The Anaphylatoxin Receptor C5aR is Expressed in Bone Cells in vitro and During Fracture Healing in vivo

INTRODUCTION:
There is clinical evidence that fracture healing is delayed in polytrauma patients with significant additional injuries. The reasons remain unidentified, but may at least in part be caused by the systemic inflammatory response after severe trauma. One of the key systems involved in the inflammatory response is the complement system, a complex protein cascade consisting of over 30 different proteins. The influence of complement on bone and on fracture healing has barely been investigated. A few studies have demonstrated the presence of complement components in bone cells, clearly indicating that there is an interaction of the complement system with bone cells. However, to date their function has remained unknown. In vivo data on the expression of complement components in bone cells is completely lacking. The aim of our study was, therefore, to investigate the presence of the anaphylatoxin receptor C5aR in bone derived cells in vitro and in vivo during fracture healing. We examined the expression of C5aR in human mesenchymal stem cells (MSC) during osteogenic differentiation, as well as in osteoblasts and osteoclasts. To elucidate one of the possible functions of C5aR, the migratory response of osteoblasts to C5a was investigated. Furthermore, the expression of C5aR during fracture healing was investigated in rats.

METHODS:
C5aR expression in MSC, osteoblasts, and osteoclasts:
The isolation of human cells was approved by the Ethical Committee of the University of Ulm, Germany. MSC were isolated from bone marrow aspirates by density gradient centrifugation and characterized by the typical expression profile of CD markers (presence of CD9, CD54, CD90, CD166, STRO-1; absence of CD34, CD45) and by the differentiation potential towards the osteoblastic, chondroblastic or adipogenic lineages. For osteogenic differentiation MSC were treated with 0.1 µM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM ascorbate-2-phosphate. Osteogenic differentiation was proven by von Kossa and alkaline phosphatase staining. Human osteoblasts were isolated from bone biopsies by collagenase digestion and cultivated for 21 days in medium containing 0.1 µM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM ascorbate-2-phosphate. Human osteoclasts were generated from peripheral blood mononuclear cells by treatment with 20 ng/ml M-CSF (Millipore, USA). Expression of C5aR was investigated in MSC after 6, 14, 21 and 28 days osteogenic differentiation, in osteoblasts and in osteoclasts. C5aR expression was measured on the mRNA level with real-time RT-PCR on an iCycler system and on the protein level by C5aR immunostaining (anti-CD88; Santa Cruz Biotechnology Inc., USA). The migratory response of MSC and osteoblasts to C5a was investigated by a chemotaxis assay in a Boyden chamber. C5aR expression during fracture healing in rats:
The animal experiment was conducted according to international animal protection guidelines after approval by the German Government (Reg.-No. 814). A transverse fracture of the right tibia and fibula was created by a chemotaxis assay in a Boyden chamber. Human osteoblasts were isolated from peripheral blood mononuclear cells by treatment with 20 ng/ml M-CSF and 0.2 mM ascorbate as well as in osteoblasts and osteoclasts. To elucidate one of the possible functions of C5aR, the migratory response of osteoblasts to C5a was investigated. Furthermore, the expression of C5aR during fracture healing was investigated in rats.

RESULTS:
C5aR expression in MSC, osteoblasts, and osteoclasts:
In non-differentiated MSC C5aR mRNA was barely detectable. After 14 days of culture in osteogenic differentiation medium C5aR expression increased twelve-fold. After 3 and 4 weeks C5aR expression increased further reaching statistically significant levels compared to day 0. To confirm osteogenic differentiation, mineralization and alkaline phosphatase activity was positively proven. In primary osteoblasts isolated directly from bone, mRNA expression of C5aR was lower in comparison to differentiated MSC but was increased compared to undifferentiated cells. The expression of C5aR was confirmed on the protein level by immunostaining. C5aR was also expressed in osteoclasts. Migratory response to C5a:
Under basal conditions migration of 25±4 MSC and 30±8 primary osteoblasts (M±SD), respectively could be observed. In comparison to these basal values, C5a ranging from 10-1000 ng/ml induced a significant concentration dependent migratory response of human primary osteoblasts (273±111 migrated cells when 1000 ng/ml C5a was used). Osteogenic differentiation of MSC led to a more enhanced migratory response to 100 ng/ml C5a. In order to confirm the direct functional involvement of the C5aR, a pre-incubation with a specific C5aR inhibitor was performed which completely abolished the migratory response to 100 ng/ml C5a. C5aR expression during fracture healing in rats:
After day 1 a hematoma was observed within the fracture gap containing platelets and inflammatory cells, many of the latter being stained positively for C5aR. Distal to the fracture proliferation of the periosteum was initiated, where occasionally positive cells were observed. After 3 days large numbers of unstained fibroblasts were present at the fracture site. Distal to the fracture thickening of the periosteum had occurred where bone was already starting to be formed by positively stained osteoblasts. Osteocytes within the cortex also stained positive for C5aR. After 7 days cartilaginous tissue was formed by chondroblast-like cells also positively stained for C5aR, while chondroblasts undergoing cell death showed loss of such staining. After 2 weeks little fibrous tissue remained in the vicinity of the fracture. The C5aR pattern of chondroblast-like cells was similar to day 7. Osteoblasts within the newly formed bone tissue again exhibited C5aR expression, with osteocytes there also stained. Positively stained osteoblasts were observed in regions where new bone was replacing calcified cartilaginous tissue. After 4 weeks the majority of the callus was transformed to bone with an expanding marrow. The C5aR expression pattern was similar to day 14.

DISCUSSION:
In vitro C5aR mRNA was barely detectable in human MSC but was up-regulated during osteogenic differentiation and also found in osteoblasts and osteoclasts. C5aR induced a strong dose dependent chemotactic activity in osteoblasts, which could be completely abolished by a specific C5aR antagonist. A significantly reduced chemotactic activity was found in MSC reflecting their lower level of C5aR expression. C5aR was also abundantly detected in osteoblasts, chondroblasts and osteoclasts during all stages of fracture healing in rats. It can be suggested, therefore, that the complement systems plays a role in bone biology and that it has a stage-dependent impact on cells from the osteogenic lineage. The expression of C5aR in osteoblasts and osteoclasts and the induction of chemotaxis by C5a in osteoblastic cells indicate that C5a might play a regulative role in these processes. A stimulation of local osteoblast recruitment by C5a during the course of fracture healing may support a functional role of osteoimmunologic processes in bone tissue regeneration.

REFERENCES: