Effect of a high dose of glucosamine on systemic and tissue inflammation in an experimental model of atherosclerosis aggravated by chronic arthritis

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Atherosclerosis is a major cause of mortality in the twenty-first century. Although the mechanisms contributing to atherogenesis are complex, it has recently been suggested that all of the cardiovascular risk factors act by aggravating an underlying inflammatory process (25). Monocytes and macrophages play a key role in the origin and development of atherosclerosis, and, initially, the monocytes in the vessel wall differentiate into macrophages that accumulate intracellular modified low-density lipoprotein (LDL) cholesterol to generate fatty streak lesions (12). Endothelial cells then produce adhesion molecules and chemoattractant cytokines, mainly CCL2 (monocyte chemoattractant protein-1, MCP-1), thereby promoting the migration of inflammatory cells (monocytes, T cells) to atherosclerotic plaques (13, 23). In turn, adhesion molecules are upregulated by several cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL)-1, and IL-6. TNF-α and IL-6 trigger the synthesis of C-reactive protein (CRP), and circulating CRP is a marker of systemic inflammation that is associated with an increased risk of cardiovascular events in the general population (19). Moreover, CRP is present in atherosclerotic lesions, and its local concentration may be related to plaque stability (17). Significantly, these mediators are also considered to be key pathogenic factors of rheumatoid arthritis (RA) (26). Indeed, chronic inflammation, as determined by the mean CRP levels in individuals with long-standing RA, has been associated with the development of both subclinical atherosclerosis manifested by increased carotid artery intima-media thickness (9) and cardiovascular events (8).

RA is a chronic disease characterized by persistent synovial and systemic inflammation, which leads to structural changes in joints and disability. Epidemiological studies have shown a significantly higher mortality rates among RA patients than in age- and sex-matched control populations, with a notable increase in fatal cardiovascular events associated with atherosclerosis (32, 47). The rheumatoid synovium produces large amounts of inflammatory cytokines, increasing their serum concentrations severalfold and facilitating their access to distal tissues and atherosclerotic plaques. Therefore, sustained systemic inflammation in RA seems to favor and aggravate endothelial disease, increasing the susceptibility of these patients to atherosclerosis-related cardiovascular events (40).

A number of genes involved in the pathogenesis of both atherosclerosis and RA have nuclear factor-kB (NF-kB)-binding sites in their promoters (30). Although the activation of this ubiquitous transcription regulator is essential for normal processes in healthy individuals, its prolonged activation has been associated with atherosclerosis (27) and RA (41). Indeed, NF-kB and upstream mediators that lead to its activation are considered attractive candidates as therapeutic targets to treat these conditions.

Glucosamine sulfate (GS) is a natural amino monosaccharide found in the glycosaminoglycans, proteoglycans, and hyaluronan of the cartilage and connective tissues, contributing to their strength, flexibility, and elasticity (46). This amino monosaccharide is currently being employed widely to treat human osteoarthritis symptoms due to its reported beneficial effects in the control of pain and the structural abnormalities associated with this clinical condition (15, 33). In addition to its chondroprotective activity, GS is also thought to produce anti-inflammatory effects in local cells from joints (18), and it may have some symptomatic effects on RA (33). Although the anti-inflammatory effect of GS has been observed in several...
cell culture (10, 21) and animal models (50), its mode of action is still to be fully elucidated. However, because GS partially inhibits NF-κB activation in vitro (21), this pathway might explain the effects of this compound in vivo to some extent (15). Accordingly, we have studied the effect of glucosamine on systemic and local inflammation in a rabbit model of induced atherosclerosis aggravated by chronic arthritis.

