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The CO-releasing molecule CORM-3 protects against articular degradation in the K/BxN serum transfer arthritis model

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ABSTRACT

Carbon monoxide-releasing molecules can counteract inflammatory responses. The aim of this study was to investigate whether tricarbonylchloro(glycinate)ruthenium (II) (CORM-3) is able to control the effector phase of experimental arthritis. Arthritis was induced in C57Black-6 mice by an intraperitoneal injection of serum from arthritic K/BxN mice. CORM-3 was administered intraperitoneally at 10 mg/kg/day (5 mg/kg twice a day) from days 0 to 10 and animals were sacrificed on day 11. Serum levels of osteocalcin and prostanoids were measured by enzyme-linked immunosorbent assay and radioimmunoassay. Gene expression was determined by real-time PCR. Histological analysis was performed and protein expression was examined by immunohistochemistry. Treatment with CORM-3 reduced the macroscopic score in hind paws, the migration of inflammatory cells and erosion of cartilage and bone. CORM-3 increased the levels of osteocalcin in the serum and reduced PGD_2 levels, whereas PGE_2 and 6-ketoPGF$_{1\alpha}$ were not affected. In synovial tissues, we also observed a significant reduction in gene expression of interleukin-1β, receptor activator of nuclear factor κB ligand (RANKL), matrix metalloproteinase (MMP)-9 and MMP-13. CORM-3 induced HO-1 expression in joint tissues but inhibited high mobility group box 1 (HMGB1), hematopoietic-prostaglandin D_2 synthase (H-PGDS) and lipocalin-type prostaglandin D_2 synthase (L-PGDS), as well as RANKL and intercellular adhesion molecule-1. COX-2 expression was not affected by CORM-3 treatment. We have shown that CORM-3 decreases the inflammatory response and protects against the degradation of cartilage and bone in the arthritic mice. Pharmacological CO delivery represents a novel strategy to regulate the effector phase of arthritis.

1. Introduction

CO is produced in biological systems during the oxidative catabolism of heme by heme oxygenase (HO). Many biological functions of HO, such as regulation of vessel tone and anti-inflammatory effects have been attributed to its enzymatic product, CO (Choi and Otterbein, 2002; Ryter and Otterbein, 2004). Recently, CO-releasing molecules, which carry and liberate small amounts of CO in biological systems, have been synthesized as pharmacological tools to mimic the bioactivity of endogenously generated CO (Motterlini et al., 2002). A number of studies have demonstrated that CO-releasing molecules are a novel drug class able to exert anti-inflammatory effects (reviewed in Alcaraz et al., 2008), stressing the dual role of CO in biological systems. Interestingly, we have previously shown that the water–soluble metal complex tricarbonylchloro(glycinate)ruthenium (II) (CORM-3) (Fig. 1) suppresses both the clinical and histopathological manifestations of collagen-induced arthritis (Ferrándiz et al., 2008). In that model, it was observed that the treatment of animals with CORM-3 resulted in decreased levels of anti-collagen II antibodies which play a critical role in the initiation of disease (Luross and Williams, 2001).

The K/BxN T cell receptor-transgenic mouse line spontaneously develops a joint disorder sharing many of the features of rheumatoid arthritis in humans. The development of disease depends on the reactivity of T lymphocytes bearing the transgene-encoded T cell receptor KRN, and B lymphocytes to the self antigen glucose-6-phosphate isomerase (Kouskoff et al., 1996; Matsumoto et al., 1999). In this spontaneous inflammatory arthritis, the effector phase of the disease is provoked by binding of immunoglobulins to joint surfaces (Ji et al., 2002). Transfer of serum from K/BxN transgenic mice into healthy animals induces an autoimmune and inflammatory response mediated by IgG1 autoantibodies (Matsumoto et al., 2002; Maccioni et al., 2002). This model of arthritis using the transfer of serum from

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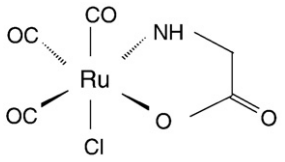

Tricarbonylchloro(glycinate)ruthenium(II)
$[Ru(CO)_3Cl(glicinate)]$
CORM-3

Fig. 1. Chemical structure of CORM-3.

K/BxN mice bears a number of similarities to human rheumatoid arthritis, such as synovial hypertrophy and pannus formation, as well as erosion of cartilage and bone (Wipke and Allen, 2001).

To further our understanding of the mechanisms involved in the anti-arthritic effects of CO delivery via CORM-3, we have examined the possible regulatory role of this strategy during the effector phase of arthritis subsequent to autoantibody accumulation. Transfer of serum from K/BxN transgenic mice provided a good opportunity to address this issue, as this autoantibody-mediated model of rheumatoid arthritis bypasses the initiation phase of arthritis.

2. Materials and methods

2.1. Induction of arthritis-serum transfer model

K/BxN mice were generated by crossing KRN-TCR-transgenic mice (B10.BR genetic background) with NOD mice. Arthritis was induced in male C57Black-6 mice by intraperitoneal injection of 200 µl serum from arthritic K/BxN mice on day 0 and 2. C57BL/6 mice were obtained from Janvier (Le Genest St Isle, France). All animal procedures were approved by the institutional ethics committee. All mice were maintained in cages with a 12-h light/dark cycle and free access to standard diet and water. Mice were housed and cared for by the veterinary staff in accredited facilities and were routinely screened for health status. The mice used were between 10 and 12 weeks of age.

2.2. Protocol and animal treatment

CORM-3 (Hemocorm Ltd., Harrow, UK) was administered at 10 mg/kg/day i.p. (5 mg/kg twice a day) from days 0 to 10. This dose was selected due to its activity and lack of toxicity in collagen-induced arthritis (Ferrándiz et al., 2008). CORM-3 was freshly prepared before each experiment by dissolving the compound in saline (0.2 ml per 20 mg mouse). The same amount of saline was injected in the control group. On day 11, serum samples were obtained and animals were sacrificed by cervical dislocation and hind paws were amputated for histological analyses.

2.3. Arthritis score

The clinical severity of arthritis (arthritis score) was macroscopically graded on a scale of 0 (no inflammation of the ankle) to 2 (severe inflammation) for each hind paw. This macroscopic grading system assessed the extent of changes in redness and swelling of the paws. Scoring was performed by two independent observers without knowledge of the experimental groups.

2.4. Measurement of inflammatory markers

To determine the levels of prostaglandins PGD_2 and 6 keto$PGF_{1\alpha}$ in serum samples (dilution 1/2000 and 1/40, respectively, final volume 50 µl), enzyme-linked immunosorbent assay kits were used (Cayman Chemical, Ann Arbor, MI, USA). PGE_2 levels in serum samples (6 µl) were measured by radioimmunoassay (Moroney et al., 1988). Osteocalcin levels in serum samples (1/50 dilution, final volume 25 µl) were determined by the LINCOplex™ system (Millipore Iberica, Madrid, Spain). The sensitivity of this kit was 10–40,000 pg/ml.

2.5. Histology

For standard histological assessment, isolated right ankles were kept in 10% formalin for 4 days, decalcified in 5% formic acid, and subsequently dehydrated and embedded in paraffin. Standard frontal sections (7 µm) of the joint tissue were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany). Hematoxylin and eosin staining was performed to study joint inflammation. The severity of inflammation in the joints was scored on a scale of 0–3 (0 = no cells, 1 = mild cellularity, 2 = moderate cellularity, and 3 = maximal cellularity). To study proteoglycan depletion from the cartilage matrix, sections were stained with Safranin O, followed by counterstaining with fast green. Depletion of proteoglycan was determined using an arbitrary scale of 0–3, ranging from normal, fully stained cartilage to destained cartilage that was fully depleted of proteoglycan. Bone destruction was graded on a scale of 0–3, ranging from no damage to the complete loss of bone structure. Histopathologic changes were scored on 3 semiserial sections of the joint, with sections spaced 70 µm apart. Scoring was performed in a blinded manner.

2.6. Immunohistochemistry

For immunohistologic analyses, isolated left ankles were fixed for 4 days in 10% formalin, decalcified in EDTA (10%), and subsequently dehydrated and embedded in paraffin. Tissue sections (7 µm) were deparaffinized, rehydrated, and treated with 2% H_2O_2 for 10 min at room temperature. Sections were incubated for 15 min with goat antiserum (1:10), and thereafter incubated for 2 h with rabbit anti-mouse antibodies against cyclooxygenase-2 (COX-2), hematopoietic-prostaglandin D_2 synthase (H-PGDS), lipocalin-type prostaglandin D_2 synthase (L-PGDS) (Cayman Chemical, Ann Arbor, Michigan, USA), HO-1 (Stressgen, Victoria, British Columbia, Canada), high mobility group box 1 (HMGB1) (Upstate, Millipore Iberica) or goat antibodies against receptor activator of nuclear factor κB ligand (RANKL) (Santa Cruz Biotechnology, Santa Cruz, California, USA) and intercellular adhesion molecule-1 (ICAM-1) (R&D Systems, Abingdon, UK). Rabbit or goat Ig antibody (Dako, Glostrup, Denmark) was used as control. After rinsing, sections were incubated for 30 min with the correspondent secondary biotinylated antibody: swine anti-rabbit antibody (Dako) or anti-goat antibody (R&D Systems), followed by labeling with streptavidin–horseradish peroxidase (Dako). Development of the peroxidase staining was performed with diaminobenzidine (Sigma Aldrich, St. Louis, MO, USA). Sections were counterstained with hematoxylin for 1 min. Positive cells were counted in five random high-power fields by two independent observers.

2.7. RNA isolation from synovial tissue

On day 11, synovium samples were obtained in a standardized manner using a 3-mm biopsy punches (Stiefel, Wachtersbach, Germany). Synovial tissue was homogenized with a MagNALyser Instrument (Roche Applied Science, Almere, The Netherlands), and total RNA was extracted using an RNeasy Mini kit (Qiagen, Venlo, The Netherlands). To obtain cDNA, 1 µg DNase-treated total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase, oligo(dT) primers and dNTPs (Invitrogen).

2.8. Quantitative PCR

Quantitative real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The quantitative PCR amplification protocol was as follows:

2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, with data collection during the last 30 s. Primer sequences for the reference gene GAPDH and the genes of interest were as follows: for *GAPDH*, forward 5′-GGC-AAA-TTC-AAC-GGC-ACA-3′ and reverse 5′-GTT-AGT-GGG-GTC-TCG-CTC-CTG-3′; for *TNF-α*, forward 5′-CAG-ACC-CTC-ACA-CTC-AGA-TCA-TCT-3′ and reverse 5′-CCT-CCA-CTT-GGT-GGT-TTG-CTA-3′; for *IL-1β*, forward 5′-GGA-CAG-AAT-ATC-AAC-CAA-CAA-GTG-ATA-3′ and reverse 5′-GTG-TGC-CGT-CTT-TCA-TTA-CAC-AG-3′; for *RANK*, forward 5′-GCC-CCA-GTC-TCA-TCG-TTC-TGC-3′ and reverse 5′-GCA-AGC-ATC-ATT-GAC-CCA-ATT-C-3′; for *RANKL*, forward 5′-CTG-AGG-CCC-AGC-CAT-TTG-3′ and reverse 5′-GTT-GCT-TAA-CGT-CAT-GTT-AGA-GAT-CTT-G-3′; for *MMP-3*, forward 5′-TGA-AGC-CAC-CAA-CAT-CAG-GA-3′ and reverse 5′-TGG-AGC-TGA-TGC-ATA-AGC-CC-3′; for *MMP-9*, forward 5′-GGA-ACT-CAC-ACG-ACA-TCT-TCC-A-3′ and reverse 5′-GAA-ACT-CAC-ACG-CCA-GAA-GAA-TTT-3′; for *MMP-13*, forward 5′-AGA-CCT-TGT-GTT-TGC-AGA-GCA-CTA-C-3′ and reverse 5′-CTT-CAG-GAT-TCC-CGC-AAG-AG-3′. Quantitative PCR was performed in a total volume of 20 µl, which contained 4 µl of cDNA (diluted 1:20) or water for NTC, 1.2 µl of forward primer (5 µM), 1.2 µl of reverse primer (5 µM), 10 µl of SYBR Green Master Mix (Applied Biosystems), and 3.6 µl of distilled H_2O. For each sample, differences in threshold cycle (ΔC_t) values were calculated by correcting the C_t of the gene of interest to the C_t of the reference gene GAPDH. Relative gene expression was expressed as ΔΔCt with respect to naïve animals.

2.9. Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M.). Differences between experimental groups were tested using the one-tailed Mann–Whitney *U* test, unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, as appropriate. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effect of CORM-3 on arthritis

After serum transfer, animals treated with CORM-3 developed a less severe disease than did control mice (Fig. 2A). Although the progression of the disease was similar in both groups, at the end of the experiment (day 11) the macroscopic score in hind paws was significantly reduced from 3.43 ± 0.27 (arthritic control) to 2.59 ± 0.31 (CORM-3) ($n = 8$, $P < 0.05$, Mann–Whitney *U* test). Histomorphometric examination on day 11 of inflammation and cartilage and bone erosion confirmed the clinical findings. Active inflammation with synovial hyperplasia and leukocytic infiltration, as well as joint destruction could be seen on histology sections taken on day 11 from arthritic control animals. Fig. 2B shows the protective effect mediated by CORM-3. Histological analysis of joint ankles indicated that CORM-3 significantly reduced cell infiltration, with a score value of 1.50 ± 0.30, compared to arthritic control mice (2.35 ± 0.13). In addition, treatment with CORM-3 resulted in lower bone erosion (0.37 ± 0.06) and proteoglycan loss (1.63 ± 0.29) versus arthritic control (0.59 ± 0.05 and 2.50 ± 0.15, respectively) ($n = 8$, $P < 0.05$, Mann–Whitney *U* test). The protective effect of CORM-3 on bone erosion was confirmed by measuring osteocalcin levels in serum at the end of the experiment. Arthritis progression in control animals resulted in reduced osteocalcin levels (107.81 ± 7.04 ng/ml, $n = 8$, $P < 0.01$) with respect to non-

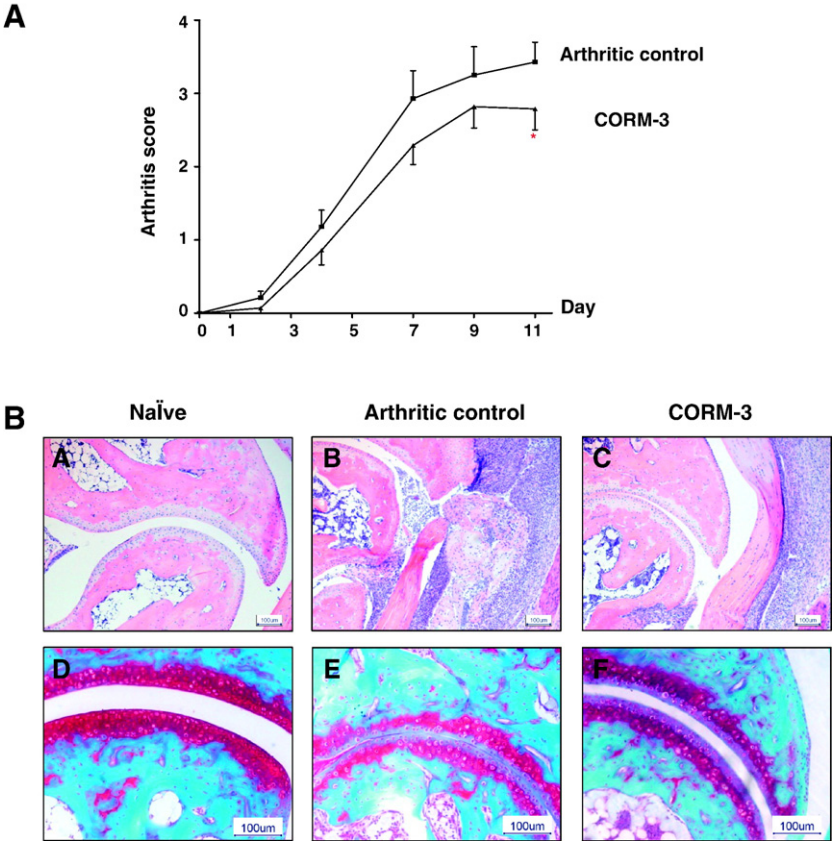

Fig. 2. Effect of CORM-3 on the K/BxN serum transfer arthritis. A, time course of the arthritis macroscopic score. B, histological analysis on day 11. A–C, hematoxylin and eosin-stained frontal sections of ankle joints on day 11. A, naïve mouse. B, arthritic mouse (control group). C, arthritic mouse treated with CORM-3 (10 mg/kg/day). Original magnification ×100. D–F, safranin O-stained frontal sections of ankle joints on day 11. D, naïve mouse. E, arthritic mouse (control group). F, arthritic mouse treated with CORM-3 (10 mg/kg/day). Original magnification ×100. Cellular infiltration, bone erosion and proteoglycan depletion were scored on a scale of 0–3.

Table 1
Relative mRNA expression in synovial tissue.

	ΔΔCt versus naïve animals	
	Arthritic control	CORM-3
IL-1β	8.80 ± 0.37	4.33 ± 2.01[a]
TNF-α	2.11 ± 0.38	0.75 ± 0.72
RANK	3.05 ± 0.45	0.92 ± 1.00
RANKL	9.43 ± 0.46	4.03 ± 2.05[a]
MMP-3	7.16 ± 0.59	4.35 ± 1.43
MMP-9	6.78 ± 0.72	2.77 ± 1.01[b]
MMP-13	11.23 ± 0.66	5.95 ± 1.87[a]

Values are mean ± S.E.M., $n = 4–5$. [a]$P<0.05$, [b]$P<0.01$, with respect to arthritic control, unpaired Student's *t*-test.

arthritic animals (199.57 ± 5.76 ng/ml). Administration of CORM-3 significantly modified this parameter in arthritic animals, with osteocalcin levels of 138.66±10.27 ng/ml ($n = 8$, $P<0.05$ with respect to the arthritic control group, Dunnett's *t*-test).

3.2. Effect of CORM-3 on the expression of inflammatory genes

Consistent with the important contribution of pro-inflammatory cytokines in promoting arthritis, we found in synovial tissues of arthritic mice a significant increase (relative to naïve animals) in mRNA levels of interleukin(IL)-1β and to a lesser extent of tumour necrosis factor-α (TNFα) (Table 1). Higher levels in the expressions of RANK, RANKL and matrix metalloproteinase (MMP)-3, MMP-9 and MMP-13 were also observed. Interestingly, in animals treated with CORM-3 we found a significant reduction in the relative expressions of IL-1β, RANKL, MMP-9 and MMP-13.

3.3. Effect of CORM-3 on HO-1 and HMGB1 protein expressions

As some of the protective effects of CO may be dependent on HO-1 induction, we examined whether CORM-3 was able to induce HO-1 in this arthritis model. As shown in Fig. 3, immunohistochemical analysis of ankle sections obtained from arthritic mice on day 11 revealed that HO-1 expression was weakly augmented during arthritis (14.60 ± 3.30), compared with naïve mice (4.14 ± 2.01). Treatment of arthritic animals with CORM-3 increased the number of cells positive for HO-1 (25.60 ± 3.33, $P<0.05$). The expression of this protein was higher in synovial cells and infiltrate than in chondrocytes. HMGB1 was detected by immunohistochemistry in synovial cells, infiltrate and to a lesser extent in chondrocytes. For the arthritic control group, we observed a significant increase ($P<0.05$) in HMGB1 positive cells (72.16 ± 7.32) with respect to naïve animals (34.50 ± 3.07), whereas the treatment with CORM-3 significantly reduced the immunoreactivity for this protein (37.88 ± 4.49, $P<0.01$).

3.4. Effect of CORM-3 on RANKL and ICAM-1 protein expression

The effect of CORM-3 on RANKL and ICAM-1 protein expression was determined by immunohistochemistry of ankle joint sections (Fig. 4). The number of cells positive for RANKL increased from 31.33 ± 4.91 (naïve animals) to 67.81±5.12 ($P<0.01$) in arthritic controls. Administration of

