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Role of the chemokines CCL3/MIP-1α and CCL5/RANTES in sponge-induced inflammatory angiogenesis in mice

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ABSTRACT

Objective: We examined the potential contribution of CCL3 and CCL5 to inflammatory angiogenesis in mice. *Methods:* Polyester-polyurethane sponges were implanted in mice and blood vessel counting and hemoglobin, myeloperoxidase and N-acetylglucosaminidase measurements used as indexes for vascularization, neutrophil and macrophage accumulation, respectively. *Results:* CCL3 and CCL5 were expressed throughout the observation period. Exogenous CCL3 enhanced angiogenesis in WT, but angiogenesis proceeded normally in *CCL3*$^{-/-}$ mice, suggesting that endogenous CCL3 is not critical for sponge-induced angiogenesis in mice. CCL5 expression was detected at day 1, but levels significantly increased thereafter. Exogenous CCL5 reduced angiogenesis in WT mice possible via CCR5 as CCL5 was without an effect in *CCR5*$^{-/-}$ mice. Treatment of WT with the CCR1/CCR5 antagonist, Met-RANTES, prevented neutrophil and macrophage accumulation, but enhanced sponge vascularization. *Conclusion:* Thus, endogenous CCL3 appears not to play a role in driving sponge-induced inflammatory angiogenesis in mice. The effects of CCL5 were anti-angiogenic and appeared to be mediated via activation of CCR5.

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Introduction

The chemokine system is involved in the regulation of inflammatory and angiogenic responses at various levels, including the control of migration, activation, proliferation and apoptosis of leukocytes and endothelial cells (Coscia and Biragyn, 2004; Rot and von Andrian, 2004). These effects of chemokines may influence the outcome of wound healing, inflammatory diseases and tumor growth. Members of the CXC chemokine family are the best studied examples of angiogenic or angiostatic chemokines, but there is now emerging evidence that members of the CC chemokine family also appear to play a role in the modulation of angiogenesis (Hwang et al., 2004, 2005; Salcedo et al., 2001; Strasly et al., 2004).

The CC chemokines CCL3/MIP-1α and CCL5/RANTES have been shown to be involved in several chronic inflammatory conditions, but their role during angiogenesis is less well understood (Appay et al., 1999; Bernardini et al., 2003). While anti-CCL3 antiserum has been shown to decrease angiogenic activity in a wound repair model (DiPietro et al., 1998), wound-associated angiogenesis proceeded normally in CCL3-deficient mice (Low et al., 2001). The injection of anti-CCL2/JE or anti-CCL3 antibodies alone did not affect ischemia-induced retinal neovascularization, but simultaneous injection of both antibodies was able to interfere with blood vessel formation (Yoshida et al., 2003). We are not aware of published studies evaluating directly the role of CCL5 in angiogenesis.

The chemokine receptors CCR1 and CCR5 appear to mediate most of the actions of CCL3 and CCL5 in vivo. Despite the well-established pro-inflammatory role of both CCR1 and CCR5 (Gao and Murphy, 1995; Olbrich et al., 1999), their role on angiogenesis has not been established. Previous studies have shown that CCL23/MIP-3 is able to promote neovascularization in the chick chorioallantoic membrane via CCR1 receptor (Hwang et al., 2005). Nevertheless, CCR1 was not involved in angiogenesis either in a myocardial infarction (Liehn et al., 2008) or in a wound healing model (Kaesler et al., 2004). On the other hand, CCR5 appears to be a component in the development of corneal neovascularization (Ambati et al., 2003) and, more recently, has been implicated in ischemia-induced angiogenesis (Westerweel et al., 2008).

To examine the potential role of CCL3 and CCL5 in inflammatory angiogenesis, initial experiments evaluated the production of these chemokines during the development of neovascularization after implantation of polyether-polyurethane sponges in the dorsum of mice. Further studies then evaluated the ability of both chemokines to modify sponge-induced inflammatory angiogenesis when added exogenously. Finally, the endogenous role of CCL3 and the receptor

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CCR5 were evaluated by using gene-deficient mice and/or the antagonist, Met-RANTES.