MATERIALS AND METHODS

Experimental model of induced atherosclerosis in rabbits with chronic arthritis. In this study, 45 white adult New Zealand male rabbits (Granja San Bernardo, Navarra, Spain) were used with a mean ± SE weight of 3.0 ± 0.3 kg. A detailed report describing the experimental animal model employed here has recently been published (22). Briefly, all rabbits were allowed to adapt to the facilities for 1 wk, and they were then separated into three groups of 15 animals each: a control group of healthy rabbits fed with standard chow (control); a group of rabbits with chronic antigen-induced arthritis and atherosclerosis (AIA-AT); and a group of rabbits (AIA-AT + GS) with chronic induced arthritis and atherosclerosis that received GS with their food (GS, 500 mg kg⁻¹·day⁻¹, Xicil; Rottapharm). To induce atherosclerosis, the rabbits were fed a hypercholesterolemic diet of 2% cholesterol and 6% peanut oil throughout the study (Letica, Barcelona, Spain). After starting the diet (2 wk), an endothelial lesion was induced in both femoral arteries by the intravascular instillation of nitrogen gas under general anesthesia (22). To induce AIA, animals were given two intradermal injections of 4 mg ovalbumin in Freund’s complete adjuvant, 14 days apart, beginning the same week as the hyperlipidemic diet. After the second injection (5 days), 1 ml of ovalbumin (5 mg/ml in 0.9% NaCl) was injected in both knee joints on a weekly basis over the following 4 wk. Animals were then killed 6 wk after the beginning of the atherogenic diet (a scheme detailing the experimental model of arthritis and atherosclerosis is shown in Fig. 1). At the end of the study, 10 ml of blood were obtained under general anesthesia, and then animals were killed with an overdose of pentobarbital. Both the femoral arteries and the thoracic aortas were removed and fixed in 4% buffered paraformaldehyde, and they were dehydrated and embedded in paraffin. Alternatively, a different piece of femoral artery was snap-frozen and stored at −70°C for molecular biology studies. Furthermore, both synovial membranes were removed and divided into two pieces to be processed for histological analysis or for molecular biology studies. All experiments were performed in accordance with Spanish regulation and the Guidelines for the Care and use of Laboratory Animals drawn up by the National Institutes of Health. The protocol was approved by the Institutional Ethics Committee.

Biochemical measurements. Blood (10 ml) was obtained for serum extraction, and the total serum cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were determined by enzymatic methods using commercial kits (Sigma Chemicals). Specific commercial enzyme-linked immunosorbent assays were used to determine CRP (Alpha Diagnostic International) and IL-6 (R&D Systems) levels.

Isolation of peripheral mononuclear cells. At the time of death, 30 ml of blood were collected to isolate peripheral blood mononuclear cells (PBMC) using lymphoprep (22). Thereafter, cells were washed twice in PBS and treated with either a hypotonic solution to obtain the nuclear extracts (21) or with Trizol Reagent (Roche) to extract RNA from the lysates.

Electrophoretic mobility shift assay. Protein extracts pooled from mononuclear cells were prepared according to the protocol described previously (22), and the protein concentration in each sample was quantified by the BCA method (Thermo Scientific). A consensus oligonucleotide for NF-κB (Promega) was end-labeled with ³²P using 10 units of T4 polynucleotide kinase (Promega), and the nuclear extracts were then equilibrated for 10 min in binding buffer before adding the labeled probe (21). The specificity of the assay was tested by preincubating the samples with a 100-fold excess of unlabeled probe. Samples were resolved on 4% non-denaturing acrylamide gels in Tris-borate buffer, which were dried and exposed to X-ray film to determine the NF-κB reaction activated by the radioactivity bound to the nuclear proteins present in the nuclear extracts.

RNA extraction and real-time PCR. Tissue from femoral arteries or knee synovial membranes was homogenized in liquid nitrogen, and total RNA was extracted from the resulting powder using Trizol (Roche) according to the manufacturer’s instructions. Total RNA was also extracted from PBMC following the same protocol. First-strand cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions (Applied Biosystems, Stockholm, Sweden). PCR primers and probes were designed by Applied Biosystems (available on request), and the endogenous control in these assays was the

