Avocado/Soybean Unsaponifiables Increase Aggrecan Synthesis and Reduce Catabolic and Proinflammatory Mediator Production by Human Osteoarthritic Chondrocytes

YVES E. HENROTIN, CHRISTELLE SANCHEZ, MICHELLE A. DEBERG, NATHALIE PICCARDI, GEORGES BERNARD GUILLOU, PHILIPPE MSIKA, and JEAN-YVES L. REGINSTER

ABSTRACT. ***Objective.*** To investigate the effects of avocado (A)/soybean (S) unsaponifiables on the metabolism of human osteoarthritic (OA) chondrocytes cultured in alginate beads over 12 days.

Methods. Enzymatically isolated OA chondrocytes were cultured in alginate beads in a well defined culture medium for 12 days, in the presence or not of 10^{-10} M interleukin 1ß (IL-1ß). DNA content was measured using a fluorometric method. Production of aggrecan (AGG), stromelysin-1 (MMP-3), tissue inhibitor of metalloproteinases-1 (TIMP-1), macrophage inflammatory protein-1ß (MIP-1ß), IL-6, and IL-8 were assayed by specific enzyme amplified sensitivity immunoassays. Prostaglandin (PG) E_2 was measured by a specific radioimmunoassay and nitrite by a spectrophotometric method based on the Griess reaction. A commercial avocado and soybean mixture of unsaponifiables (A1S2) and each component separately were tested in a range of 0.625 to 40.0 µg/ml.

Results. After 12 days' incubation, A1S2 increased AGG synthesis and accumulation in alginate beads in a dose and time dependent manner. A1S2 promoted the recovery of aggrecan synthesis after 3 days of IL-1ß treatment. A1S2 was a potent inhibitor of basal and IL-1ß stimulated MMP-3 production. The procedure also weakly reversed the inhibitory effect of IL-1ß on TIMP-1 production. A1S2 inhibited basal production of MIP-1ß, IL-6, IL-8, NO•, and PGE_2 by OA chondrocytes and partially counteracted the stimulating effect of IL-1 on PGE_2. Compared to avocado or soybean added separately, the mixture had a superior effect on NO• and IL-8 production.

Conclusion. A1S2 stimulated aggrecan production and restored aggrecan production after IL-1ß treatment. In parallel, A1S2 decreased MMP-3 production and stimulated TIMP-1 production. These results suggest A1S2 could have structure-modifying effects in OA by inhibiting cartilage degradation and promoting cartilage repair. (J Rheumatol 2003;30:1825–34)

Key Indexing Terms:
AVOCADO SOYBEAN CARTILAGE CYTOKINES OSTEOARTHRITIS

Osteoarthritis (OA) is a complex disease characterized by bone remodeling, synovium inflammation, and cartilage loss. The progression of cartilage degradation is not linear but is interrupted by attempts at repair, suggesting that chondrocytes keep potential synthetic capacities even at endstage of the disease[1-3]. The mechanisms leading to cartilage failure have been largely investigated. They primarily involve excessive production of metalloproteinases, including collagenases (MMP-1, 8, 13)[4-6], aggrecanases (ADAM-TS4 and TS5)[7], stromelysin-1 (MMP-3)[8,9], and gelatinases (MMP-2, 9)[10,11] by chondrocytes and neighboring cells. Metalloproteinase production is upregulated by interleukin 1ß (IL-1ß), a cytokine found to be elevated in OA cartilage[12-14]. IL-1ß also contributes to cartilage degradation by inhibiting matrix component synthesis (aggrecan and type II collagen)[15,16]. It also stimulates the synthesis of proinflammatory cytokines [IL-6, IL-8, macrophage inflammatory protein (MIP)] and reactive oxygen species (NO•, O_2^-, H_2O_2) that contribute to the sequence of synovium inflammation[17-19].

Promotion of cartilage repair reaction and/or reduction of metalloproteinase synthesis/activity constitute important research objectives for treatment of OA. The unsaponifiable elements of avocado (A) and soybean (S) oils mixed in a ratio 1:2 (A1S2, Piascledine®, Laboratories Expanscience,

From the Bone and Cartilage Metabolism Research Unit, University Hospital, CHU Sart-Tilman, Liège, Belgium.