Materials and methods

Animals

Eight to 10 weeks old male C57Bl/6J (WT) were obtained from Centro de Bioterismo (CEBIO) of the Universidade Federal de Minas Gerais (UFMG). CCL3- or CCR5-deficient mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and bred in the animal house of the Department of Biochemistry and Immunology (UFMG, Brazil). After sponge implantation, animals were maintained in individual cages with food/water ad libitum and in a controlled environment (temperature and humidity) in the Laboratory of Angiogenesis at the Department of Physiology and Biophysics (UFMG, Brazil).

Preparation of cannulated sponge discs and implantation

Polyether-polyurethane sponge discs 5 mm thick and 8 mm diameter (Vitafoam Ltd., Manchester, UK) were used as the matrix for fibrovascular tissue growth. Twelve-mm polyvinyl tubing (PE 20, Biovida, Brazil) was secured with silk sutures (Ethicon Ltd, UK) to the center of each disc in such a way that the tube was perpendicular to the disc face. The open-end cannula was sealed with removable plugs. The cannulated sponge discs were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 min before the implantation surgery. The animals were anesthetized (Tribromoethanol — Sigma — 2.5% v/v, 1 mL/100 g body weight, i.p.), the dorsal hair shaved and the skin wiped with 70% ethanol. The cannulated sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. The cannula was exteriorized through a small incision in a subcutaneous neck pouch.

Quantification of angiogenesis by hemoglobin measurement

Animals were anaesthetized and killed by cervical dislocation and the sponge implants were carefully excised, released from the cannula, and weighed. Each implant was homogenized (Tekmar TR-10, Ohio, USA) in 2.0 mL of Drabkin Reagent (Labtest, São Paulo, Brazil) and centrifuged at 10,000 *g* for 15 min. The supernatants were filtered through a 0.22 μm filter (Millipore). Hemoglobin in the samples was quantified colorimetrically at 540 nm in a spectrophotometer (E max — Molecular Devices). The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. The results were expressed as μg Hb/mL/mg of wet tissue. Previous studies have shown that hemoglobin detection correlated well with other methods for the detection and quantification of angiogenesis in tissue (Hu et al., 1995; Machado et al., 2000).

Morphometric analysis and blood vessel quantification

To examine the degree of neovascularization in the implants of control (vehicle) and CCR5 antagonist Met-RANTES (1.5 mg/kg/day) treated mice, a total of 6 sponge discs (three for each group) was harvested and stained with Hematoxylin–Eosin. Microscopic images of cross-sections (5 μm) were analyzed with a reticulum inserted in one eyepiece of a binocular microscope (Olympus CHK, Japan) at 400× magnification (0.053 mm^2 per view field). The images were digitized through a microcamera Optronics DEI-470 (Optronics, California) and processed through the software Image-Pro Plus (Media Cybernetics, Inc., version 4.5.1.22). A countable microvessel was defined as a structure with a lumen that contained red blood cells or not as described by Ferreira et al. (2004). The results were expressed as mean ± SEM of the total number of vessels/view field.

Quantification of neutrophil or macrophage tissue accumulation

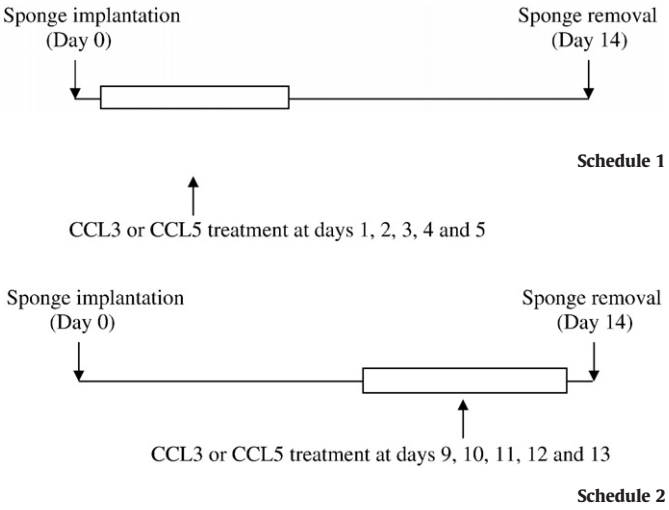
Pellets from centrifugation of sponge homogenates (see hemoglobin measurement method) were divided into two portions and suspended with different buffers specific for measurement of myeloperoxidase (MPO) or N-acetylglucosaminidase (NAG) activities used as neutrophil and macrophage accumulation indexes respectively, as described previously (Barcelos et al., 2005).

