British Journal of Pharmacology (2008) 154, 843–851
© 2008 Nature Publishing Group All rights reserved 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Effect of chondroitin sulphate in a rabbit model of atherosclerosis aggravated by chronic arthritis

G Herrero-Beaumont[1], ME Marcos[1], O Sánchez-Pernaute[1], R Granados[2], L Ortega[3], E Montell[4], J Vergés[4], J Egido[1] and R Largo[1]

[1]*Joint and Bone Research Unit and Vascular Research Laboratory, Fundación Jiménez Díaz, Universidad Autónoma, Madrid, Spain;* [2]*Department of Pathology, Hospital Universitario de Getafe, Madrid, Spain;* [3]*Department of Pathology Hospital Clínico San Carlos, Madrid, Spain and* [4]*Clinical Pharmacology Research Unit, Scientific Medical Department, Bioibérica S.A, Barcelona, Spain*

Background and purpose: Among the agents employed to manage osteoarthritis, chondroitin sulphate (CS) is a natural glycosaminoglycan with an anti-inflammatory effect on joint cells. CS might also influence the inflammatory component of atherosclerosis. Our aim was to examine the effect of CS administration on vascular injury and on markers of systemic inflammation in a rabbit model of atherosclerosis aggravated by systemic inflammation provoked by chronic antigen-induced arthritis.

Experimental approach: Atherosclerosis was induced in rabbits by maintaining them on a hyperlipidaemic diet after producing an endothelial lesion in the femoral arteries. Simultaneously, chronic arthritis was induced in these animals by repeated intraarticular injections of ovalbumin in previously immunized rabbits. A group of these rabbits were treated prophylactically with CS (100 mg $kg^{-1}day^{-1}$) and when the animals were killed, serum and peripheral blood mononuclear cells (PBMC) were isolated. Furthermore, femoral arteries and thoracic aorta were used for gene expression studies and histological examination.

Key results: CS administration reduced the concentration of the proinflammatory molecules C-reactive protein and IL-6 in serum. Likewise, CS inhibited the expression of CCL2/monocyte chemoattractant protein (MCP)-1 and cyclooxygenase (COX)-2 in PBMC, and reduced the nuclear translocation of nuclear factor-κB. In the femoral lesion, CS also diminished the expression of CCL2 and COX-2, as well as the ratio of the intima/media thickness. Moreover, CS decreased the percentage of rabbits with atherosclerosis and chronic arthritis that developed vascular lesions in the aorta.

Conclusions and implications: These findings suggest that CS treatment may to some extent impede the progression of atherosclerosis.

British Journal of Pharmacology (2008) **154**, 843–851; doi:10.1038/bjp.2008.113; published online 21 April 2008

Keywords: chondroitin sulphate; atherosclerosis; chronic inflammation; arthritis; rabbit model

Abbreviations: AT, atherosclerosis; cAiA, chronic antigen induced arthritis; CCL2, monocyte chemoattractant protein-1; COX, cyclooxygenase; CRP, C-reactive protein; CS, chondroitin sulphate; IL, interleukin; NF-kB, nuclear factor-kappa B; PBMC, peripheral blood mononuclear cells

Introduction

Atherosclerosis is an inflammatory disease that involves the recruitment of monocytes into the arterial wall, a process driven by adhesion molecules and chemoattractant cytokines, mainly CCL2 (monocyte chemoattractant protein-1, MCP-1) (Hernández-Presa *et al*., 1997; Libby, 2002). These inflammatory cells express metalloproteinases that weaken the fibrous cap of the lesions making them prone to rupture and subsequent thrombosis, and also causing an acute coronary syndrome (Nomoto *et al*., 2003). Tumour necrosis factor α (TNFα), interleukin (IL)-1 and IL-6 promote atherosclerotic lesions and upregulate adhesion molecules, the latter being an important step in the migration of inflammatory cells to atherosclerotic plaques. These mediators are also considered key factors in promoting rheumatoid arthritis (RA) (Manzi and Wasko, 2000).

RA is a chronic systemic disease that primarily affects the joints, and is marked by inflammatory changes in the synovial membranes and articular structures. Epidemiological studies have shown a significantly higher mortality rate among RA patients than in age- and sex-matched control populations (Isomaki *et al*., 1975; Mutru *et al*., 1989; Wolfe

Correspondence: Dr R Largo, Joint and Bone Research Unit, Department of Rheumatology, Fundación Jiménez Díaz, Avenida Reyes Católicos 2, Madrid 28040, Spain.
E-mail: rlargo@fjd.es
Received 21 December 2007; revised 30 January 2008; accepted 13 February 2008; published online 21 April 2008

et al., 1994), with a notable increase in deaths associated with cardiovascular diseases related to atherosclerosis. The rheumatoid membrane produces large amounts of inflammatory cytokines that leak into the blood increasing their serum concentrations several fold and reaching other tissues such as atherosclerotic plaques. Therefore, it seems that the sustained systemic inflammatory activity in RA favours and aggravates endothelial lesions, increasing the susceptibility of these patients to cardiovascular events associated with atherosclerosis (Sattar *et al*., 2003).

Chondroitin sulphate (CS) is a natural glycosaminoglycan (GAG) that is predominantly found in the extracellular matrix, especially in cells surrounding the cartilage, skin, blood vessels, ligaments and tendons (Wight and Merrilees, 2004; Theocharis *et al*., 2006). The therapeutic effect of CS in patients with osteoarthritis of the knee (Leeb *et al*., 2000; Volpi, 2004) is probably the result of its anti-inflammatory activity at the level of chondrocytes and synovial cells. Accordingly, CS reduces nitric oxide release, the activation of nuclear factor- κB (NF-κB) and the synthesis of different proinflammatory mediators involved in the pathogenesis of atherosclerosis (Ronca *et al*., 1998; Bali *et al*., 2001; Álvarez-Soria *et al*., 2005; Monfort *et al*., 2007).

The objective of this study was to examine the anti-inflammatory properties of CS on atherosclerotic lesions in a rabbit model of induced atherosclerosis. In this model, chronic arthritis was experimentally induced at the same time as atherosclerosis to produce a systemic inflammation to aggravate the vascular lesions.