Fig. 1. A: schematic representation of the experimental model. OVA, ovalbumin; B: lipid profile and glycemic values of the rabbits studied. Chol, cholesterol; HDL, high-density lipoprotein; AIA-AT, antigen-induced arthritis and atherosclerosis; GS, glucosamine sulfate. C: effect of GS administration to AIA-AT rabbits on serum concentrations of interleukin (IL)-6 (open bars) and C-reactive protein (CRP) (filled bars). *P < 0.01 vs. controls; #P < 0.05 vs. AIA-AT rabbits; n = 15 animals/group.
eukaryotic 18S rRNA. Thermal cycling and florescence detection were performed in an ABI Prism 7500 sequence detection system with ABI Prism 7000 SDS software (Applied Biosystems), and thermal cycling was carried out for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Gene expression values were calculated using $2^{-\Delta\Delta Ct}$, where the $\Delta Ct$ value of each sample was determined by subtracting the average Ct value of the reference gene from the average Ct value of the target gene. The $\Delta\Delta Ct$ value was then calculated by subtracting the $\Delta Ct$ of the sample with the highest expression (i.e., that with the lowest $\Delta Ct$ value) from the $\Delta Ct$ value of the sample. Target gene expression relative to the sample with highest expression was then estimated by the $\Delta\Delta Ct$ method.

**Western-blot analysis.** Tissues from femoral arteries or from knee synovial membranes were homogenized in liquid nitrogen, and total protein was extracted from the resulting powder by the Trizol method (Roche). Once precipitated in ethanol, the proteins were solubilized in 1% SDS and resolved on 10% acrylamide-SDS gels. After transfer to PVDF membranes were blocked in 5% skimmed milk in PBS-Tween (Cruz Biotech) and $\alpha$-tubulin (Sigma Chemicals) (21). Briefly, the PVDF membranes were blocked in 5% skimmed milk in PBS-Tween 20 for 1 h at room temperature and then incubated overnight at 4°C with the primary antibodies. Antibody binding was detected by enhanced chemoluminescence using peroxidase-conjugated secondary antibodies, and the results were expressed as arbitrary densitometric units normalized to the $\alpha$-tubulin levels.

**Histopathological analysis of the vascular and synovial lesions.** Each femoral artery was cut transversely into four equal fragments and embedded in a single paraffin block. Serial sections (4 μm thick) were cut from these blocks, and multiple sections from each block were chosen at regular intervals and stained with hematoxylin-eosin or orcein. This procedure was also carried out on the tissue from each thoracic aorta. The sections were then analyzed qualitatively to identify the zone with the most severe stenosis where morphometric and immunohistochemical studies were then performed. Morphometry was performed using the Olympus semiautomatic analytic system with Micro Image software (version 1.0 for Windows), and the images of the preparations were digitized with an Olympus microscope (BH-2) connected to a video camera device (22, 45). Intima and media thickness were measured, and the results were expressed as intima-to-media thickness ratios.

Paraffin-embedded knee synovial sections were prepared and stained with hematoxylin-eosin, and synovitis was evaluated in each sample according to the Krenn scale (20), as described previously (1).

**Immunohistochemistry in femoral lesions.** We identified macrophages at the site of maximal stenosis in the femoral lesions using a monoclonal anti-rabbit macrophage antibody (RAM11; Dako) and according to a protocol described previously (22, 45). The antibody was detected with a biotinylated goat anti-mouse IgG that was visualized with an horseradish peroxidase/ABCComplex using 3,3’-diaminobenzidine tetra-hydrochloride as the chromagen (Dako). The tissues, previously counterstained with hematoxylin, were mounted in Pertex (Medite), and the stained area was analyzed in digital photomicrographs and expressed as a percentage per square millimeter of tissue (22, 45). The negative controls involved detection with an IgG isotype.

**Statistical analysis.** The lipid values and the data from the morphometric analysis, global synovitis score, immunohistochemistry, electrophoretic mobility shift analysis (EMSA), Western blots, and real-time PCR are all expressed as the means ± SE, and they were analyzed using the Mann-Whitney U-test. Where multiple comparisons were performed, the Kruskal-Wallis test was used. The null hypothesis was rejected in each statistical test when the $P$ value was $<0.05$. All statistical analyses were performed using Windows SPSS version 11.0 software (SPSS, Chicago, IL).