ELISA for cytokines/chemokines

The supernatants from centrifugation of sponge homogenates (see hemoglobin measurement method) were used to examine the levels of VEGF, TNF-α, CXCL1–3/KC, CCL2/JE, CCL3/MIP-1α, and CCL5/RANTES produced in sponge implants by ELISA. The assays were performed using Kits from R and D systems and according to the manufacturer's instructions. The threshold of sensitivity for each cytokine/chemokine was 7.5 pg/mL.

Experimental protocol of chemokine treatments

Mice received intrasponge treatment with either murine recombinant CCL3 (PeproTechec Ltd) (100 ng/sponge/day), CCL5 (PeproTechec Ltd) (100 ng/sponge/day) or PBS (50 μl/sponge/day) for 5 consecutive days. We performed the treatment using two schedules: (1) at days 1, 2, 3, 4 and 5 after sponge implantation and (2) at days 9, 10, 11, 12 and 13 after sponge implantation (see diagram below). The effects of treatment on angiogenesis and on the neutrophil and macrophage accumulation were assessed on day 14 after sponge implantation.



Experimental protocol for CCR1 and CCR5 antagonist treatment

Mice received subcutaneous treatment with Met-RANTES (0.5 or 1.5 mg/kg/day), a CCR1 and CCR5 antagonist (Proudfoot et al., 1996; Yun et al., 2004), or PBS vehicle (200 μl/animal/day). The antagonist was given 30 min before the sponge implantation (considered day 0) and daily thereafter till the day before the excision of the sponge. The effects of treatment on angiogenesis and on neutrophil and macrophage accumulation were assessed on day 14 after sponge implantation and the effects on cytokine/chemokines production were assessed on day 7. These time points have been shown to be optimal to determine angiogenesis/cell influx and cytokine/chemokine levels in the sponges (Barcelos et al., 2005).

Statistical analysis

Results are presented as the mean ± SEM. Comparisons between two groups were carried out using Student's *t* test for unpaired data. For three or more groups, comparisons were carried out using one-way analysis of variance (ANOVA) and differences between groups assessed using an appropriate post-test (as indicated). A *P*-value less than 0.05 was considered significant.

Results

Exogenous CCL3 and CCL5 have opposite effects on sponge-induced inflammatory angiogenesis in WT mice

Both CCL5 and CCL3 proteins were detected throughout the sponge-induced angiogenic process in WT mice (Fig. 1A). The levels of CCL3 were greater than those of CCL5 and the chemokine was detected at high levels from the first day after implantation. Thereafter, the levels of CCL3 remained stable, but levels of the CCL5 rose significantly and reached the highest levels at day 7, and remained stable till day 14.