Methods

Experimental model

Studies were conducted on 40 white adult New Zealand male rabbits (3.02 ± 0.04 kg body weight). All animal handling and experimentation was performed in accordance with Spanish regulations and the Guidelines for the Care and use of Laboratory Animals drawn up by the National Institutes of Health (USA). All the procedures carried out here were approved by the Institutional Ethical Committee. After a 1-week period of adaptation to the facilities, chronic antigen-induced arthritis and atherosclerosis were synchronized as outlined in Figure 1. To initiate atherosclerosis, rabbits were fed a hyperlipidaemic diet consisting of 2% cholesterol and 6% peanut oil, which was maintained throughout the study. Two weeks after this diet had commenced, an endothelial lesion was induced in both femoral arteries by the infusion of gaseous nitrogen inside the femoral arteries under general anaesthesia (LeVeen *et al*., 1982; Hernández-Presa *et al*., 1997, 2002). This experimental model has been extensively employed to test the ability of different drugs to modify the progression of atherosclerosis (Bocan *et al*., 1993, 1998; Mehta *et al*., 1996; Hernández-Presa *et al*., 2002; Jùzwiak *et al*., 2005; Hasegawa *et al*., 2006; Vidal *et al*., 2007). Antigen-arthritis was induced as described previously with some modifications that were intended to produce a more severe phenotype (Howson *et al*., 1986; Palacios *et al*., 1998; Benito *et al*., 2000; Sánchez-Pernaute *et al*., 2003). In brief, animals were given two intradermal injections of 4 mg mL^{-1} ovalbumin (OVA) in Freund's complete adjuvant 14 days apart, at the start of the same week as the animals first received the hyperlipidaemic diet (Figure 1). One week after the first OVA injection, the animals were randomly allocated into two groups; one group received 100 mg kg^{-1} day^{-1} of CS while still on the atherogenic diet ($n = 10$, CS group) and the other was given no treatment ($n = 15$; NT group). All rabbits received intraperitoneal injections of powdered CS dissolved in 0.9% NaCl or the vehicle alone over 5 weeks. Five days after the second injection, 1 mL of OVA (5 mg mL^{-1}) was injected into the knee joints on a weekly basis over the following 4 weeks. Additionally, 15 rabbits that were fed standard chow and spared from any experimental intervention were used as healthy controls. At the end of the study, the rabbits were bled from their marginal ear vein under general anaesthesia and then killed with an overdose of pentobarbital. The femoral arteries and the thoracic aortae were removed and fixed in 4% buffered paraformaldehyde, dehydrated and embedded in paraffin. A piece of femoral artery was snap frozen and stored at –70 °C for molecular biology studies.

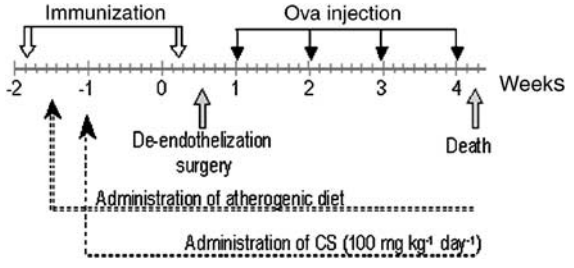

Figure 1 Schematic representation of the experimental model.

Serum chemistry

Ten millilitres of blood were employed for serum extraction. Total and HDL cholesterol and triglycerides were measured using enzymatic assays. Specific ELISA kits were used to determine the concentrations of C reactive protein (CRP) and interleukin (IL)-6.

Studies in femoral and aortic arteries

Once fixed, the arterial specimens from each animal were cut transversally into four fragments that were placed together in a single paraffin block. The blocks were cross-sectioned at 4 μm and serial sections were chosen at regular intervals and stained with haematoxylin–eosin or orcein. The sections were then scanned to identify the most severe stenosis to conduct the morphometric studies.

Morphometric analysis was performed using the Olympus semiautomatic analytic system with Micro Image software (version 1.0 for Windows). The images of the preparations were digitized with an Olympus microscope (BH-2), connected to a video camera device (Hernández-Presa *et al*., 2002; Vidal *et al*., 2007). The size of the plaque and the vessel lumen, and the intima/media thickness ratio were measured in the selected regions.

To identify macrophages in femoral lesions, a monoclonal antibody against rabbit macrophages (RAM11) was used as described previously (Hernández-Presa *et al*., 2002; Vidal *et al*., 2007). The antibody was detected with a biotinylated goat anti-mouse IgG which was visualized with ABComplex/horseradish peroxidase and 3,3′-diaminobenzidine tetrahydrochloride. Tissues were counterstained with haematoxylin and mounted in Pertex. The area stained (per square millimetre of tissue) was assessed in digital photomicrographs and expressed as a percentage (Hernández-Presa *et al*., 2002; Vidal *et al*., 2007). The evaluation was performed at the site of maximal stenosis and negative controls were carried out using an IgG isotype.

Isolation of peripheral mononuclear cells

At death, 30 ml of blood was obtained and used to isolate peripheral mononuclear cells (PBMC) (Hernández-Presa *et al*., 2002). PBMC were extracted, washed two times with cold PBS, pelleted and resuspended in either hypotonic buffer to isolate nuclear proteins (Largo *et al*., 2003) or in Trizol Reagent for RNA extraction.

Electrophoretic mobility shift assay

Protein extracts pooled from mononuclear cells were prepared as described previously (Hernández-Presa *et al*., 2002) and the concentration of each sample was quantified by the BCA method. A consensus oligonucleotide for NF-κB was end labelled with ^{32}P using 10 μl of T4 polynucleotide kinase and the nuclear extracts were equilibrated for 10 min in binding buffer before the labelled probe was added (Largo *et al*., 2003). The specificity of the assay was tested by preincubating the samples with a 100-fold excess of unlabelled probe. Samples were resolved on 4% non-denaturing acrylamide gels in Tris–Borate buffer, which were then dried and exposed to X-ray film.

RNA extraction and real-time polymerase chain reaction

Total RNA from the femoral arteries or the PBMC was obtained by the Trizol method and 1 μg of this RNA was reverse-transcribed with the high capacity cDNA kit following the manufacturers' recommendations. The GeneBank rabbit CCL2 and COX-2 cDNA sequences were used to design assay on demand primers and probes with the primer express software (Vidal *et al*., 2007). The expression of eukaryotic 18S rRNA (VIC/TAMRA Probe) was used as the endogenous control. Quantitative PCR was carried out in a 7500 Real-Time PCR system using the default amplification protocol (95 °C for 10 min followed by 40 PCR cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). Relative quantification was performed with the Prism 7000 System SDS programme and the results are expressed in terms of the n-fold increase in expression with regard to a reference sample, using the $\Delta\Delta C_t$ method.

Western-blot analysis

For COX-2 protein studies, total protein from the femoral arteries was isolated with Trizol and resolved on 10% acrylamide-SDS gels. After transfer to polyvinylidene difluoride membranes, antibodies against COX-2 and α-tubulin were used to probe the lysates (Largo *et al*., 2003). In brief, the membranes were blocked in 5% skimmed milk in PBS-Tween-20 for 1 h at room temperature, and incubated overnight at 4 °C with the primary antibodies. Antibody binding was detected by enhanced chemoluminiscence using peroxidase-labelled secondary antibodies and the results are expressed in arbitrary densitometric units normalized to the α-tubulin levels.

