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The Anaphylatoxin Receptor C5aR Is Present During Fracture Healing in Rats and Mediates Osteoblast Migration In Vitro

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Abstract

Background—There is evidence that complement components regulate cytokine production in osteoblastic cells, induce cell migration in mesenchymal stem cells, and play a regulatory role in normal enchondral bone formation. We proved the hypothesis that complement might be involved in bone healing after fracture.

Methods—We investigated the expression of the key anaphylatoxin receptor C5aR during fracture healing in rats by immunostaining after 1, 3, 7, 14, and 28 days. C5aR expression was additionally analyzed in human mesenchymal stem cells (hMSC) during osteogenic differentiation, in human primary osteoblasts, and osteoclasts by reverse transcriptase polymerase chain reaction and immunostaining. Receptor functionality was proven by the migratory response of cells to C5a in a Boyden chamber.

Results—C5aR was expressed in a distinct spatial and temporal pattern in the fracture callus by differentiated osteoblast, chondroblast-like cells in cartilaginous regions, and osteoclasts. In vitro C5aR was expressed by osteoblasts, osteoclasts, and hMSC undergoing osteogenic differentiation. C5aR was barely expressed by undifferentiated hMSC but was significantly induced after osteogenic differentiation. C5aR activation by C5a induced strong chemotactic activity in osteoblasts, and in hMSC, which had undergone osteogenic differentiation, being abolished by a specific C5aR antagonist. In hMSC, C5a induced less migration reflecting their low level of C5aR expression.

Conclusions—Out in vitro and in vivo results demonstrated the presence of C5aR in bone forming osteoblasts and bone resorbing osteoclasts. It is suggested that C5aR might play a regulatory role in fracture healing in intramembranous and in enchondral ossification, one possible function being the regulation of cell recruitment.

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Keywords

Complement system; C5aR (CD88); Mesenchymal stem cells; Osteoblast; Fracture healing

There is strong clinical evidence that fracture healing is delayed in trauma patients with significant additional injuries.¹⁻³ The reasons remained unidentified, but one reason could be the posttraumatic systemic inflammatory response influencing bone healing locally. One of the key systems of the systemic inflammatory reaction is the complement system. It is a crucial part of the innate immunity, consisting of more than 30 proteins. Its functions are the opsonization of antigens, the cytolysis of microorganisms, the support of phagocytosis, and the induction and modulation of the inflammatory reactions.⁴ After trauma, the injured tissue activates complement by four well-established pathways: the classical, the lectin, the alternative, and the extrinsic pathway.⁵ All pathways lead in the end to the activation of the anaphylatoxin receptors C3aR and C5aR, inducing important inflammatory functions such as cell migration, vasodilatation, mast cell destabilization, cytokine release, and regulation of apoptosis.⁶

In contrast to its beneficial effects in the immune defense system., the excessive activation of complement can provoke detrimental side effects. Because of its potent inflammatory profile, C5a seems to be the most dangerous molecule and may lead for example to immunoparalysis and multiorgan dysfunction.⁶⁻⁸ Because of its proinflammatory effects, excessive complement activation might contribute to the delayed fracture healing observed in polytraumatic patients.

However, little is known about the role of complement in bone biology and regeneration. Some in vitro studies demonstrated the expression of complement-related genes in bone cells. Osteoblasts have been shown to produce C3 in response to vitamin D3.⁹ C3aR, C1q, C4, C1r, C1s, C1 inhibitor, properdin, and factor H were found to be differentially expressed during osteogenic differentiation in vitro.^{10,11} Functionally active C5aR was present in osteoblastic MG 63 cells and modulated IL-6 production.¹² Recently, it was demonstrated that C3a and C5a were chemoattractants for mesenchymal stem cells.¹³ Therefore, we speculated that C3a and C5a might contribute to stem cell recruitment in injured tissues in vivo.

In vivo C1s was found in hypertrophic chondrocytes during enchondral ossification, possibly participating in cell disintegration and matrix degradation.¹⁴ C3, C5, C9, and factor B were expressed in a distinct pattern in the growth plate, suggesting that complement proteins may be important in cartilage bone transformation.¹⁵ These in vitro and in vivo studies provided the first evidence that complement components might play a role in bone regeneration, for instance, by the regulation of cell migration and cytokine release, and in normal enchondral bone formation. To our knowledge, the involvement of complement in fracture healing has not yet been investigated.

To address this question, we investigated the time-dependent expression of the key anaphylatoxin receptor C5aR during fracture healing in rats. C5aR expression was additionally analyzed in vitro in human mesenchymal stem cells (hMSC) during osteogenic differentiation, in osteoblasts, and in osteoclasts. Receptor functionality was proven by the migratory response of hMSC and osteoblasts to C5a.

MATERIALS AND METHODS

The isolation of human primary cells was approved by the Ethical Committee of the University of Ulm, Germany. The animal experiment was strictly conducted according to

national and international animal protection guidelines and approved by the German Government (Reg. No. 814).

Cultivation of hMSC, Osteoblasts, and Osteoclasts

hMSC were prepared from bone marrow aspirates obtained from surgical procedures on five donors during anterior cruciate ligament replacement or pelvic osteotomies (men, aged, 20–38 years), according to established protocols.¹⁶ hMSC were cultured in Dulbecco's Modified Eagle Medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). The cells were characterized by their typical expression profile of CD markers (presence of CD9, CD54, CD90, CD166, STRO-1; absence of CD34, CD45) and by their differentiation potential toward the osteoblastic, chondroblastic, or adipogenic lineages.^{17,18} Cells from passages 2 or 3 were used for the experiments. To induce osteogenic differentiation, 1×10^4 hMSC/cm² were seeded into 24-well tissue culture plates (Nunc, Wiesbaden, Germany) in DMEM supplemented with 10% FCS, 0.1 μ mol/L dexamethasone, 10 mmol/L β -glycerophosphate, and 0.2 mmol/L ascorbate-2-phosphate (all from Sigma-Aldrich, Taufkirchen, Germany). To confirm successful osteogenic differentiation, calcium deposition was analyzed by von Kossa staining, and staining for alkaline phosphatase (86R-1KT, Sigma-Aldrich, Taufkirchen, Germany) was performed after 21 days of culture. The capacity to differentiate toward the osteogenic lineage was also proven in the additional presence of 100 ng C5a in the differentiation medium.

Primary human osteoblasts were isolated from bone samples of five male donors undergoing operation for fracture repair (aged 27–78 years) by collagenase digestion and cultured in DMEM supplemented with 10% FCS as previously described.^{19,20} Cells from passages 3 or 4 were used for the experiments. The cells isolated by this procedure express osteogenic markers, e.g., alkaline phosphatase and osteocalcin, and were, therefore, regarded as osteoblasts.^{19,20}

Human osteoclasts were generated from peripheral blood mononuclear cells (PBMNC) obtained from the blood of anonymous healthy donors (Red Cross Blood Bank Baden-Württemberg, Ulm, Germany) as previously described.²¹ After 20 days, osteoclasts were identified as tartrate resistant acid phosphatase positive multinucleated cells. Their characteristic resorption activity was confirmed on dentin (pit-assay).²¹

C5aR mRNA Expression

To investigate mRNA expression of C5aR, total RNA was isolated from undifferentiated hMSC, from hMSC treated with osteogenic differentiation medium for 14, 21, and 28 days, and from primary osteoblasts, subjected to reverse transcription reactions and analyzed by real-time reverse transcriptase polymerase chain reaction as previously described.²² For C5aR amplification, 5'-CTC AAC ATG TAC GCC AGC AT-3' (sense primer) and 5'-CAG GAA GGA GGG TAT GGT CA-3' (antisense primer) were used. The amount of the respective amplification product was determined relative to the housekeeping gene GAPDH.

C5aR Immunostaining

Immunostaining of hMSC, osteoblasts, osteoclasts, and of paraffin sections from the fracture callus of rats and intact human bone was performed by the use of a polyclonal goat anti-C5aR antibody (anti-CD88; Santa Cruz Biotechnology, CA) at 4 μ g/mL diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 60 minutes at room temperature (overnight at 4° for fracture sections), followed by biotinylated rabbit anti-goat antibody (Invitrogen, Carlsbad, CA) for 15 minutes (30 minutes for tissue sections). For negative controls, the primary antibody was substituted with goat immunoglobulin (IgG:

I5256, Sigma-Aldrich, Taufkirchen, Germany). Endogenous peroxidase activity was stopped with 3% H₂O₂ and unspecific binding was blocked with 2% bovine serum albumin and 0.1% Triton X-100 in tris buffer. Biotin was detected using ZytoChem-Plus streptavidin-horse radish peroxidase technology and 3-amino-9-ethylcarbazol as the chromogen (Zytomed Systems, Berlin, Germany). Finally, counterstaining with hematoxylin was performed.

Chemotaxis Assay

The chemotactic response of nondifferentiated hMSC, and hMSC that were differentiated toward to osteogenic lineage for 21 days, and primary osteoblasts was measured with a modified Boyden chamber assay using a 48-well microchemotaxis chamber (NeuroProbe, Baltimore, MD) and as migration filters, polycarbonate filters with 8- μ m pores (Whatman Biometra, Göttingen, Germany).²³ The lower wells of the chemotaxis chamber were filled with DMEM without (negative control) or with 10, 100, or 1,000 ng/mL human recombinant C5a (Sigma-Aldrich, Taufkirchen, Germany) and covered by the migration filter. About 1×10^4 cells in 50 μ L DMEM of each cell type were individually added to the upper wells. After 4-hour incubation, the filter was carefully removed. Nonmigrated cells on the upper side were eliminated by rinsing the filter with cold PBS and scraping it over a rubber wiper. The remaining migrated cells on the lower side of the filter were fixed with 4% formaldehyde and Giemsa stained for cell counting. In addition, the migration of osteoblasts in response to 100 ng/mL C5a was tested with and without preincubation with a C5aR antagonist AcF[OPdChaWR] at a concentration of 1 μ g/mL for 1 hour. This C5aR antagonist has been shown to specifically block C5a-mediated effects in various rodent disease models.^{24,25} A checkerboard analysis was performed with osteoblasts by adding 100 ng/mL C5a to either only the lower well or to both the upper and lower wells to distinguish between chemotaxis and random migration.²³

Fracture Healing Experiments in Rats

The fracture healing experiments were performed as previously described²⁶ in 30 male Wistar rats weighing 250 g to 300 g (Charles River, Sulzfeld, Germany). Briefly, a transverse fracture of the right tibia was created and stabilized by a Kirschner wire (7 mm; Synthes, Umkirch, Germany) inserted in the intramedullar cavity of the tibia. Six animals each were killed after 1, 3, 7, 14, and 28 days postfracture. The tibia was fixed in sodium phosphate-buffered 4% formalin (pH, 7.0–7.4), decalcified more than 18 days using 25% ethylenediamine tetra-acetic acid (pH adjusted from 7.2 to 7.4), and embedded in paraffin. Sections of 6 μ m to 8 μ m were cut and then stained using Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany) for histologic observation. C5aR immunostaining was performed as described above and qualitatively evaluated in the different zones of the fracture callus over the healing time.

C5aR Expression in Intact Human Bone

With informed consent, a bone specimen of the proximal humerus of a male donor was obtained and processed as described above. C5aR immunohistochemistry was performed as described above.

Statistical Analysis

The cell culture experiments were performed in three to six independent experiments, which were performed in triplicates or quadruplicates. Data were expressed as mean $\pm$ standard error of the mean. Statistical analysis was performed using a nonparametric Mann-Whitney *U* test (SPSS Version 10.1.3; SPSS, Chicago, IL). Results with $p \leq 0.05$ were considered significant. The immunostaining was evaluated qualitatively.

RESULTS

C5aR Expression in Bone Cells and During Osteogenic Differentiation In Vitro

In undifferentiated hMSC, C5aR mRNA was barely detectable, whereas its expression was significantly induced after 14 days of culture in the presence of osteogenic differentiation medium (Fig. 1). C5aR mRNA expression increased slightly further during ongoing osteogenic differentiation up to day 28. Successful osteogenic differentiation was confirmed by positive matrix mineralization and alkaline phosphatase activity (Fig. 2, A–D). The addition of C5a to the cell culture medium did influence the successful differentiation toward the osteogenic lineage determined by von Kossa staining and staining of alkaline phosphatase activity (Fig. 2, E and F). C5aR mRNA was also expressed in primary human osteoblasts (Fig. 1).

C5aR was also expressed at the protein level as demonstrated by immunostaining (Fig. 3). Undifferentiated hMSC were minimally stained (Fig. 3, A), whereas the receptor was expressed if osteogenic differentiation was induced (Fig. 3, C), confirming the results at the mRNA level. Osteoblasts and osteoclasts were also strongly positively stained for C5aR (Fig. 3, D and E). In controls with nonspecific IgG, no positive staining was observed (Fig. 3, B and F).

Effect of C5a on the Migration of hMSC and Osteoblasts

Under basal conditions, the migration of 25 ± 2 MSC and 30 ± 4 primary osteoblasts, respectively, was observed. C5a at concentrations ranging from 10 ng/mL to 1,000 ng/mL induced a significant migratory response of human primary osteoblasts at all the tested concentrations that was concentration dependent (Fig. 4). In nondifferentiated hMSC, cell migration was significantly induced only by the highest C5a concentration of 1,000 ng/mL (87 ± 12 migrated cells) (Fig. 4). In contrast, in vitro differentiation of hMSC toward the osteogenic phenotype led to a significantly enhanced migratory response to 100 ng/mL C5a (74 ± 11 migrated cells) in comparison with nondifferentiated hMSC (31 ± 3 migrated cells).

To confirm the direct functional involvement of the C5aR, a preincubation with a specific C5aR inhibitor was performed, which completely abolished the migratory response of primary osteoblasts to 100 ng/mL C5a (Fig. 5). To rule out the possibility that C5a induced undirected migration (chemokinesis), a checkerboard analysis was performed with primary osteoblasts using 100 ng/mL C5a. The results clearly indicated that the migratory response only occurred in the presence of a positive concentration gradient (Fig. 5).

C5aR Expression During Fracture Healing in Rats

The callus formation during fracture healing in the rat tibia over time was described in a previous study of our group by quantitatively evaluating the relative amounts of newly formed bone, cartilage, and soft tissue.²⁶ In this study, we qualitatively evaluated the spatial and temporal pattern of C5aR expression in the fracture callus (Fig. 6).

After 1 day, a hematoma was formed within the fracture gap containing platelets and inflammatory cells. Most of the inflammatory cells (neutrophils, PBMNC) were positively stained for C5aR. Distal and proximal to the fracture proliferation of the periosteum was initiated, where occasionally positively stained cells were observed. Some osteocytes within the cortex near the fracture also expressed C5aR. In adjacent control sections with nonspecific IgG, no positive staining was observed at this or any other time point (results not shown).

After 3 days, the amount of inflammatory cells in the fracture gap decreased, with significant numbers again being positively stained for C5aR (Fig. 6, day 3, A). The dominating tissue was fibrous mesenchymal tissue, which occupied the callus area and contained large numbers of fibroblast-like cells, which did not express C5aR. At the same time, new bone started to be formed along the periosteal surface at some distance from the fracture gap. Most of the osteoblasts in this newly formed bone strongly expressed C5aR (Fig. 6, B). Osteocytes within the cortex near the fracture gap also stained positive for C5aR but to a lesser extent compared with the osteoblasts. At the endosteal surface of the cortex, strongly stained active osteoclasts were observed.

After 7 days, only a few positively stained inflammatory cells remained. Fibrous connective tissue reached its maximum amount (Fig. 6, day 7). Fibroblast-like cells again did not express C5aR. At the same time, intramembranous bone formation took place distal and proximal from the fracture gap. Nearly, all the osteoblasts in this newly formed bone were strongly stained for C5aR (Fig. 6, D). Close to the fracture gap, cartilage was formed and enchondral ossification occurred. Most of the chondroblastic cells in these zones were strongly stained for C5aR (Fig. 6, C). C5aR staining appeared less intensive in hypertrophic chondroblastic cells during enchondral ossification. At the endosteal bone surface active osteoclasts were again observed expressing C5aR.