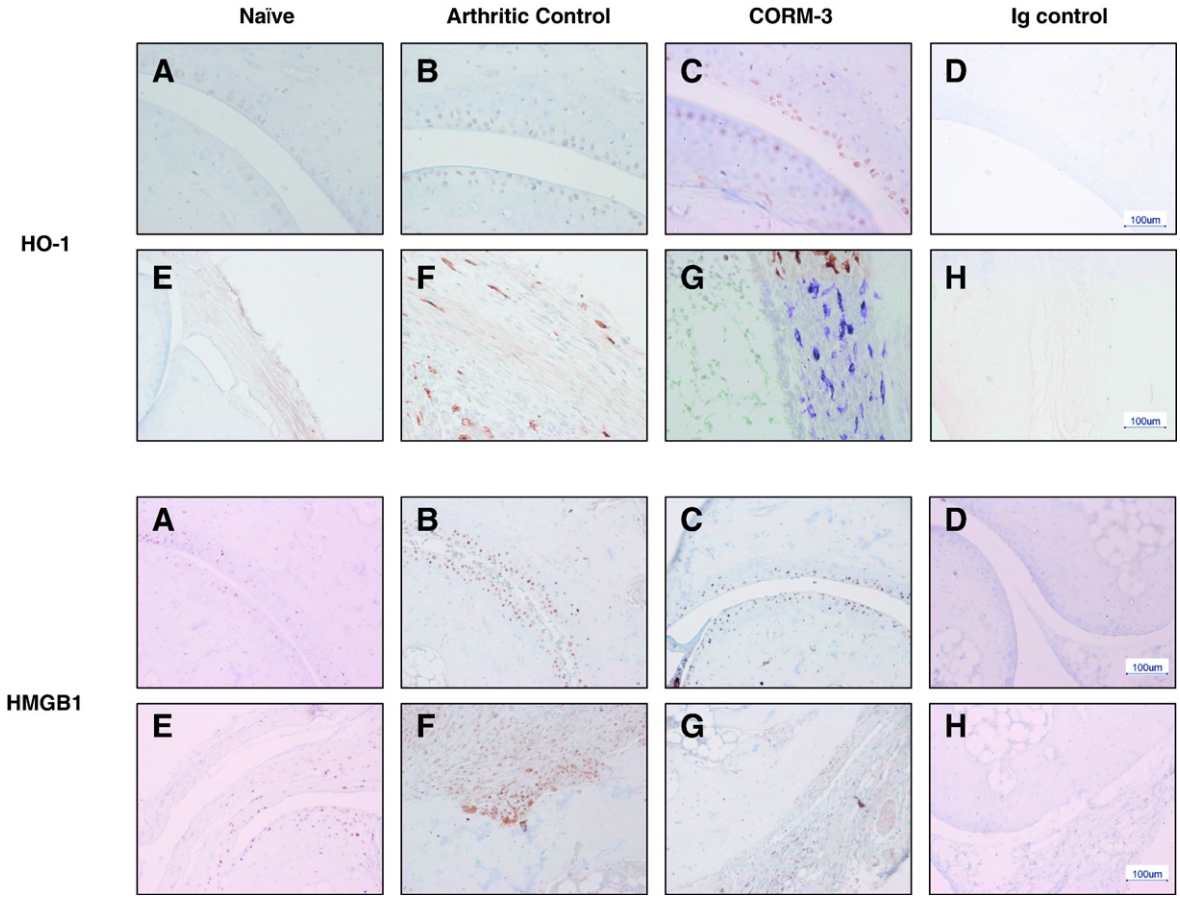

Fig. 3. Effect of CORM-3 on HO-1 and HMGB1 expressions on day 11. HO-1 and HMGB1 expressions were analyzed by immunohistochemistry. Ankle sections were treated with a specific anti-HO-1 antibody (A–C, E–G), a specific anti-HMGB1 antibody (A–C, E–G) or IgG control antibody (D–H). A, E, sections from a naïve mouse. B, F, sections from an arthritic control mouse. C, G, sections from an arthritic mouse treated with CORM-3 (10 mg/kg/day). Original magnification ×200.