Materials

Rabbits were obtained from Granja San Bernardo, Navarra, Spain. The hyperlipidaemic diet was from Letica, Barcelona, Spain. Ovalbumin for rabbit immunization (Sigma Chemicals, St Louis, MO, USA) was dissolved in 0.9% NaCl (Sigma, St Louis, MO, USA) and Freund's complete adjuvant (Difco, Detroit, MI, USA). CS (Condrosan) was obtained from Bioibérica SA, Barcelona, Spain. Different enzymatic ELISA assays were used: total and HDL cholesterol and triglycerides determination kits were from Sigma Chemicals, CRP ELISA was from Alpha Diagnostic International (San Antonio, TX, USA) and IL-6 ELISA was from R&D Systems (Minneapolis, MN, USA). Different primary antibodies were used: RAM11 (Dako, Glostrup, Denmark); COX-2 (polyclonal antiserum from Santa Cruz Biotech, Heidelberg, Germany) and α-tubulin (monoclonal antibody from Sigma Chemicals). AS secondary antibodies used were: a biotinylated goat anti-mouse IgG (Amersham, Buckinghamshire, UK), a peroxidase-labelled secondary antibody (Amersham) and an ABComplex/horseradish peroxidase (Dako). Diaminobenzidine tetra-hydrochloride (Dako) was used as chromogen and immunohistochemical sections were mounted in Pertex (Medite, Burgdorf, Germany). For RNA and protein isolation from the tissues we used Trizol Reagent (Roche, Mannheim, Germany). For protein quantification, a BCA method by Pierce was carried out. The consensus oligonucleotide for NF-κB was obtained from Promega, and it was labelled with 10 μl of T4 polynucleotide kinase (Promega, Madison, WI, USA). All the reagents for Real-time PCR experiments were purchased from Applied Biosystems, and quantitative PCR was carried out in a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The statistical analysis was carried out using the SPSS package (version 11.0 for Windows). Lipid values, morphometric analysis, global synovitis score, immunohistochemistry, electrophoretic mobility shift assay (EMSA), western blot and real time PCR data are expressed as mean ± s.e.mean, and they were analyzed using the Mann–Whitney *U*-test. Where multiple comparisons were performed, the Kruskal–Wallis test was used. Statistical significance was set at $P<0.05$.

Results

One rabbit from the CS group died prematurely 2 days after the vascular surgery. Consequently, data from only 39 rabbits were available: 15 animals for the NT group, nine animals for the CS group and 15 healthy controls.

Serum parameters

The atherogenic diet increased total cholesterol in serum whereas it decreased the amount of HDL. A clear trend towards an increase of serum triglyceride concentrations was also observed in NT rabbits (Table 1). The induction of the combined model of atherosclerosis and chronic arthritis decreased the serum glucose content, an effect probably related to the inflammatory status of the animals. CS administration induced a weak reduction in total cholesterol in the serum when compared with the NT animals, as well as in the triglyceride content. By contrast, CS administration did not alter the serum glucose concentration in these animals when compared with NT group (Table 1).

With regard to acute phase reactant content in serum, both CRP and IL-6 concentrations were higher in NT animals than in the healthy controls ($P<0.001$, Figure 2). The administration of CS to rabbits with atherosclerosis plus chronic arthritis diminished the concentration of both CRP and IL-6 in serum ($P=0.011$ and $P=0.001$, respectively, vs NT). In fact, serum IL-6 content in CS rabbits was undistinguishable from that measured in healthy animals (Figure 2).

Table 1 Serum parameters

(mg dl^{-1})	*Healthy* (n = *15*)	*NT* (n = *15*)	*CS* (n = *9*)
Total cholesterol	49 ± 5	1842 ± 81*	1596 ± 55*,#
HDL	36 ± 4	19 ± 2*	18 ± 1*
Triglycerides	77 ± 13	90 ± 13	50 ± 3*,#
Glucose	254 ± 6	185 ± 6*	170 ± 5*

Data are shown as mean ± s.e.mean. *$P<0.05$ vs Healthy; #$P<0.05$ vs NT.

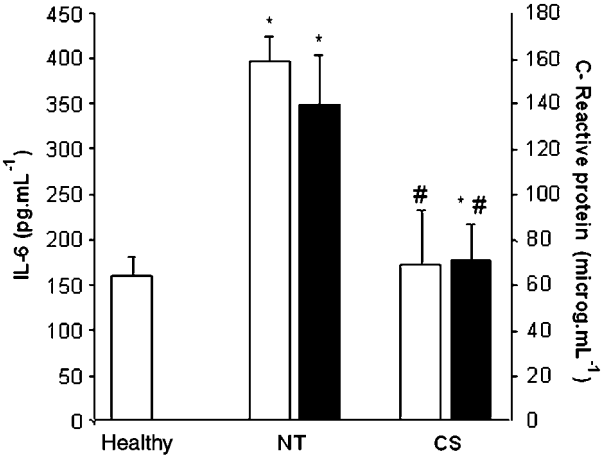

Figure 2 Effect of chondroitin sulphate (CS) administration to rabbits with atherosclerosis plus chronic arthritis on IL-6 (open columns) and C-reactive protein (solid columns) concentration in serum. *$P<0.01$ vs healthy controls; #$P<0.05$ vs NT rabbits.

COX-2 and CCL2 gene expression in PBMC

The untreated rabbits subjected to the combined model showed an increase in both COX-2 and CCL2 gene expression when compared with the healthy rabbits (Figure 3). The administration of CS reduced this increase in the gene expression of the genes encoding these mediators in PBMC ($P=0.045$ and $P=0.003$ vs NT, respectively; Figures 3a and b).