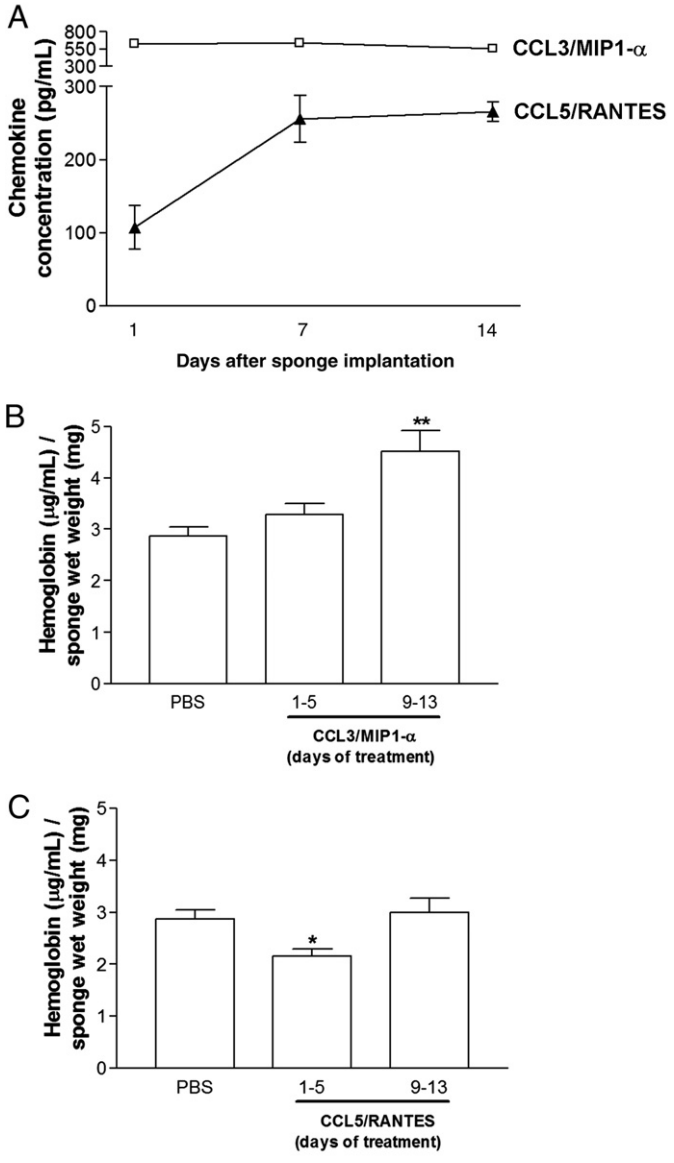


Fig. 1. CCL3/MIP-1α and CCL5/RANTES in sponge-induced inflammatory angiogenesis. (A) ELISA was carried out with specific monoclonal antibodies for CCL3/MIP-1α (opened square) and CCL5/RANTES (filled triangle); (B) rmCCL3/MIP-1α and (C) rmCCL5/RANTES (100 ng/sponge/day) were given locally at the indicated times. Inflammatory angiogenesis was evaluated at day 14 after sponge implantation by measuring tissue hemoglobin content. Results are expressed as the mean ± SEM of 6–8 animals for each group. Statistical analysis was assessed by Student–Newman–Keuls multiple comparisons test. * for $P<0.05$ and ** for $P<0.01$ when compared to PBS-treated sponges.

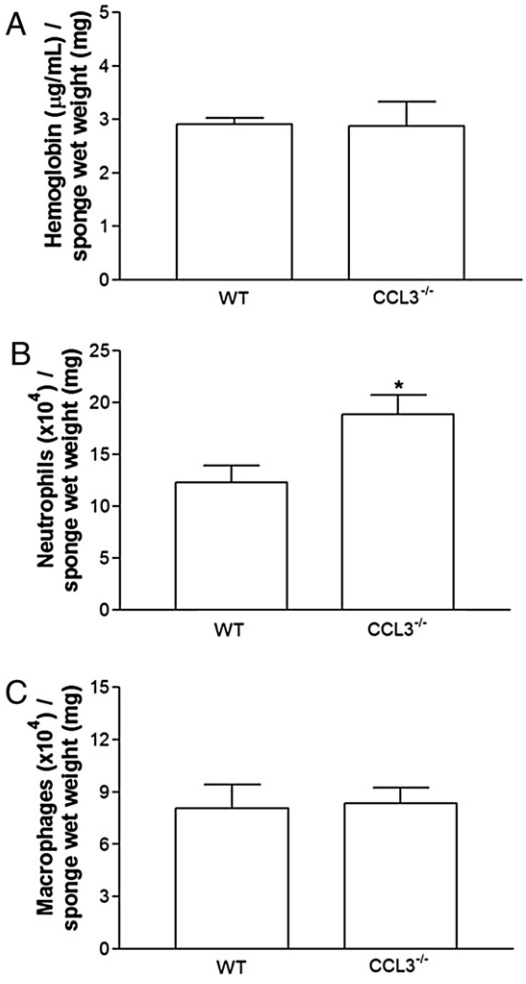


Fig. 2. Sponge-induced inflammatory angiogenesis in CCL3/MIP-1α-deficient mice. (A) Angiogenesis and (B) neutrophil and (C) macrophage influx evaluated at day 14 after sponge implantation. Angiogenesis was evaluated by measuring tissue hemoglobin concentration and neutrophil and macrophage recruitment by evaluating MPO and NAG activities, respectively. Results represent the mean ± SEM of 6–8 animals for each group. Statistical analysis was assessed by Student *t* test. * for $P<0.05$ vs. WT sponges.

Exogenous CCL3 given into the implant from days 9–13, but not from days 1–5, significantly increased blood vessel formation at day 14 after implantation (Fig. 1B). In contrast, CCL5 given from days 1–5 induced a significant decrease of blood vessel formation at day 14 after implantation (Fig. 1C).