**RESULTS**

**Lipid profile and the circulating levels of IL-6 and CRP.** As expected, the lipid profile in rabbits fed with an atherogenic diet (AIA-AT and AIA-AT + GS groups) was characterized by higher levels of total cholesterol and triglycerides and by lower concentrations of HDL-cholesterol than in control rabbits (Fig. 1B). Moreover, oral glucosamine treatment affected the triglyceride levels significantly, preventing the increase in serum triglyceride levels induced by the atherogenic diet. By contrast, GS administration did not modify the glucose concentration that remained similar to that in AIA-AT rabbits.
With regard to the serum concentrations of proinflammatory mediators, both CRP and IL-6 levels increased in the AIA-AT rabbits when compared with their levels in control animals. However, these increases were inhibited in the animals that received glucosamine (Fig. 1C).

**NF-κB activation in PBMC.** The nuclear translocation of NF-κB observed by EMSA was augmented in the PBMC from rabbits in the AIA-AT and AIA-AT + GS groups with regard to the control animals (Fig. 2A). However, this increase in NF-κB translocation was smaller in the AIA-AT + GS group compared with the AIA-AT animals ($P < 0.05$).

**COX-2 and CCL2 gene expression in PBMC.** In the AIA-AT rabbits, there was an increase in the expression of both COX-2 and CCL2 genes in PBMC compared with the PBMC from control rabbits (Fig. 2, B and C). This increase in COX-2 and CCL2 gene expression was inhibited by the administration of GS.

**Morphometric analysis of the femoral arteries.** All rabbits in the AIA-AT and AIA-AT + GS groups developed a stenotic lesion in the femoral arteries that was characterized by a hyperplasia of the intima and by foam cell infiltration (Fig. 3, A and B). By contrast, there was no evidence of lesions in the vessel wall of control animals. However, lower intima-to-media thickness ratios were detected at the site of maximal stenosis in the AIA-AT + GS group than in the AIA-AT group ($1.1 \pm 0.2$ vs. $2.0 \pm 0.5$, $P < 0.05$; Fig. 3C).

**Evaluation of the presence of macrophages in the femoral arteries.** Macrophages were detected in the neointima of all the rabbits subjected to femoral surgery, and administration of GS did not significantly affect the infiltration of macrophages in the neointima of the femoral artery (Fig. 3F).

**COX-2 and CCL2 expression in femoral arteries.** A significant increase in COX-2 mRNA expression and protein levels was evident in AIA-AT rabbits compared with control rabbits (Fig. 4, A and B), although administration of GS totally or partially attenuated these increases in COX-2 expression and accumulation ($P < 0.05$). Expression of the CCL2 gene (Fig. 4C) also increased significantly in the femoral arteries of AIA-AT rabbits compared with control animals, although the...
increase observed in the AIA-AT + GS animals was significantly lower than that in the AIA-AT group ($P < 0.05$).

**Immunohistochemistry of the synovial tissue.** In representative photomicrographs of synovial tissues from rabbits in each group, it was evident that AIA-AT rabbits displayed widespread lesions involving intimal hyperplasia and mononuclear cell infiltration at the subintima, lesions that were frequently associated with aggregate formation (Fig. 5, A, C, and F). In addition, foam cells were abundant in both layers (Fig. 5, C and F). In these animals, the characteristic adipose-rich interstitium of the healthy synovium (Fig. 5, E and J) was replaced by the cells recruited and by areas of fibrosis (Fig. 5, F and H). While in general all of these lesions were also found in AIA-AT + GS rabbits, they were not so severe (Fig. 5, B, D, and G). In this respect, the intimal hyperplasia was focal (Fig. 5D), and the mononuclear cell infiltrates were smaller (Fig. 5G). Accordingly, the semiquantitative synovitis score showed significant differences between both groups ($P < 0.05$; Fig. 5K).

**Gene expression evaluation in synovial tissue.** Interestingly, an effect of GS treatment on CCL2 and COX-2 gene expression at the inflamed joint was also evident. In this regard, the expression of both genes increased in AIA-AT rabbits compared with the expression in control synovial tissues. In
turn, COX-2 and CCL2 gene expression was diminished by GS treatment \((P < 0.05)\), although COX-2 protein accumulation in synovial membranes did not appear to be affected by GS.