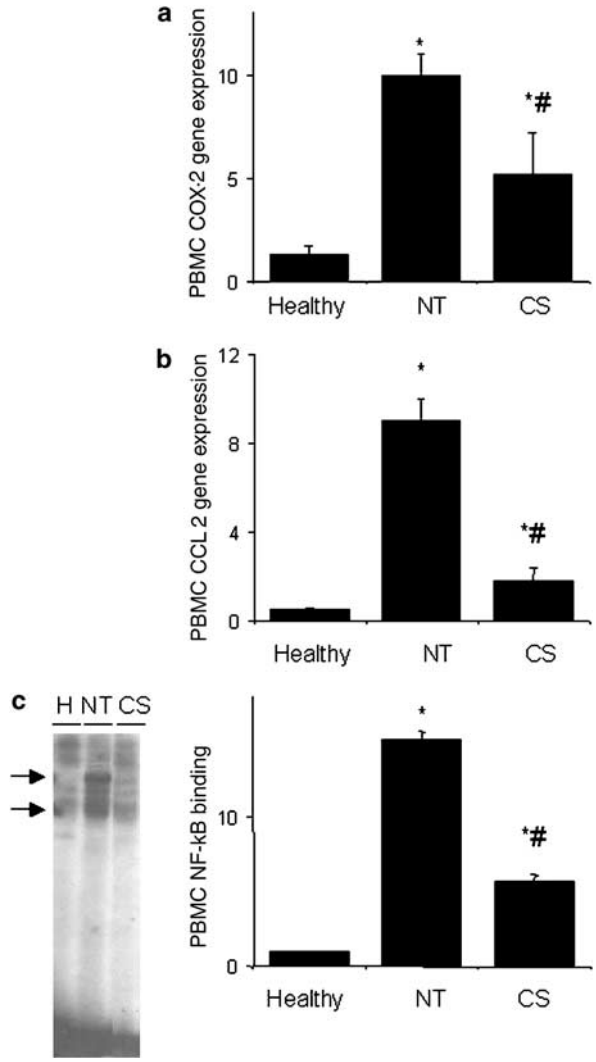

Figure 3 Effect of CS on peripheral blood mononuclear cells (PBMC). COX-2 (**a**) and CCL2 (**b**) gene expression in the PBMC cells extracted at the time of death from all the experimental animals. (**c**) Left panel, a representative example of an electrophoretic mobility shift assay of radioactive NF-κB bound to the nuclear proteins extracted from the PBMC; right panel, densitometric analysis of EMSA experiments. *$P<0.05$ vs healthy controls; #$P<0.05$ vs NT rabbits.

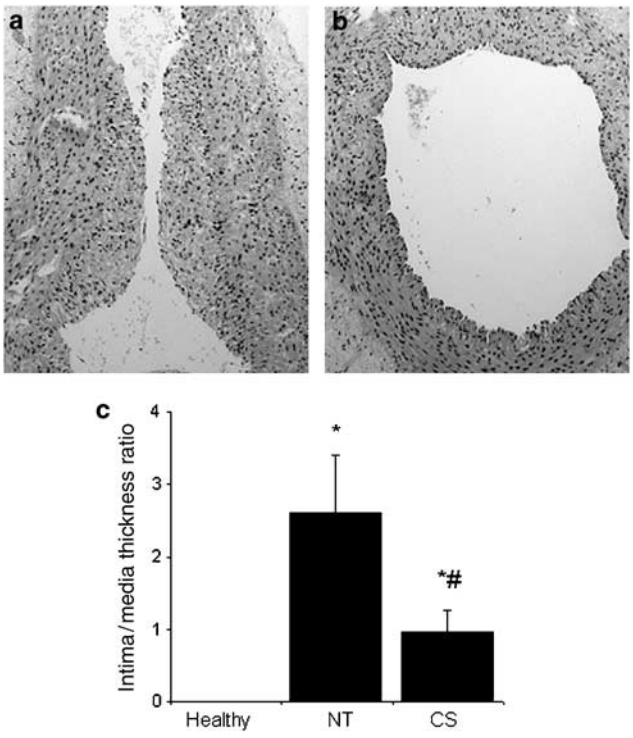

Figure 4 Analysis of the femoral vascular lesions. Top, photomicrographs of representative haematoxylin-eosin stained arterial sections of femoral arteries. Representative femoral sections of NT and CS treated rabbits are shown. (**a**) NT rabbit; (**b**) CS treated rabbit (magnification $\times 100$). (**c**) Intima/media thickness ratio in the arterial sections of all the groups studied. $^{*}P<0.05$ vs healthy controls; $^{\#}P<0.05$ vs NT rabbits.

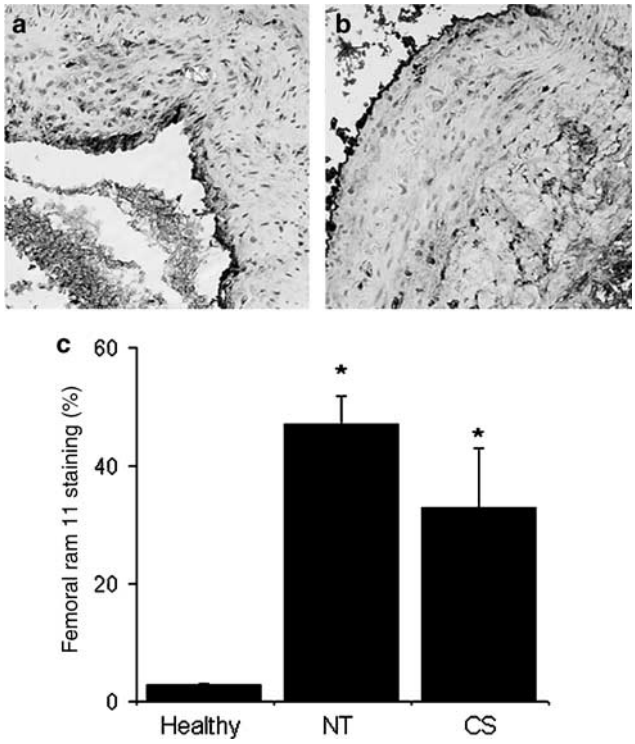

Figure 5 Macrophage detection by immunohistochemistry in sections of femoral arteries. Top, photomicrographs of arterial sections stained with the RAM11 antibody, specific for rabbit macrophages. Representative sections of NT- and CS-treated rabbits are shown: (**a**) NT rabbit; (**b**) CS-treated rabbit (magnification $\times 200$). (**c**) Quantification of positive staining in the neointimal area in all the rabbits studied. $^{*}P<0.05$ vs healthy controls.

NF-κB activation in PBMC

EMSA was carried out to determine the NF-κB activation, reflected by the radioactivity bound to the nuclear proteins in extracts obtained from the rabbits' PBMC (Figure 3c, left panel). The densitometric analysis of these gels indicated that there was an increase in the NF-κB activity in NT rabbits when compared with healthy rabbits, whereas the administration of CS produced a reduction in NF-κB activation in PBMC when compared with that in NT animals (Figure 3c).

Morphometric analysis of the femoral arteries

All of the animals subjected to femoral surgery (both in the NT and CS groups) developed a stenotic lesion in the femoral artery characterized by a hyperplasic transformation of the intima and foam cell infiltration (Figure 4). In contrast, no lesions were found in the femoral artery of the healthy rabbits. The intima/media thickness ratio was quantified in each animal at the site of maximal stenosis. CS reduced this intima/media ratio in the injured femoral arteries when compared with NT animals (0.9 ± 0.3 vs 2.5 ± 0.5, $P<0.05$; Figure 4c).