After 14 days, some fibrous tissue remained in the immediate vicinity of the fracture (Fig. 6, day 14). The amount of fibrous cartilage reached its maximum. Chondroblastic cells were intensively stained for C5aR as on day 7. Newly formed bone continuously replaced the cartilage by enchondral ossification. Osteoblasts at the bone-forming surfaces and within the newly formed bone tissue exhibited C5aR expression, as did osteocytes both here and in the cortex (Fig. 6, E and F). Positive osteoclasts were observed in regions where new bone was replacing calcified cartilaginous tissue (Fig. 6, F).

After 28 days, cartilaginous tissue was significantly reduced, with the majority of the callus having been transformed to bone (Fig. 6, day 28). Staining of chondroblastic cells resembled that of day 7 (Fig. 6, G). The majority of the callus was transformed to bone. The fracture gap was closed with bone containing strongly positively stained osteoblasts (Fig. 6, H). The developing osteocytes within the callus appeared less frequently stained. C5aR-stained osteoclasts were again present at the endosteal cortex and within the cartilaginous tissue or bone region in the callus.

C5aR Expression in Human Bone

Consistent with the results from the cell culture experiments, a strong positive staining of bone cells in nonfractured healthy human bone was observed. In addition to a strong staining of osteoblasts on the surface of the trabeculae, osteocytes as well as multinucleated osteoclasts and peripheral blood nucleated cells in the bone marrow were also positively stained for C5aR (Fig. 6, human bone, I, J).

DISCUSSION

There is evidence that complement components can regulate cytokine production in osteoblastic cells,¹² induce cell migration in mesenchymal stem cells,¹³ and mediate cell disintegration and matrix degradation in normal enchondral bone formation,^{14,15} suggesting that complement might play a role in bone biology and formation. We proved the hypothesis that complement components might be involved in bone healing after fracture. This study demonstrated for the first time the presence of the key receptor C5aR in a distinct spatial and temporal pattern in the zones of intramembranous and enchondral bone formation of the fracture callus. Furthermore, we clearly showed that functional C5aR was minimally

expressed in undifferentiated mesenchymal stem cells but was strongly induced during osteogenic differentiation, this receptor mediating the migration of differentiated osteoblasts in vitro.

In this study, we focused on the anaphylatoxin receptor C5aR because of its key function in complement-mediated cell reactions. In inflammatory cells C5aR activation induces, for example, chemoattraction, cytokine release, mast cell destabilization and respiratory burst induction, and regulating apoptosis.⁶⁻⁸ However, little is known about the expression and function of C5aR in bone cells. One study demonstrated that human osteoblast-like osteosarcoma cells (MG-63) express functional C5aR and respond to C5a by IL-6 release.¹² Recently, it was reported that C5aR and C3aR are expressed by hMSCs and mediate cell migration.¹³ In vivo data on C5aR expression in bone was lacking.

Our in vitro results revealed C5aR expression both at the mRNA and protein levels by human primary osteoblasts, osteoclasts, and by hMSC undergoing osteogenic differentiation. C5aR was barely expressed in hMSC but was significantly induced after differentiating these cells toward the osteogenic lineage. Other studies similarly demonstrated differential regulation of complement-related genes during osteogenic differentiation.^{10,11} In primary murine osteoblasts, C1q, C4, C3aR, properdin, C1 inhibitor, and complement factor H were among those genes displaying the greatest induction after osteogenic differentiation in an Affymetrix Gene-Chip array. The authors proposed that, because of their strong upregulation, complement proteins might play a significant role in osteoblast development and function but without further investigating possible mechanisms.¹⁰ In contrast to this study, Billiard et al. found a strong down-regulation of C1r, C1s, and factor H during osteogenic differentiation in a human osteoblast cell line. On the basis of the observation that C3 increased osteoclast development in vitro,²⁷ we speculated that the observed decrease in the expression of complement-related genes in mature osteoblasts could result in suppressed osteoclast formation during new bone formation.¹¹