Endogenous CCL3 is not essential for the development of sponge-induced inflammatory angiogenesis

To examine the role of endogenous CCL3, neovascularization and inflammatory cell accumulation were evaluated in sponges implanted in CCL3-deficient ($CCL3^{-/-}$) and wild-type control mice (WT) at day 14 post implantation. Angiogenesis, as assessed by hemoglobin content, and macrophage accumulation (NAG activity) into sponges were comparable in $CCL3^{-/-}$ and WT mice (Figs. 2A and C). There was an increase in neutrophil accumulation in $CCL3^{-/-}$ mice (Fig. 2B). Despite the increased neutrophil influx, the levels of the neutrophil-active chemokine CXCL1–3/KC was similar in implants from $CCL3^{-/-}$ and WT mice (417 ± 94, WT versus 344 ± 90 pg/mL, $CCL3^{-/-}$). The

levels of the pro-angiogenic molecules CCL2 (900 ± 111, WT versus 1025 ± 275 pg/mL, *CCL3*$^{-/-}$), TNF-α (382 ± 90, WT versus 506 ± 95 pg/mL, *CCL3*$^{-/-}$) and VEGF (390 ± 84, WT versus 602 ± 107 pg/mL, *CCL3*$^{-/-}$) were also comparable in WT and *CCL3*$^{-/-}$ mice. Thus, although exogenous CCL3 enhances the process, our data suggest that inflammatory angiogenesis proceeds normally in the absence of endogenous CCL3.

Simultaneous blockade of CCR1 and CCR5 receptors enhances sponge-induced inflammatory angiogenesis in WT mice

To characterize the role of signaling through CCR1 and CCR5 in the sponge model, animals were treated daily with Met-RANTES (0.5 or 1.5 mg/kg, s.c.). Mice treated with Met-RANTES exhibited a significant increase of neovascularization, as assessed by hemoglobin content (Fig. 3A) and number of blood vessel (Fig. 3B), compared with vehicle-treated mice. This increase in vascularization was accompanied by a significant fall in the content of neutrophils (Fig. 3C) and macrophages (Fig. 3D). Histological sections of the implants confirmed the biochemical findings and show less cellular accumulation and increased number of blood vessels in implants from Met-RANTES-treated mice (Fig. 3E).

To gain insight into potential mechanisms of action of Met-RANTES on sponge-induced inflammatory angiogenesis, we examined whether this antagonist altered the levels of VEGF (383 ± 81, vehicle versus 299 ± 176 pg/mL, Met-RANTES), TNF-α (75 ± 13, vehicle versus 56 ± 3 pg/mL, Met-RANTES), CXCL1–3 (190 ± 25, vehicle versus 176 ± 23 pg/mL, Met-RANTES) and CCL2 (730 ± 90, vehicle versus 673 ± 145 pg/mL, Met-RANTES). Treatment with Met-RANTES failed to affect significantly the levels of these mediators.

Angiogenesis and inflammatory cell accumulation in sponges were then examined at day 14 post implantation in *CCR5*$^{-/-}$ and WT mice. There was no difference in the hemoglobin content in sponges of *CCR5*$^{-/-}$ and WT mice (Fig. 4), suggesting that neovascularization proceeded normally in these animals. In contrast, neutrophil ($24 \pm 3 \times 10^4$ cells/mg sponge, WT versus $15 \pm 2 \times 10^4$, *CCL5*$^{-/-}$) and macrophage ($12 \pm 1 \times 10^4$ cells/mg sponge, WT versus $7 \pm 1 \times 10^4$, *CCL5*$^{-/-}$) accumulation were reduced in *CCR5*$^{-/-}$ mice.