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Y.E. Henrotin, PhD; C. Sanchez, BSc; M.A. Deberg, PhD, Bone and Cartilage Metabolism Research Unit, University Hospital, Liège; N. Piccardi, PhD; G.B. Guillou, MD; P. Msika, PhD, Laboratoires Expanscience, Courbevoie, France; J-Y.L. Reginster, MD, PhD, Bone and Cartilage Metabolism Research Unit, University Hospital, CHU Sart-Tilman, Liège.

Address reprint requests to Dr. Y. Henrotin, Bone and Cartilage Metabolism Research Unit, Institute of Pathology, CHU Bat B23, B-4000 Liège, Belgium. E-mail: yhenrotin@ulg.ac.be

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Courbevoie, France) have been used to treat OA for several years[20-22]. Clinical studies have described the beneficial effects of avocado/soybean unsaponifiables (A1S2) on OA symptoms[20-22]. In a prospective clinical trial, A1S2 treatment showed significant symptomatic efficacy over placebo in the treatment of hip OA, acting from month 2 and showing a persistent effect for at least 2 months after a 6 month period of treatment[21]. The authors concluded that A1S2 was a slow-acting symptomatic drug with a persistent effect and an excellent safety record. Further, A1S2 has shown potential structure-modifying effects in animal and clinical studies. Indeed, a 2 year placebo controlled trial has recently reported evidence of reduced joint space narrowing (JSN) in OA hip in a group treated with A1S2, but only in the subgroup of patients with severe JSN (under the median value of joint space width) at baseline[23].

In animal models, oral A1S2 treatment following meniscectomy appeared to confer a subtle but statistically significant effect in maintaining articular cartilage proteoglycan content and thickness and reducing subchondral bone sclerosis[24]. There is also *in vitro* evidence that A1S2 is capable of stimulating matrix production and reducing the deleterious effect of IL-1, possibly via the production of transforming growth factor-ß (TGF-ß), an important family of growth factors in terms of cartilage homeostasis[25]. We also reported that A1S2 possesses antiinflammatory properties by inhibiting IL-6 and IL-8 production by human chondrocytes[26]. However, no study concerning the effect of A1S2 on aggrecan synthesis and deposition has been published so far. Thus, studying the effect of A1S2 on chondrocytes cultured in alginate beads would be helpful to assess its potential "structure-modifying" effects. This model is particularly suitable to investigate matrix deposition and organization and allows stability of the chondrocyte phenotype for at least 12 days[27]. We investigated the effect of A1S2 on human articular OA chondrocytes over 12 days.

MATERIALS AND METHODS

Chondrocyte culture in alginate beads. Cartilage was obtained from the knees of cadavers with primary OA immediately after death, excised from the superficial and medium layers of cartilage and avoiding the calcified layer. Upon dissection, the femoral, patellar, and tibial articular surfaces were evaluated for gross pathological cartilage modifications according to a personal scale. The severity of pitting was recorded for each sample. Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow-grey area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; and III, ulcers penetrating to the subchondral bone on one or more articular surfaces. Experiments were performed with OA cartilage from 6 different donors with a mean age of 42 years (range 35–52). All donors showed OA cartilage lesions of grade I or III.

Cartilage was cut into small fragments and then subjected to enzymatic digestion sequentially with hyaluronidase, pronase, and collagenase (3 g cartilage/10 ml enzyme solution) as described[28]. Cell viability was estimated by Trypan blue exclusion test, and in all cases was > 95%. Chondrocytes were suspended in alginate beads as described[27] and maintained in culture for 12 days in Dulbecco's modified Eagle's medium supplemented with 1% ITS+ (ICN Biomedicals, Asse-Relegem, Belgium), 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml), 200 µg/ml glutamine (Biowhittaker Europe, Verviers, Belgium), 50 µg/ml ascorbic acid (Sigma-Aldrich, Bornem, Belgium), and 2 mM proline (Invitrogen, Merelbeke, Belgium). ITS+ is a premixed cell growth system containing per 1 ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 µg selenious acid, 0.125 g bovine serum albumin (BSA), and 0.535 mg linoleic acid. Cells remained in this culture medium (washout medium) for 48 h as a precaution against *in vivo* contamination with drugs that donors might have taken before death. After this washout period, culture medium was changed and the various drugs added. Culture medium was changed every 3 days and the collected supernatants were kept at –20°C until analysis.