Presence of macrophages in the femoral lesion

The presence of macrophages at the neointima was assessed by using the RAM11 antibody. We detected macrophages in the neointima of the femoral arteries in all animals subjected to femoral surgery (Figure 5a). Moreover, there was an increase in the proportion of positive staining with the RAM11 antibody in the NT group when compared with the healthy rabbits (Figure 5b). CS appeared to attenuate macrophage infiltration in the neointimal area of the femoral arteries when compared with the NT group, although this difference was not significant (33 ± 10 vs 47 ± 5, $P=0.1$; Figure 5c). As expected, macrophages were not detected in the vessels of the healthy rabbits.

COX-2 expression in femoral arteries

The expression of the COX-2 gene and protein was studied in the femoral arteries of all the rabbits by quantitative RT-PCR and western blot methods, respectively. Both COX-2 gene and protein expression increased in NT animals when compared with the healthy rabbits (Figures 6a and b). The administration of CS diminished both COX-2 gene and protein expression in the damaged femoral arteries (Figures 6a and b).

CCL2 expression in femoral arteries

The induction of the combined disease model (NT rabbits) induced an increase in CCL2 gene expression when compared with healthy rabbits (Figure 6c), although the

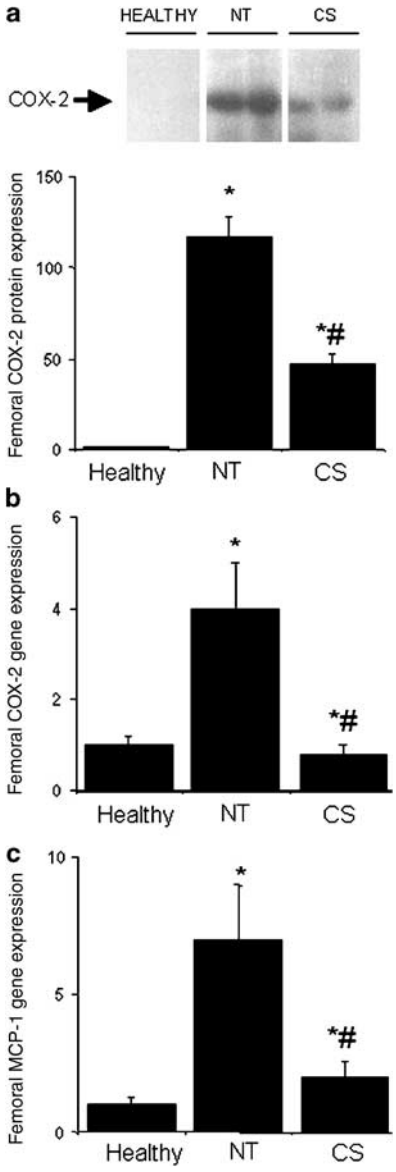

Figure 6 COX-2 protein (**a**) and mRNA (**b**) expression in the femoral artery in the rabbits studied. (**a**) Top, a representative western blot of COX-2 in femoral arteries. (**a**) Bottom, densitometric analysis of western blot studies. (**b** and **c**) Analysis of COX-2 (**b**) and CCL2 (**c**) mRNA expression measured by real-time PCR method. $^{*}P<0.05$ vs healthy controls; $^{\#}P<0.05$ vs NT rabbits.

expression of the CCL2 gene in the animals subjected to the surgery was downregulated following CS administration (2.0 ± 0.6 vs 7 ± 2, $P<0.05$). Furthermore, CCL2 gene expression in the femoral artery was positively correlated to the percentage of macrophage staining ($r=0.603$, $P=0.013$).

Presence of atherosclerotic lesions in the aorta

At the end of the study, the thoracic aortae were recovered from the animals and serial sections were stained to explore the appearance of vascular lesions. The experimental induction of the combined atherosclerosis and chronic arthritis model only evoked aortic lesions in a proportion of the rabbits studied. Thus, to ensure the presence or absence of atherosclerotic lesions, at least 20 different aortic sections were evaluated from all the rabbits in each group. When present, the lesions in the aorta were highly variable in size and appearance, and they consisted of lipid-rich deposits adhering to the aortic wall that were infiltrated with foam cells and other mononuclear cells (Figure 7b). All of these alterations are characteristic of early atherosclerotic lesions (Libby, 2002). The larger ones contained elastic fibres, fibroblast-like cells and collagen deposits. In some cases, fatty streaks were found inside the media layer, which could be seen to be associated with the ruptured elastic fibres, when observed with orcein. No aortic lesions were discovered in any of the healthy rabbits (Figure 7a), whereas visible atherosclerotic lesions were found in nine of the 15 NT rabbits (60%) (Figure 7c, $P<0.05$ vs healthy controls). Interestingly, only one of the nine CS-treated rabbits developed an aortic lesion ($P=0.001$ vs NT rabbits).

Discussion

Systemic inflammation is thought to be an independent risk factor for the development of atherosclerotic vascular damage (Blake and Ridker, 2001). In the present study, we showed that CS can reduce some markers of systemic inflammation and decrease the pro-inflammatory activation of the PBMC. Moreover, CS diminished the size of the femoral neointima and the content of some pro-inflammatory mediators in re-stenotic lesions. Additionally, only 11% of CS-treated animals developed early atherosclerotic lesions in the aorta, in comparison to 60% of the untreated rabbits.

The presence of a systemic inflammatory component, for example chronic arthritis, to the atherosclerosis model allowed us to assess the effect of CS on the inflammatory component of atherosclerosis. CS has different anti-inflammatory effects on human articular chondrocytes and synoviocytes *in vitro*. As such, CS counteracts the effect of proinflammatory mediators, such as IL-1, on nitric oxide and prostaglandin E_2 (PGE_2) release, as well as the synthesis of metalloproteinases and COX-2 by joint cells (Sadowski and Steinmeyer, 2002; Álvarez-Soria *et al*., 2005; Chan *et al*., 2005, 2006).

The beneficial effect of CS in controlling the systemic inflammatory response and in improving the inflammatory response at the atherosclerotic plaque might share similar mechanisms either in the circulating PBMC, the vascular neointima or affecting the osteoarthritic cells of the joint.