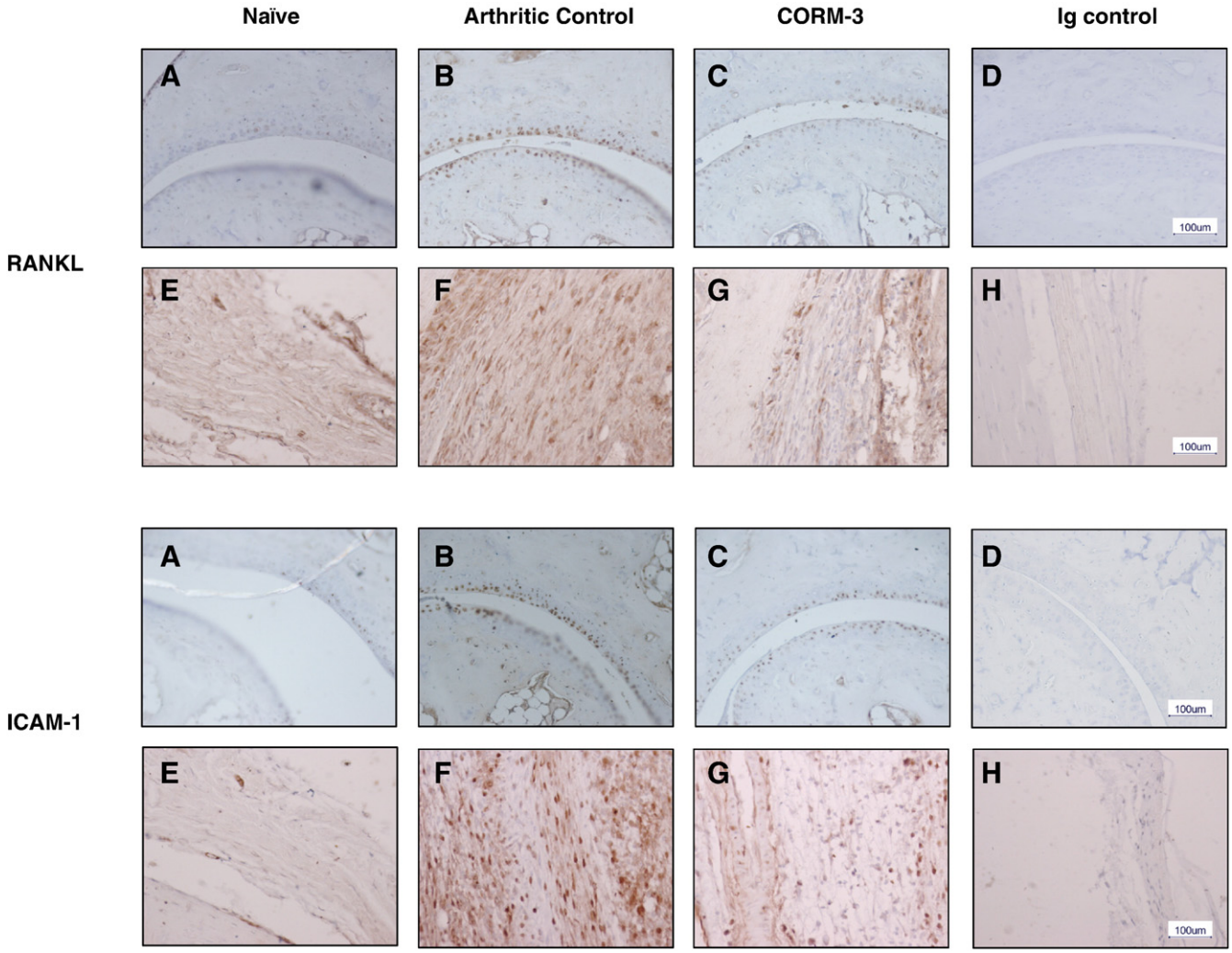

Fig. 4. Effect of CORM-3 on RANKL and ICAM-1 expressions on day 11 analyzed by immunohistochemistry. Ankle sections were treated with a specific anti-RANKL antibody (A–C, E–G), a specific anti-ICAM-1 antibody (A–C, E–G) or IgG control antibody (D–H). A, E, sections from a naïve mouse. B, F, sections from an arthritic control mouse. C, G, sections from an arthritic mouse treated with CORM-3 (10 mg/kg/day). Original magnification ×200.

CORM-3 resulted in levels of protein expression similar to non-arthritic animals (36.85 ± 3.70, $P<0.01$). In addition, CORM-3 significantly decreased (50.94 ± 7.40, $P<0.01$) the up-regulation in ICAM-1 expression observed in the K/BxN arthritis (85.07 ± 7.20 in arthritic controls versus 22.20 ± 5.30 in naïve animals, $P<0.01$).

3.5. Effect of CORM-3 on prostanoid production

Increased prostanoid production has been shown to contribute to both the induction and perpetuation of arthritis in the K/BxN serum transfer arthritis (Chen et al., 2008). We have measured prostanoid levels in serum and found that the PGI_2 metabolite 6 ketoPGF$_{1\alpha}$ and PGD_2 are significantly increased in the serum of arthritic mice on day 11, compared with naïve animals, whereas PGE_2 showed a modest increase that did not reach statistical significance (Table 2). Interestingly, treatment with CORM-3 exerted a weak effect on 6 ketoPGF$_{1\alpha}$ but significantly lowered PGD_2 levels. COX-1 and COX-2 convert arachidonic acid to PGH_2, which is the precursor for terminal prostanoids (DuBois et al., 1998). PGD_2 is synthesized from PGH_2 by two enzymes, L-PGDS and H-PGDS (Urade and Eguchi, 2002). Since our studies demonstrated increasing levels of PGD_2 in serum of arthritic animals, we explored the contribution of H-PGDS and L-PGDS. Fig. 5 shows that H-PGDS is constitutively expressed in chondrocytes from naïve animals (54.71 ± 7.53) and up-regulated in arthritic controls (87.24 ± 4.87, $P<0.01$), where a strong expression was also observed in synovial cells. In contrast, L-PGDS was weakly detected in naïve mice (3.00 ± 3.00) but it was strongly up-regulated after arthritis development, mainly in chondrocytes (74.60 ± 6.98% in arthritic controls, $P<0.01$). Our results indicate that CORM-3 treatment was able to down-regulate the increased expression of both proteins in chondrocytes, with values of 53.22 ± 5.38 ($P<0.01$) and 51.60 ± 5.62 ($P<0.05$) for H-PGDS and L-PGDS, respectively. The expression of COX-2 protein increased from 19.33 ± 2.31 (naïve animals) to 47.34 ± 3.99 (arthritic controls) but CORM-3 treatment was unable to significantly modify the expression of this protein (50.37 ± 4.80).

Table 2
Levels of prostanoids in serum (day 11).