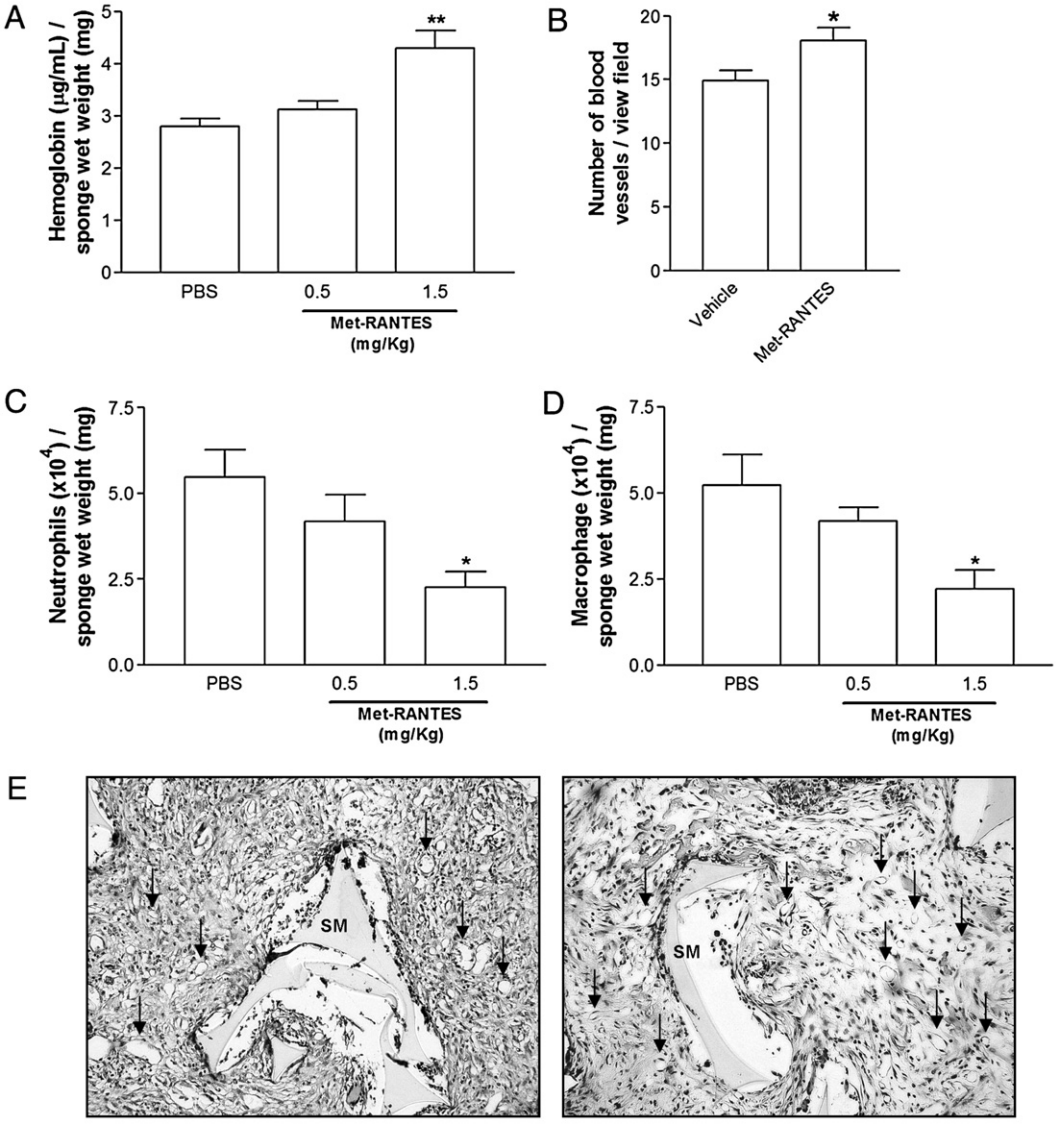


Fig. 3. CCR1/CCR5 antagonism enhances inflammatory angiogenesis in sponge implants. Met-RANTES (0.5 or 1.5 mg/kg/day) or PBS vehicle were given subcutaneously as described in Materials and methods. Angiogenesis in implants was quantified by hemoglobin concentration (A) and number of blood vessels (B). Neutrophil (C) and Macrophage (D) accumulation in implants were measured by indirect methods based on myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) activities, respectively. Values represent the mean ± SEM of 6–8 animals for each group. Statistical analysis was assessed by Student–Newman–Keuls multiple comparisons test. * for $P<0.05$ and ** for $P<0.01$ when compared to PBS-treated sponges. Hematoxylin and eosin-stained histological sections (E). Symbols indicate blood vessels (narrows) and sponge matrix (SM). 400× magnification.