NF-κB is a key mediator that regulates the expression of many genes involved in the pathophysiology of tissue inflammation and cell recruitment (Barnes and Karin, 1997; Tak and Firestein, 2001). CS reduces NF-κB activation in the PBMC of rabbits in which the combined model of atherosclerosis and chronic arthritis was induced. We have previously demonstrated that CS can inhibit the activation of NF-κB induced by IL-1 in synovial cells in culture (Álvarez-Soria *et al*., 2005). Furthermore, other glicosaminoglycans with similar structures also inhibit COX-2 synthesis and PGE_2 release through the inhibition of the NF-κB pathway in different cell types, such as chondrocytes, fibroblasts and macrophages (Largo *et al*., 2003; Álvarez-Soria *et al*., 2005;

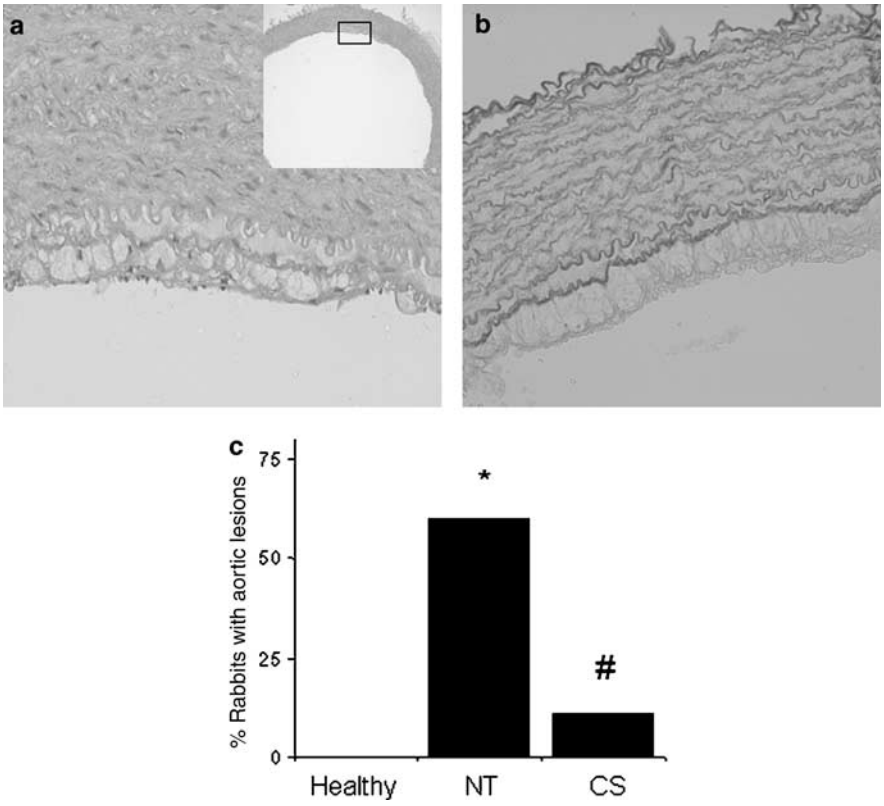

Figure 7 Presence of atherosclerotic lesions in the aorta of the animals. All the aortae were examined as detailed in the methods section. (**a**) Haematoxylin-eosin section from a NT rabbit (magnification $\times$ 200). A complete section of the vessel is shown in each inset ($\times$ 40). (**b**) Orcein staining of an aortic lesion from a NT rabbit, magnification $\times$ 100. (**c**) Percentage of rabbits with an atherosclerotic plaque in the aorta. *$P<0.05$ vs healthy controls; $^{\#}P<$ 0.05 vs NT rabbits.

Rafi *et al*., 2007). Similarly, heparin and hyaluronan also exert significant anti-inflammatory effects by inhibiting NF-κB activation in human monocytes and synovial fibroblasts (Hiramitsu *et al*., 2006; Hochart *et al*., 2006). Accordingly, CS treatment may decrease the expression of COX-2 and CCL2 not only in PBMC but also in the injured femoral artery due to the local inhibition of NF-κB activation.

In the current experimental model, CS reduced the intima/media thickness ratio in the arteries of injured rabbits. Therefore, CS could interfere with the neointimal growth process linked to vascular injury. Indeed, some GAGs have been shown to play an important role in the formation of the neointima, as well as in vascular endothelial and smooth muscle cell proliferation (Duan *et al*., 2005). Although we only observed a tendency for CS to diminish neointimal macrophage infiltration, it is conceivable that the total amount of infiltration may be reduced due to the decrease in the intima/media ratio evoked by CS. In this regard, CS and other soluble GAGs block the chemokine interaction at cell surfaces, thus reducing monocyte binding to the endothelial cells (Mack *et al*., 2002; Duan *et al*., 2005). We have also shown that the effect of CS on inflammatory cell recruitment to the neointima of the femoral artery could also be mediated through the inhibition of CCL2, which participates in the atherosclerotic process by recruiting monocytes to the arterial wall.

In atherosclerotic lesions, macrophages that express scavenger receptors, such as CD36, on their surface aggressively take up the oxidized LDL deposited on the inside of the blood vessel wall, and they are transformed in foam cells. In this sense, there is evidence that the macrophage scavenger receptor CD36 might be regulated by GAG (Sobal and Sinzinger, 2002). Thus, some of the lipoprotein uptake pathways in the foam-cell-forming macrophages might depend on the presence of CS (Halvorsen *et al*, 1998; Sobal and Sinzinger, 2002).

The mild reduction in total cholesterol levels that we observed in CS-treated rabbits does not seem to account for the beneficial effects of this drug on systemic and local inflammation that we observed. Indeed, cholesterol levels remained 30-fold higher in these rabbits than in controls.

Furthermore, COX-2 inhibition decreases CCL2 expression in vascular cells (Wang *et al*., 2005; Vidal *et al*., 2007). Thus, it is tempting to speculate that the inhibitory effect of CS on CCL2 expression might be mediated not only by NF-κB but also through COX-2, since CS evoked a decrease in COX-2 expression and synthesis. The inhibition of COX-2 induced by CS deserves additional attention in the light of the increase in cardiovascular death provoked by treatment with COX-2 inhibitors (McGettigan and Henry, 2006). Although the mechanisms associated with this phenomenon remain unclear, it seems that the extent to which PGE_2 synthesis is impaired by each drug, or their different anti-inflammatory potential, could be a crucial factor (Álvarez-Soria *et al*., 2006; Gislason *et al*., 2006). In this respect, we recently demonstrated that partial inhibition of COX-2 expression by

atorvastatin improved the progress of atherosclerosis in experimental atherosclerosis (Hernández-Presa *et al*., 2002). Furthermore, intensive treatment with atorvastatin in patients with carotid atherosclerotic plaques decreased both local and PBMC expression of COX-2 and the inflammatory activity of PBMC (Martin-Ventura *et al*., 2005; Gómez-Hernández *et al*., 2006).

It should be noted that the present results are difficult to extrapolate to humans due to several limitations. Although this experimental model of atherosclerosis has been criticized, it has been used extensively to test the ability of different drugs to modify the progression of atherosclerosis (Bocan *et al*., 1993; Mehta *et al*., 1996; Bocan *et al*., 1998; Hernández-Presa *et al*., 2002; Jùzwiak *et al*., 2005; Hasegawa *et al*., 2006; Vidal *et al*., 2007). Moreover, although the re-stenotic lesions described in injured femoral arteries do not exactly reflect all the findings in human atherosclerosis, the spontaneous lesions observed in intact aortae are similar to those observed in early human atherosclerotic lesions (Libby, 2002). Additionally, CS is administered orally to patients, and in this study it was administered intraperitoneally, and the doses given were much higher than those used for the treatment of osteoarthritis. Finally, CS was employed as a prophylactic agent, to detect a potential therapeutic effect (as is usual in these type of studies). Once this effect has been demonstrated, it must be confirmed in a designed therapeutic study. However, the present results are relevant as they can be used to design studies to test the effects of CS in human atherosclerosis.