	PGD_2 (ng/ml)	6-ketoPGF$_{1\alpha}$ (ng/ml)	PGE_2 (ng/ml)
Naïve animals	155.30 ± 0.10	0.98 ± 0.01	0.79 ± 0.02
Arthritic control	780.20 ± 85.92^a	1.65 ± 0.21^a	1.09 ± 0.13
CORM-3	444.30 ± 61.17^b	1.28 ± 0.10	1.52 ± 0.22

Values are mean ± S.E.M., $n=8$. $^aP<0.01$ with respect to naïve animals, $^bP<0.01$ with respect to arthritic control. One-way ANOVA followed by Dunnett's *t*-test.

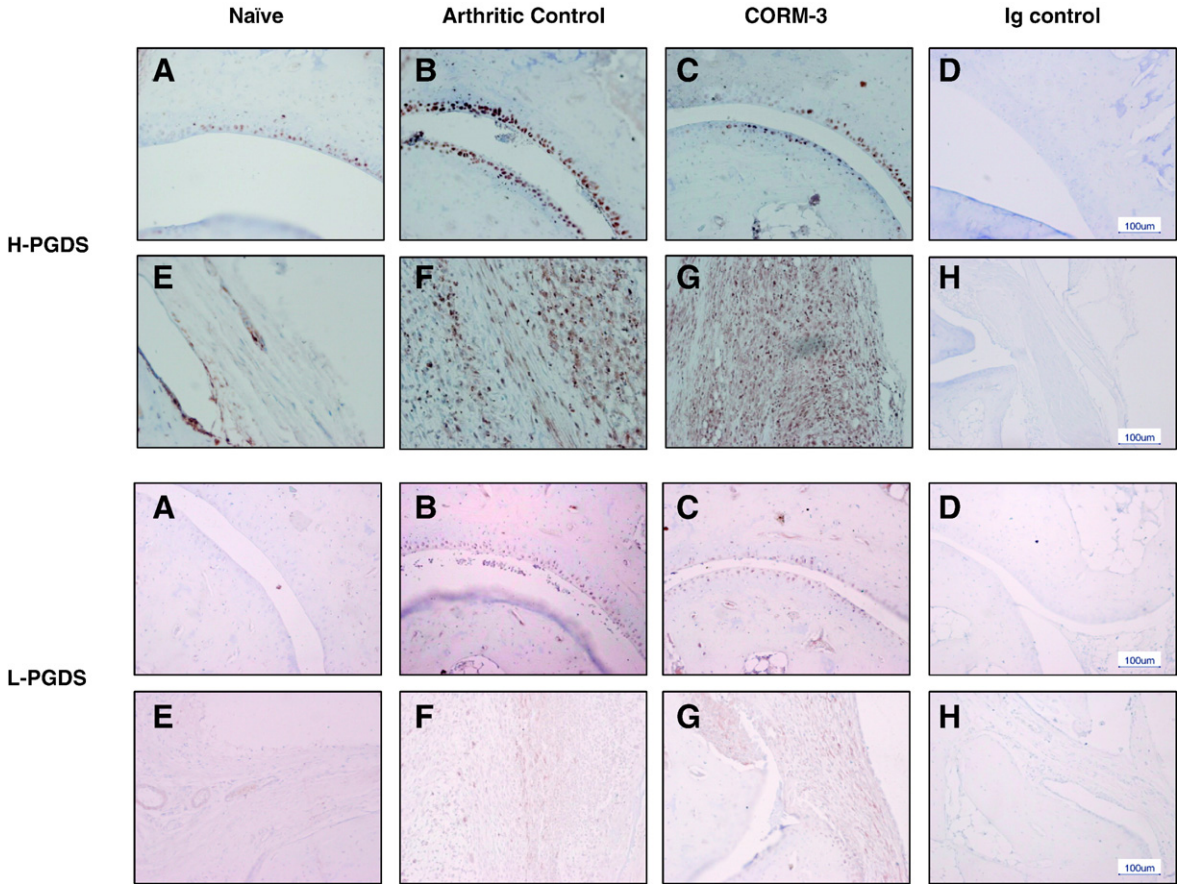

Fig. 5. Effect of CORM-3 on H-PGDS and L-PGDS expressions on day 11 analyzed by immunohistochemistry. Ankle sections were treated with a specific anti-H-PGDS antibody (A–C, E–G), a specific L-PGDS antibody (A–C, E–G) or IgG control antibody (D–H). A, E, sections from a naïve mouse. B, F, sections from an arthritic control mouse. C, G, sections from an arthritic mouse treated with CORM-3 (10 mg/kg/day). Original magnification ×200.

4. Discussion

Induction of HO-1 during inflammatory conditions could be part of an adaptative mechanism to limit cytotoxicity via scavenging of reactive oxygen or nitrogen species, regulation of cell proliferation and prevention of apoptosis (reviewed in Alcaraz et al., 2003). Induction of HO-1 can exert anti-inflammatory effects in vivo (Devesa et al., 2005). In line with the view that CO-releasing molecules mimic the biological actions of CO derived from HO activity (Motterlini et al., 2002), we have observed previously the in vivo anti-inflammatory effect of CORM-3 in the collagen-induced arthritis model (Ferrándiz et al., 2008). In the present work, we show that CORM-3 is effective in protecting against the articular destruction in autoantibody-induced arthritis. In the K/BxN serum transfer model of arthritis there is a predominance of infiltrating neutrophils in synovial tissues and fluid which play a key role in inducing arthritis (Wipke and Allen, 2001). Activated macrophages contribute to inflammation and joint destruction and they are associated with articular lesions in rheumatoid arthritis (van den Berg and van Lent, 1996) and the pathology of the K/BxN model (Solomon et al., 2005). Histological evaluation of joints revealed that CORM-3 limited the migration of leukocytes into the joints leading to a significant reduction in the extent of inflammation and associated cartilage and bone erosion.