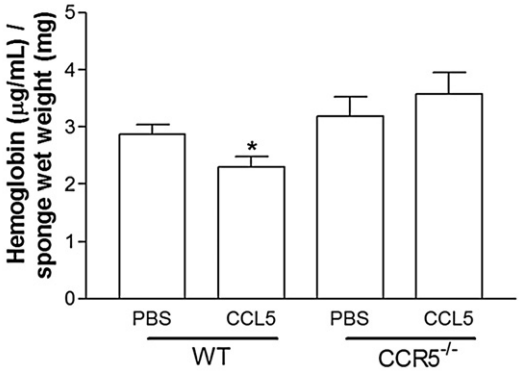


Fig. 4. CCL5/RANTES-mediated anti-angiogenic activity was lost in *CCR5*$^{-/-}$ mice. Angiogenesis was evaluated at day 14 after sponge implantation by measuring tissue hemoglobin concentration. Results represent the mean ± SEM of 6–8 animals for each group. Statistical analysis was assessed by Student *t* test. * for $P<0.05$ when compared to PBS-treated sponges from WT mice.

CCL5-mediated anti-angiogenic activity is dependent on the CCR5 signaling

Next, we determined whether exogenous CCL5-mediated anti-angiogenic activity was mediated by signaling through the CCR5, one of the main receptors for CCL5. In the absence of CCR5, i.e., in *CCR5*$^{-/-}$ mice, the reduction of hemoglobin content induced by exogenous CCL5 was not anymore observed (Fig. 4). These findings suggest that CCR5 is the main receptor involved in the anti-angiogenic effects of exogenous CCL5.

Discussion

In addition to promoting the recruitment of leukocytes to sites of inflammation, there has been much interest in the understanding of the role of chemokines in angiogenesis. It is now clear that chemokines may affect neovessels formation by directly activating endothelial cells, but may also induce the production of angiogenic molecules, including VEGF, by leukocytes (Griffioen and Molema, 2000; Romagnani et al., 2004). Here, we have investigated the expression, effects and putative role of the chemokines CCL3 and CCL5 during the development of neovascularization after implantation of polyether-polyurethane sponges in the dorsum of mice.

As an experimental animal system, the sponge implantation technique for the study of the inflammatory and fibrovascular components of wound healing provides an environment of defined dimensions in which the influence of epithelial cells, hair follicles, sweet glands and the process of wound contraction are not necessary (Andrade et al., 1997; Campos et al., 2006, 2008; Belo et al., 2005; Bradshaw et al., 2001; Kyriakides et al., 2001). In addition, sponge implants can be easily manipulated and examined at defined time points and thus facilitate the kinetic tracing of different cell lineages (Barcelos et al., 2004, 2005; Opalenik and Davidson, 2005).

Using the above system, we found a significant expression of CCL3 in the sponges, which appeared as early as 1 day after implantation and persisted throughout the observation period. Although exogenous CCL3 enhanced angiogenesis in WT mice, the constant levels of CCL3 would suggest this chemokine is not the major endogenous contributor to the angiogenic process in sponge implants. Interestingly, our data in CCL3-deficient mice showed that angiogenesis proceeded normally when compared to their wild-type controls. This is in agreement with the study of Low et al. (2001) who showed that skin wound angiogenesis was similar in *CCL3*$^{-/-}$ and WT mice. Altogether, these studies suggest that endogenous CCL3 does not play a major role in inflammatory angiogenesis in mice.

The chemokine CCL5 is expressed by, and may act on, endothelial cells both in vitro and in vivo (Berger et al., 1999; Kotani et al., 2002; Shahrara et al., 2003). In addition, CCL5 expression is associated with and may play a role in chronic inflammation (Appay et al., 1999; Shahrara et al., 2003), wound repair (Frank et al., 2000) and with the progression of some angiogenesis-dependent tumors (Azenshtein et al., 2002; Kondo et al., 2004). Moreover, CCL5 is capable of inducing the influx and activation of leukocytes (Appay et al., 1999; Appay and Rowland-Jones, 2001; Bonecchi et al., 1999; Pan et al., 2000). These effects of CCL5 are consistent with a role for the chemokine in the process of inflammatory angiogenesis. In our model of sponge-induced inflammatory angiogenesis, there was a dynamic expression of the chemokine CCL5 that paralleled neovascularization and inflammatory cell accumulation. Surprisingly, exogenous CCL5 in the early stages of the process actually prevented angiogenesis. CCR1 and CCR5 are the main receptors for CCL5 in rodents (Baltus et al., 2003), thus deletion of CCR5 alone will still allow CCL5 to act on CCR1. However, in our experiments, exogenous CCL5 no longer modified angiogenesis in *CCR5*$^{-/-}$ mice, showing that in our model CCL5 signals mainly through CCR5. Altogether our results clearly demonstrate that the activation of CCR5 by exogenous CCL5 may initiate a cascade of events leading to inhibition of sponge-induced angiogenesis.