The presence of atherosclerotic lesions in the aorta. The sections of the thoracic aorta were evaluated microscopically to assess any atherosclerotic lesions. The piece of thoracic aorta obtained from each animal at death was cut transversely into four equal fragments that were embedded in the same paraffin block and, thus, each paraffin section contained four different fragments of the thoracic aorta. We obtained 100 consecutive 4-μm-thick paraffin sections for each animal, of which one in five was evaluated. Therefore, 20 paraffin sections from the aortas of each control, AIA-AT, and AIA-AT + GS rabbit were evaluated. Although there were no appreciable aortic lesions in any of the control rabbits (Fig. 6), visible aortic wall lesions were detected in 9 of the 15 (60%) AIA-AT rabbits \((P < 0.05\) vs. controls). Interestingly, no aortic lesions were identified in any of the 15 AIA-AT + GS rabbits \((P = 0.05\) vs. AIA-AT rabbits).
DISCUSSION

Atherosclerosis and RA are diseases with an important chronic inflammatory component that have been linked to inappropriate and prolonged NF-κB activation (27, 41). Activated NF-κB has been detected in blood and plaques from atherosclerotic patients (27), and in synovial cells from patients with RA (41, 36). Although this inappropriate activation is not the causal factor in these pathologies, it could be a pathogenic factor and a potential therapeutic target. NF-κB plays a key role in regulating a number of genes encoding cytokines, chemokines, and adhesion molecules, which are involved in the origin and development of atherosclerosis and RA. In recent years, several studies have focused on identifying drugs that can prevent the inflammatory process underlying these pathologies at the level of gene expression. In this context, aspirin (6), statins (13), parthenolide (24), losartan (ANG II receptor antagonists) (49), acetyl-11-keto-β-boswellic acid (5), and magnolol (4) have all been shown to exert beneficial effects on atherosclerosis in animal models by inhibiting NF-κB activation. Here, we show that glucosamine inhibited NF-κB activation in PBMC isolated from AIA-AT rabbits, suggesting that this might be one mode of action for sulfated glucosamine. These results are in agreement with those obtained from the heart and isolated cardiomyocytes of a T-H shock model in rats (50), and in human chondrocytes and synoviocytes (21).

We have also shown that GS reduced the systemic inflammation observed in AIA-AT rabbits by significantly lowering circulating CRP and IL-6 concentrations. The mechanisms underlying the association between markers of systemic inflammation and a higher risk of atherogenesis have not yet been fully elucidated. It was recently reported that CRP induced the nuclear translocation of NF-κB in human monocytes (11), and it enhanced CCL2 and tissue factor expression (39). Monocytes are involved in the initiation and progression of atherosclerosis, and NF-κB is a key factor that regulates the expression of many genes involved in the pathophysiology of tissue inflammation and cell recruitment (2, 35, 42). In accordance with these observations, GS prevented the increase in gene expression of COX-2 and CCL2 observed in AIA-AT rabbits, two genes that are regulated by NF-κB (34) and that are involved in atherosclerosis. Although the principal source of circulating CRP is the hepatocyte, CRP is also synthesized within atherosclerotic lesions by vascular smooth muscle cells and macrophages (3). At a local level, administration of GS also inhibits COX-2 and CCL2 gene expression in the femoral arteries. Thus our results suggest that the beneficial effect of GS on systemic inflammation and on the formation of atherosclerotic plaques might be mediated by NF-κB. It was recently shown that glucosamine could have a cardioprotective effect, at least in part due to attenuating the activation of the NF-κB pathway through an increase in protein O-linked-N-acetylglu-
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tetion on synovial tissue. Oral administration of glucosamine lesions are induced through common mechanisms.