Leukocyte recruitment to the inflamed synovium is a requirement for the process of arthritis. In rheumatoid arthritis, leukocyte migration is facilitated by the up-regulation of integrin counterreceptors such as ICAM-1 in inflamed endothelium, synovial macrophages and lining cells of rheumatoid arthritis joints (Szekanecz et al., 1994). ICAM-1 plays an important role in the K/BxN serum transfer arthritis, as deficient mice show a delay in onset and initial severity (Watts et al., 2005). Consistent with our results in collagen-induced arthritis (Ferrándiz et al., 2008), CORM-3 decreased ICAM-1 expression in articular joints, which may contribute to the reduction of leukocyte migration observed in the animals treated with this agent.

Inflammatory cytokines are known to be involved in human inflammatory arthritis including rheumatoid arthritis (Feldmann and Maini, 1999). In the K/BxN serum transfer arthritis, IL-1β is absolutely necessary for joint inflammation, whereas there was variability in the requirement for TNFα and IL-6 did not play an important role (Ji et al., 2002). Our data indicate that CORM-3 inhibited IL-1β production in the affected joints, with a small reduction in TNFα. Several lines of evidence indicate that HMGB1 plays a role either as a cytokine or potentiating the effects of pro-inflammatory cytokines (Andersson et al., 2002; Sha et al., 2008). This protein can be secreted by inflammatory cells or released by necrotic cells and is involved in the inflammatory events of chronic arthritis (Palmblad et al., 2007). Interestingly, HMGB1 is up-regulated in rheumatoid arthritis synovial tissues (Taniguchi et al., 2003) and the inhibition of its activity exerts anti-arthritic effects in the collagen-induced arthritis model (Kokkola et al., 2003). We provide evidence that HMGB1 is involved in the K/BxN arthritis and that CO down-regulates the expression of this protein in the inflamed joint. Recent data in animal models of sepsis would support these observations (Takamiya et al., 2009; Tsoyi et al., 2009).

CORM-3 not only decreased the inflammatory response but also protected against the degradation of cartilage and bone. These effects of CORM-3 could be related to the inhibition of expression of pro-inflammatory cytokines, RANKL and MMPs. These observations are of relevance in arthritis where the production of pro-inflammatory cytokines results in the up-regulation of RANKL, an essential regulator of bone remodeling and bone resorption (Boyce and Xing, 2008) and

MMPs, which can degrade all components of extracellular matrix (Burrage et al., 2006).

It has been demonstrated a prominent contribution of prostaglandins to the initiation and propagation of chronic inflammation in human disease and animal models. In rheumatoid arthritis, the coordinated induction of COX-2 and microsomal PGE synthase-1 results in a marked increase in PGE_2 (Stichtenoth et al., 2001), an important mediator of inflammation in the joint (Karouzakis et al., 2006). By contrast, in the K/BxN serum transfer arthritis COX-1 and PGI_2 could contribute to joint inflammation, whereas COX-2 and PGE_2 seem to be dispensable for the initiation and perpetuation of disease in this arthritis model (Chen et al., 2008). Our data suggest the participation of another prostanoid pathway, PGD_2, in the effector phase of the K/BxN arthritis model. Little is known about the role of PGD_2 in chronic inflammation. This prostanoid has complex immunoregulatory functions (Gosset et al., 2003) and is a critical factor in asthma (Arima and Fukuda, 2008). In certain conditions, PGD_2 and its J series metabolites have been shown to exert anti-inflammatory effects (Rajakariar et al., 2007; Murakami et al., 2003). Therefore, this group of lipid mediators may have a duality of function that may contribute to inflammation and resolution of acute responses (reviewed in Herlong and Scott, 2006). We have shown that PGD_2 significantly increases in the K/BxN arthritis, which is related to the up-regulation of H-PGDS and L-PGDS in arthritic joints. H-PGDS is present in hemopoietic cells including macrophages, whereas L-PGDS is mainly detected in CNS (Urade and Hayaishi, 2000; Urade and Eguchi, 2002). Several lines of evidence indicate that L-PGDS is induced in macrophages in pathological situations such as atherosclerosis (Cipollone et al., 2004) or bacterial infection (Joo et al., 2007). Inflammatory stimuli induce COX-2 and L-PGDS to produce PGD_2 which facilitates neutrophil recruitment to the lung (Joo et al., 2007). It is also known that IL-1β induces L-PGDS and PGD_2 production in human chondrocytes (Zayed et al., 2008). Our results have shown that CORM-3 does not modify the levels of PGI_2 and PGE_2 in arthritic animals but exerted a significant inhibitory effect on PGD_2. These effects could be the result of the down-regulation of both H-PGDS and L-PGDS in arthritic tissues.

The overall data presented add novel information about the mechanisms involved in the effector phase of experimental arthritis and its regulation by CORM-3. Our results support the potential of CO-releasing molecules in the development of therapeutic strategies in inflammatory diseases.

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