From these studies and our in vitro results, it can be assumed that complement proteins might have a stage-dependent impact on cells of the osteogenic lineage and this was confirmed by this study. As C5a acts as a chemoattractant for hMSC,¹³ we performed a migration assay to demonstrate receptor functionality. C5a induced a strong dose-dependent migratory response in human primary osteoblasts and in hMSC after their differentiation toward the osteogenic lineage. Migration could be completely abolished by a specific C5aR antagonist, indicating that the effect of C5a was mediated by C5aR. Thereby, C5a specifically stimulated directed cell migration (chemotaxis) and not random migration (chemokinesis), because the effect was dependent on a positive concentration gradient. Mirroring the very low level of C5aR expression by undifferentiated hMSC, their migratory response was significantly reduced compared with differentiated MSC and to primary osteoblasts. The quantitative effect of C5a on osteoblasts was comparable to classical growth factors involved in bone metabolism like PDGF-BB, BMP-2, or IGF-I and II.²³ In conclusion, osteogenic differentiation of hMSC in vitro did not only increase C5aR expression but also chemotaxis in response to C5a, suggesting that within the osteogenic lineage more mature cells may be more responsive to C5a.

In contrast to our results, Schraufstatter et al.¹³ found that C5aR was strongly expressed in undifferentiated hMSC and mediated their migration in response to C5a. The authors suggested that C5a might contribute to stem cell recruitment in injured tissues. These contradictory findings could possibly result from differences in the differentiation status of the cells. Being a limitation, we did not confirm that the cells used actually represent undifferentiated hMSC lacking expression of osteogenic marker genes. Confirming our

results, Schmal et al.²⁸ found an induction of migration by C5a in PBMNC but not in undifferentiated hMSC.

To prove whether the differentiation potential of hMSC was affected by C5a, we treated the cells with clinically relevant concentrations during differentiation in vitro. Successful matrix mineralization and alkaline phosphatase activity were not influenced, indicating that the osteogenic potential of hMSC was not altered by C5a.

In vivo C5aR was expressed in the fracture callus of rats throughout the entire healing process in a distinct spatial and temporal pattern. Immediately after fracture, C5aR was expressed abundantly by inflammatory cells in the fracture hematoma as expected. C5a and its interaction with C5aR are crucial for the recruitment of neutrophils and PBMNC. Activation of C5aR on these cells leads to an inflammatory response, e.g., cytokine release and an increase of phagocytosis,⁶⁻⁸ modulating the early inflammatory reaction in the fracture hematoma. In the early healing phase, the hematoma was continuously replaced by connective tissue, which reached its maximum amount on day 14.²⁶ The mesenchymal fibroblast-like cells in this soft callus lacked C5aR expression, implying that C5a may not affect this type of cell during fracture healing, and this was observed during the entire healing process. This is consistent with our in vitro results demonstrating that early progenitor cells did not express C5aR.

In contrast, the receptor was abundantly expressed in differentiated osteoblasts depositing matrix at the zones of intramembraneous ossification, which started to take place at day 3 near the periosteum at some distance from the fracture gap. The zones of intramembraneous bone formation with strongly stained osteoblasts continuously extended in the direction of the fracture gap during the healing process. The osteocytcs embedded in the new mineralized matrix were also stained, but this was less intensive. This suggests that C5aR functions might be especially important during new bone formation by osteoblasts. Whether the potential role of C5aR in these cells is based on the functions observed in our and other¹² in vitro experiments, e.g., cell recruitment and cytokine release, or other unknown mechanisms, has to be investigated further.

Near the fracture gap in the peripheral callus, the connective tissue was continuously replaced by cartilage starting at day 7 and reaching its maximum amount at day 14. The fibrous cartilage was continuously mineralized and subsequently replaced by bone during enchondral ossification. C5aR was strongly expressed in chondroblastic cells in these regions during the entire healing process. Our findings suggest that C5aR mediated effects on chondroblastic cells might be important in secondary enchondral bone formation during fracture healing. The cellular effects have to be addressed in further studies. These findings support the theory of complement playing a crucial role in enchondral bone formation.^{14,15} C1s was found in hypertrophic chondrocytes at the secondary ossification center of hamster tibiae and possibly mediated cell disintegration and matrix degradation.¹⁴ C3, C5, C9, and factor B were found in a distinct pattern in the growth plate of rats, suggesting an important role in cartilage bone transformation.¹⁵