It was interesting, however, that angiogenesis proceeded normally in CCR5-deficient mice. In another model of wound healing (Kaesler et al., 2004) as well as in a model of myocardial ischemia (Liehn et al., 2008), CCR1 also appeared to be dispensable for angiogenesis. In favor of the previously suggested anti-angiogenic role of CCL5, our studies demonstrate that simultaneous blockade of both CCR5 and CCR1 by treatment with Met-RANTES greatly enhanced the sponge-induced angiogenesis. The latter results are consistent with a possible role for the activation of CCL5 receptors in modulating negatively the angiogenic process. In addition, it is worth noticing that Met-RANTES treatment did not modify the levels of pro-angiogenic molecules, including VEGF, TNF-α, and the chemokines CXCL1–3/KC and CCL2/JE. As blockade or loss of either receptors singly was insufficient for the enhancement of angiogenesis (Kaesler et al., 2004, and present report), it is likely that both receptors act coordinately and in a redundant manner to control, negatively, the angiogenic process in the sponge implants.

Support for the relevance of both chemokine receptors was provided by the results of Met-RANTES treatment which inhibited pro-inflammatory cell infiltration. Similarly, experiments in *CCR5*$^{-/-}$ mice showed that inflammatory cell influx was partially prevented whereas angiogenesis was not affected. Thus, CCR1 and CCR5 appear to be essential for inflammatory cell influx to occur during sponge-induced inflammatory angiogenesis. The effects of Met-RANTES treatment and the experiments in gene-deficient animals are consistent with the pro-inflammatory actions attributed to CCR1 and CCR5 (Ajuebor et al., 2001; Baltus et al., 2003; Yun et al., 2004). Interestingly, Met-RANTES and CCR5 deficiency have been shown to reduce neointima formation and macrophage infiltration (Schober et al., 2002; Zernecke et al., 2006). Given that neointima formation is limited by reendothelialization, the results presented here may provide an additional explanation for the effects of CCR1/CCR5 blockade after vascular injury. Although angiogenic stimuli play important role in neointima formation, the blockade of the infiltration of inflammatory cells may be enough to reduce vascular injury. On the other hand, those results are in sharp contrast with the data reported by Westerweel et al. (2008) in which Met-RANTES treated animals did not show any change in capillary density or macrophage infiltration following ischemia in rat hind limbs. One possible explanation regards the differences among models and species. The angiogenesis in sponges is based on a newly formed proliferating tissue while, in the limb ischemia model, the vessels are formed from pre-existing ones in a severe hypoxic environment.

The unexpected anti-angiogenic effect of CCL5 could have analogies with some chemokine-like proteins encoded by Kaposi's sarcoma-associated herpes virus (KSHV). Viruses appear to have adopted the chemokine system as a mechanism to subvert the immune response and favor the spread of infection (Ahuja et al., 1994). For instance, two of the chemokine-like genes encoded by KSHV, vMIP-I and vMIP-II, share significant sequence similarity with human CCL3 and CCL5 and stimulate tumor angiogenesis (Boshoff et al., 1997; Jensen and Lira, 2004). However, these chemokine-like proteins behave as CCR1 and CCR5 antagonists (Boshoff et al., 1997; Kledal et al., 1997; Navenot et al., 2001) and have anti-inflammatory activities (Chen et al., 1998), reinforcing our suggestion that the CCR1 and CCR5 may have a predominantly negative modulatory role on inflammatory angiogenesis.

In summary, the present study demonstrates that the chemokines CCL3 and CCL5 are expressed during sponge-induced inflammatory angiogenesis in mice. Whereas exogenous CCL3 enhanced sponge-induced angiogenesis, endogenous CCL3 was not essential for the process. Exogenous CCL5 negatively modulated sponge-induced angiogenesis, an effect mediated by CCR5. Simultaneous blockade of CCR1 and CCR5 with Met-RANTES, but not CCR5 deficiency alone, was necessary for facilitation of sponge-induced angiogenesis under steady-state conditions. Altogether, our studies disclose an unexpected in vivo anti-angiogenic effect of the activation of CCL5/RANTES receptors during inflammatory angiogenesis in mice.

Acknowledgments

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