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specific modes of action have not been fully elucidated. Thus until now, it remained unknown whether CS interacts with 

proteoglycans may interfere with the neointimal growth linked to vascular injury. This key result is consistent with recent observations in rats subjected to balloon injury of the carotid artery, where GS was shown to significantly reduce neointima formation, an effect probably mediated by an increase in O-GlcNAc protein modification (48). The ability of glucosamine to partially prevent atherogenesis has also been shown in apolipoprotein E null mice (7). Exogenous glucosamine is taken up by cells through the GLUT-2 and GLUT-4 glucose transporters (44), and there is evidence that GS might act directly on the vessel wall, preventing or reduc-

ing the progression of atherosclerosis by attenuating cell growth, as well as influencing the inflammatory environment 

and the coagulation system. In vascular smooth muscle cells, GS inhibits cell proliferation (7), and it provokes the synthesis of proteoglycans with shorter glycosaminoglycan chains. GS also reduces LDL efflux (43) and, consequently, the oxidation of LDL retained by the proteoglycans as well as the inflam-

matory responses precipitated by oxidized LDL. The potential effect of GS on inflammation might also be mediated by the inhibition of the inducible nitric oxide synthase (29). Furthermore, GS increases plasminogen activator inhibitor 1 expression, preventing vascular smooth muscle cell apoptosis (38), stabilizing atheroma plaques, and preventing atherothrombo-

sis. It has also been suggested that increasing the specific incorporation of sulfate into heparin sulfate proteoglycans may be related to the favorable effects of GS, since it contains hypersulfated regions structurally and functionally analogous to heparin (28). Further studies will be needed to clarify if all these effects of glucosamine are mediated by NF-κB.

We also analyzed the effects of oral glucosamine adminis-

tration on synovial tissue. Oral administration of glucosamine was reported to significantly suppress the progression of adju-

vant arthritis in rats, where it inhibited nitric oxide and PGE2 release (16). Our results indicate that GS is partly effective in controlling synovitis by inhibiting the presence of COX-2 and CCL2 in synovial tissue.

We recently showed that administering chondroitin sulfate (CS) to rabbits with AIA-AT reduced the concentration of inflammatory markers in serum and produced a clear improve-

ment in the inflammatory content of femoral atherosclerotic lesions, as well as a decrease in the aortic lesions (14). GS and CS are saccharides with a different chemical composition, and, while GS is an amino monosaccharide, CS is a glycosaminoglycan formed by the polymerization of the glucuronic acid and galactosamine disaccharides with different sulfation sites. Until now, it remained unknown whether CS interacts with cells as a polymer or if it is hydrolyzed into its different components to be biologically active. Although both com-

pounds share different effects on the control of some proinflam-

matory mediators, such as NF-κB activation, nitric oxide release, or the synthesis of different cytokines (31, 37), their specific modes of action have not been fully elucidated. Thus studies to compare the effects of both compounds would be helpful to elucidate whether their effects on atherosclerotic lesions are induced through common mechanisms.

It should be noted that there are several limitations to the study presented here. First, the doses of GS given to the rabbits were ~20 times higher than those used in humans to treat osteoarthritis. Moreover, the approach was prophylactic and aimed at detecting a potential therapeutic effect. Thus, once this effect has been demonstrated, it must be confirmed in a specifically designed therapeutic study. In this sense, similar doses have been employed in experimental models of tissue injury when addressing this hypothesis (48, 50). In addition, although the restenotic lesions described in injured femoral arteries do not exactly reflect all of the lesions found in early human atherosclerosis, the spontaneous lesions observed in intact aortas are similar to such early human atherosclerotic lesions (23). Finally, we assessed NF-κB activation in PBMC but not in arterial or synovial tissues, assuming that the mo-

lecular mechanism of GS does not differ according to cell type. Thus it is important to stress that our results will be particularly useful to design studies that will more accurately test the effect of GS in human atherosclerosis.

In summary, we have demonstrated that administering a high dose of GS to rabbits with AIA-AT prevents NF-κB activation in PBMC and the downregulation of CCL2 and COX-2 expression in PBMC, femoral arteries, and synovial tissue. Furthermore, GS produces a systemic anti-inflammatory effect, and it induces a clear decrease in the number of aortic atherosclerotic lesions. This natural compound could represent a novel therapeutic approach to regulate the expression of proinflammatory genes involved in atherosclerosis and chronic arthritis.

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