Confirming our in vitro findings, osteoclasts also strongly expressed C5aR during fracture healing. It is well known that osteoclasts play a major role in bone healing and were present during the entire healing process. In the early phase, we found osteoclasts mainly at the endosteal zones near the fracture gap. In the later healing stages, they resorb bone during callus remodeling. During all stages, osteoclasts were densely stained for C5aR. Because it has been shown that osteoclasts also expressed C3aR and that C3 could potentiate osteoclast development,²⁷ and the osteoblasts could secrete C3 in response to vitamin D3,⁹ it can be suggested that complement might regulate osteoblast-osteoclast interaction during fracture

healing. Additionally, we were able to show that C5aR was also expressed in healthy human bone. In addition to strong expression in osteoblasts on the surface of the trabeculae, osteocytes and osteoclasts were positively stained, indicating relevance also for the human situation.

In conclusion, this study demonstrated that functional C5aR was significantly upregulated during osteogenic differentiation and mediated cell migration. The receptor was also expressed by osteoclasts. C5aR was expressed in a distinct spatial and temporal pattern during fracture healing by differentiated osteoblasts, chondroblasts, and osteoclasts and might play a role in intramembranous and enchondral ossification. In ongoing studies on knockout mice with an impaired complement system, we further investigate the mechanisms of complement components in fracture healing.

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Figure 1. C5aR mRNA expression relative to the house keeping gene GADPH; o-hMSC, cells cultivated under osteogenic conditions for 14 days, 21 days, and 28 days; OB, primary osteoblasts; six independent experiments, which were performed in triplicates. * $p \leq 0.05$.

Figure 2.

Proof of successful osteogenic differentiation by von Kossa (*left side*) and alkaline phosphatase staining (*right side*); negative von Kossa (*A*) and alkaline phosphatase (*B*) staining of undifferentiated hMSC; positive von Kossa (*C*) and alkaline phosphatase (*D*) staining of hMSC cultivated under osteogenic conditions for 21 days; positive von Kossa (*E*) and alkaline phosphatase (*F*) staining of hMSC cultivated under osteogenic conditions for 21 days and treated with 100 ng/ml C5a.

Figure 3. Immunostaining of C5aR of bone cells in vitro; (A) C5aR negative undifferentiated hMSC; (B) negative control after incubation with goat IgG; (C) C5aR positive differentiated hMSC; (D) C5aR positive primary osteoblasts; (E) C5aR positive multinucleated osteoclast; (F) corresponding negative control after Incubation with goat IgG, multiple cell nuclei were counterstained with hematoxylin.

Figure 4. C5a-induced migration of undifferentiated hMSC (*white columns*) and primary osteoblasts (*grey columns*). C, basal conditions without addition of C5a. Three independent experiments, which were performed in quadruplicates. * $p \leq 0.05$.

Figure 5.

Left side, checkerboard analysis, 100 ng/ml C5a was added to the lower well alone or to both the lower and the upper wells of a Boyden chamber; only directed cell migration occurred. *Right side*, C5a induced migration could be completely abolished by preincubation with a C5aR specific antagonist (Rec-Inhib). Osteoblasts from one donor, experiments were performed in quadruplicates. * $p \leq 0.05$.

Figure 6.

Lines 1–4, Immunostaining of C5aR in the fracture callus of the rat tibia during the time course of fracture healing from day 3 until day 28. *Left side*, Overviews of the fracture callus on specified days during healing (Giemsa staining). (A–H) Detailed pictures. (A) Inflammatory cells at the fracture gap positively stained for C5aR. (B) Periosteum with osteoblasts positively stained for C5aR. (C) Chondroblast-like cells in the fracture callus positively stained for C5aR. (D) Osteoblasts in newly formed bone positively stained for C5aR. (E) Positively stained osteocytes in cortical bone. (F) Newly formed bone with osteocytes and osteoblasts positively stained for C5aR. (G) Chondroblast-like cells in the fracture callus positively stained for C5aR. (H) Positively stained cells in the fracture region. *Line 5*, Immunostaining of C5aR in a healthy human bone. *Left side*: overview. (I and J) Detailed pictures. (I) Osteocytes positively stained for C5aR. (J) Osteoblasts at the surface of a trabecular bone positively stained for C5aR.