To conclude, we have demonstrated that treatment with CS can reduce the markers of systemic inflammation in rabbits in which systemic inflammation induced by chronic arthritis aggravates vascular lesions associated with atherosclerosis. CS produced a clear improvement in the inflammatory response of atherosclerotic lesions in femoral re-stenosis, as well as a decrease in the number of aorta atherosclerotic lesions. These data indicate that clinical trials should be conducted with this compound to address the same hypothesis in human studies.

Acknowledgements

We thank Marta Pulido and Mark Sefton for editing the article. This work was partially supported by research grants from the Spanish Ministry of Education (SAF2006/2704) and Fondo de Investigaciones Sanitarias (PI06/0032; PI040259; CP03/00011).

Conflict of interest

Drs L Montell and J Vergés are employees of Bioiberica SA., whereas Drs G H-Beaumont, ME Marcos, O Sanchez-Pernaute, R Granados, L Ortega, J Egido and R Largo have no conflict of interest.

References

Álvarez-Soria MA, Largo R, Santillana J, Sánchez-Pernaute O, Calvo E, Hernández M *et al*. (2006). Long term NSAID treatment inhibits COX-2 synthesis in the knee synovial membrane of patients with osteoarthritis: differential proinflammatory cytokine profile between celecoxib and aceclofenac. *Ann Rheum Dis* **65**: 998–1005.

Álvarez-Soria MA, Largo R, Santillana J, Calvo E, Egido J, Herrero-Beaumont G (2005). Differential anticatabolic profile of glucosamine sulfate versus other anti-osteoarthritic drugs on human osteoarthritic chondrocytes and synovial fibroblast in culture. *Osteoarthritis Cartilage* **13**: S153.

Bali JP, Cousse H, Neuzil E (2001). Biochemical basis of the pharmacologic action of chondroitin sulfates on the osteoarticular system. *Semin Arthritis Rheum* **31**: 58–68.

Barnes PJ, Karin M (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* **336**: 1066–1071.

Benito MJ, Sánchez-Pernaute O, López-Armada MJ, Hernández P, Palacios I, Egido J *et al*. (2000). Cyclosporin A prevents the histologic damage of antigen arthritis without inducing fibrosis. *Arthritis Rheum* **43**: 311–319.

Blake GJ, Ridker PM (2001). Novel clinical markers of vascular wall inflammation. *Circ Res* **89**: 763–771.

Bocan TM, Mueller SB, Uhlendorf PD, Brown EQ, Mazur MJ, Black AE (1993). Inhibition of acyl-CoA cholesterol O-acyltransferase reduces the cholesteryl ester enrichment of atherosclerotic lesions in the Yucatan micropig. *Atherosclerosis* **99**: 175–186.

Bocan TM, Rosebury WS, Mueller SB, Kuchera S, Welch K, Daugherty A *et al*. (1998). A specific 15-lipoxygenase inhibitor limits the progression and monocyte-macrophage enrichment of hypercholesterolemia-induced atherosclerosis in the rabbit. *Atherosclerosis* **136**: 203–216.

Chan PS, Caron JP, Orth MW (2006). Short-term gene expression changes in cartilage explants stimulated with interleukin beta plus glucosamine and chondroitin sulfate. *J Rheumatol* **33**: 1329–1340.

Chan PS, Caron JP, Rosa GJ, Orth MW (2005). Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E(2) in articular cartilage explants. *Osteoarthritis Cartilage* **13**: 387–394.

Duan W, Paka L, Pillarisetti S (2005). Distinct effects of glucose and glucosamine on vascular endothelial and smooth muscle cells: evidence for a protective role for glucosamine in atherosclerosis. *Cardiovasc Diabetol* **4**: 16–26.

Gislason GH, Jacobsen S, Rasmussen JN, Rasmussen S, Buch P, Friberg J *et al*. (2006). Risk of death or reinfarction associated with the use of selective cyclooxygenase-2 inhibitors and nonselective non-steroidal antiinflammatory drugs after acute myocardial infarction. *Circulation* **113**: 2906–2913.

Gómez-Hernández A, Martín-Ventura JL, Sánchez-Galán E, Vidal C, Ortego M, Blanco-Colio LM *et al*. (2006). Overexpression of COX-2, Prostaglandin E synthase-1 and prostaglandin E receptors in blood mononuclear cells and plaque of patients with carotid atherosclerosis: regulation by nuclear factor-kappaB. *Atherosclerosis* **187**: 139–149.

Halvorsen B, Aas UK, Kulseth MA, Drevon CA, Christiansen EN, Kolset SO (1998). Proteoglycans in macrophages: characterization and possible role in the cellular uptake of lipoproteins. *Biochem J* **331**: 743–752.

Hasegawa H, Takano H, Ohtsuka M, Ueda K, Niitsuma Y, Qin Y *et al*. (2006). G-CSF prevents the progression of atherosclerosis and neointimal formation in rabbits. *Biochem Biophys Res Commun* **344**: 370–376.

Hernández-Presa M, Bustos C, Ortego M, Tuñón J, Renedo G, Ruiz-Ortega M *et al*. (1997). Angiotensin-converting enzyme inhibition prevents arterial nuclear factor-kappa B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early-accelerated atherosclerosis. *Circulation* **95**: 1532–1541.

Hernández-Presa MA, Martin-Ventura JL, Ortego M, Gomez-Hernández A, Tuñón J, Hernández-Vargas P *et al*. (2002). Atorvastatin reduces the expression of cyclooxygenase-2 in a rabbit model of atherosclerosis and in cultured vascular smooth muscle cells. *Atherosclerosis* **160**: 49–58.

Hiramitsu T, Yasuda T, Ito H, Shimizu M, Julovi SM, Kakinuma T *et al*. (2006). Intercellular adhesion molecule-1 mediates the inhibitory effects of hyaluronan on interleukin-1beta-induced matrix metalloproteinase production in rheumatoid synovial fibroblasts via down-regulation of NF-kappaB and p38. *Rheumatology (Oxford)* **45**: 824–832.

Hochart H, Jenkins PV, Smith OP, White B (2006). Low-molecular weight and unfractionated heparins induce a downregulation of inflammation: decreased levels of proinflammatory cytokines and nuclear factor-kappaB in LPS-stimulated human monocytes. *Br J Haematol* **133**: 62–67.

