies will need to address the nature of the stimuli leading to C5aR upregulation in keratinocytes, the relationship between the pro-inflammatory mediators C5a and IL-6 in dermatoses, and the mechanisms leading to

Figure 6. IL-6 mRNA expression in human skin from lichen planus. IL-6 mRNA was detected by *in situ* RT-PCR without nuclear counterstaining. Almost all basal and suprabasal keratinocytes from affected lichen planus express IL-6 mRNA. Original magnification, $\times 400$.

down-regulation of C5aR in older M ϕ after their migration into tissue lesions.

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