Howson P, Shepard N, Mitchell N (1986). The antigen induced arthritis model: the relevance of the method of induction to its use as a model of human disease. *J Rheumatol* **13**: 379–390.

Isomaki HA, Mutru O, Koota K (1975). Death rate and causes of death in patients with rheumatoid arthritis. *Scand J Rheumatol* **4**: 205–208.

Jùzwiak S, Wójcicki J, Mokrzycki K, Marchlewicz M, Biatecka M, Wenda-Rózewicka L *et al*. (2005). Effect of quercetin on experimental hyperlipidemia and atherosclerosis in rabbits. *Pharmacol Rep* **57**: 604–609.

Largo R, Alvarez-Soria MA, Diez-Ortego I, Calvo E, Sanchez-Pernaute O, Egido J *et al*. (2003). Glucosamine inhibits IL-1beta-induced NFkappaB activation in human osteoarthritic chondrocytes. *Osteoarthr Cartil* **11**: 290–298.

Leeb BF, Schweitzer H, Montag K, Smolen JS (2000). A metaanalysis of chondroitin sulfate in the treatment of osteoarthritis. *J Rheumatol* **27**: 205–211.

LeVeen RF, Wolf GL, Villanueva TG (1982). New rabbit atherosclerosis model for the investigation of transluminal angioplasty. *Invest Radiol* **17**: 470–475.

Libby P (2002). Inflammation in atherosclerosis. *Nature* **420**: 868–874.

Mack M, Pfirstingen J, Weber C, Weber KSC, Nelson PJ, Rupp T *et al*. (2002). Chondroitin sulphate A released from platelets blocks RANTES presentation on cell surfaces and RANTES-dependent firm adhesion of leukocytes. *Eur J Immunol* **32**: 1012–1020.

Manzi S, Wasko MC (2000). Inflammation-mediated rheumatic diseases and atherosclerosis. *Ann Rheum Dis* **59**: 321–325.

Martin-Ventura JL, Blanco-Colio LM, Gomez-Hernández A, Munoz-Garcia B, Vega M, Serrano J *et al*. (2005). Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. *Stroke* **36**: 1796–1800.

McGettigan P, Henry D (2006). Cardiovascular risk and inhibition of cyclooxygenase: a systematic review of the observational studies of selective and nonselective inhibitors of cyclooxygenase 2. *JAMA* **296**: 1633–1644.

Mehta D, Angelini GD, Bryan AJ (1996). Experimental models of accelerated atherosclerosis syndromes. *Int J Cardiol* **56**: 235–257.

Monfort J, Pelletier JP, Garcia-Giralt N, Martel-Pelletier J (2007). Biochemical basis of the effect of chondroitin sulfate on osteoarthritis articular tissues. *Ann Rheum Dis*, 20 July 2007 [e-pub ahead of print] (in press).

Mutru O, Laakso M, Isomaki H, Koota K (1989). Cardiovascular mortality in patients with rheumatoid arthritis. *Cardiology* **76**: 71–77.

Nomoto K, Oguchi S, Watanabe I, Kushiro T, Kanmatsuse K (2003). Involvement of inflammation in acute coronary syndromes assessed by levels of high-sensitivity C-reactive protein, matrix metalloproteinase-9 and soluble vascular-cell adhesion molecule-1. *J Cardiol* **42**: 201–206.

Palacios I, López-Armada MJ, Hernández P, Sánchez-Pernaute O, Gutiérrez S, Miguélez R *et al*. (1998). Tenidap decreases IL-8 and monocyte chemotactic peptide-1 (MCP-1) mRNA expression in the synovial tissue of rabbits with antigen arthritis and in cultured synovial cells. *Clin Exp Immunol* **111**: 588–596.

Rafi MM, Yadav PN, Rossi AO (2007). Glucosamine inhibits LPS-induced COX-2 and iNOS expression in mouse macrophage cells (RAW 264.7) by inhibition of p38-MAP kinase and transcription factor NF-kappaB. *Mol Nutr Food Res* **51**: 587–593.

Ronca F, Palmieri L, Panicucci P, Ronca G (1998). Anti-inflammatory activity of chondroitin sulfate. *Osteoarthr Cartil* **6** (Suppl A): 14–21.

Sadowski T, Steinmeyer J (2002). Effects of polysulfated glycosaminoglycan and triamcinolone acetonid on the production of proteinases and their inhibitors by IL-1alpha treated articular chondrocytes. *Biochem Pharmacol* **64**: 217–227.

Sánchez-Pernaute O, López-Armada MJ, Calvo E, Díez-Ortego I, Largo R, Egido J *et al*. (2003). Fibrin generated in the synovial fluid activates intimal cells from their apical surface: a sequential morphological study in antigen-induced arthritis. *Rheumatology* **42**: 19–25.

Sattar N, McCarey DW, Capell H, McInnes IB (2003). Explaining how 'high-grade' systemic inflammation accelerates vascular risk in rheumatoid arthritis. *Circulation* **16**: 2957–2963.

Sobal G, Sinzinger H (2002). Binding of [99mTc]chondroitin sulfate to scavenger receptors on human chondrocytes as compared to binding of oxidized [125I]LDL on human macrophages. *J Recept Signal Transduct Res* **22**: 459–470.

Tak PP, Firestein GS (2001). NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* **107**: 7–11.

Theocharis AD, Tsolakis I, Tzanakakis GN, Karamanos NK (2006). Chondroitin Sulfate as a key molecule in the development of atherosclerosis and cancer progression. *Adv Pharmacol* **53**: 281–295.

Vidal C, Gomez-Hernández A, Sanchez-Galan E, Gonzalez A, Ortega L, Gomez-Gerique JA *et al*. (2007). Licofelone, a balanced inhibitor of cyclooxygenase and 5-lipoxygenase, reduces inflammation in a rabbit model of atherosclerosis. *J Pharmacol Exp Ther* **320**: 108–116.

Volpi N (2004). The pathobiology of osteoarthritis and the rationale for using the chrondoitin sulfate for its treatment. *Curr Drug Targets Immune Endocr Metabol Disord* **4**: 119–127.

Wang K, Tarakji K, Zhou Z, Zhang M, Forudi F, Zhou X *et al*. (2005). Celecoxib, a selective cyclooxygenase-2 inhibitor, decreases monocyte chemoattractant protein-1 expression and neointimal hyperplasia in the rabbit atherosclerotic balloon injury model. *J Cardiovasc Pharmacol* **45**: 61–67.

Wight TN, Merrilees MJ (2004). Proteoglycans in atherosclerosis and restenosis. Key roles for versican. *Circ Res* **94**: 1158–1167.

Wolfe F, Mitchell DM, Sibley JT, Fries JF, Bloch DA, Williams CA *et al*. (1994). The mortality of rheumatoid arthritis. *Arthritis Rheum* **37**: 481–494.