Treatments. Chondrocytes were cultured 12 days in the absence or presence of 10^{-10} M IL-1ß (1.7 ng/ml or 85 U/ml; Boehringer, Mannheim, Germany) and with or without A1S2 (Piascledine; see Appendix) or A or S added separately. A1S2 was tested at concentrations ranging from 0.625 to 40.0 µg/ml. A and S unsaponifiable residues were also tested separately at concentrations of 3.3, 6.6, or 10.0 µg/ml. All drugs tested were first dissolved in tetrahydrofurane (THF; Sigma-Aldrich), and then diluted in culture medium to achieve the required final concentration. The final concentration of THF was 0.1% in all culture conditions, including the controls (i.e., IL-1ß). For the recovery experiment, chondrocytes were treated for 3 days with IL-1ß (preincubation), washed, and then cultured for 15 days with or without A1S2 (10 µg/ml). In the same experiment, each culture condition was performed in triplicate and each experiment was repeated 3 times using tissue samples from 3 different donors.

Lastly, the culture medium (S) was carefully discarded and the beads of each well were dissolved in 1 ml of 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. With this method, 2 fractions were collected: the supernatant containing macromolecules that originated from the further-removed matrix (FRM) compartment, and a pellet containing cells with their associated matrix (CM). These 2 fractions were studied separately. The cell pellets were washed with phosphate buffered saline (PBS; Biowhittaker Europe), and then homogenized in 1 ml of PBS by ultrasonic dissociation at 4°C for DNA assay. CM and FRM were kept at –20°C until analysis.

DNA assay. DNA content of the cultures was measured according to the fluorometric method[29].

Lactate dehydrogenase (LDH) assay. Cell viability was estimated by the release of LDH in the culture supernatant. In a 96-well plate, 50 µl of Tris 0.01 buffer, pH 8.5, containing 0.1% BSA and lactate (800 mM) and 50 µl of a solution of 1.6 mg/ml INT (p-iodonitrotetrazolium violet, Sigma-Aldrich), 4 mg/ml NAD (Roche Pharmaceuticals, Brussels, Belgium), and 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich) were added to 100 µl of culture supernatant (before freezing). The absorbance at 492 nm was read after 5 min incubation at room temperature. The calibration was performed with LDH isolated from rabbit muscle. The cell death score was obtained by calculating the ratio: (LDH supernatants/total LDH) × 100. Total LDH corresponded to the sum of the LDH measured in the CM, the FRM, and the culture supernatants (3 to 12 days).

PGE_2 radioimmunoassay. PGE_2 was assayed in the different compartments (culture supernatants, FRM, CM) without previous extraction using a radioimmunoassay (RIA) as described[30]. In this assay, polyclonal rabbit antiserum was used that does not crossreact with other prostanoids (thromboxane B_2, 6-keto-prostaglandin F1α, prostaglandin A_2) or with any fatty acids (arachidonic, linoleic, or oleic acids). ^{3}H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule (PGE_2) from Sigma-Aldrich. Intra and interassay coefficients of variation were 6 and 10%, respectively. The limit of detection of the RIA was 20 pg/ml.

Immunoassays for aggrecan (AGG), MIP-1ß, IL-6, IL-8, MMP-3, and TIMP-1. AGG, MIP-1ß, IL-6, IL-8, TIMP-1, and stromelysin-1 (MMP-3) were measured in the different culture compartments (S, FRM, and CM) by

specific enzyme amplified sensitivity immunoassays (EASIA; Biosource Europe, Fleurus, Belgium). The limits of detection of the AGG, MIP-1ß, IL-6, IL-8, TIMP-1, and MMP-3 immunoassays were 5.5 ng/ml, 12.5 pg/ml, 8 pg/ml, 4 pg/ml, 325 pg/ml, and 1.25 ng/ml, respectively.

The assays were based on the oligonal system in which several monoclonal antibodies (Mab) were directed against distinct epitopes of the molecules. In the case of MMP-3, the antibodies were directed against pro-MMP-3, activated-MMP-3, and MMP-3 bound to TIMP-1 and TIMP-2. Aggrecan synthesis was quantified by an EASIA using a Mab raised against the keratan sulfate region of AGG as a capture antibody, and a Mab to the hyaluronic acid binding region as a detector. Intra and interassay coefficients of variation were < 5% for all immunoassays.

Nitric oxide (NO•) assay. Nitrite and nitrate are stable endproducts of nitric oxide. Nitrate was reduced to nitrite by addition in the supernatant of nitrate reductase (0.25 U/ml, Roche) for 20 min at 37°C. Nitrite concentrations in conditioned culture supernatants were determined by a spectrophotometric method based on the Griess reaction[31]. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

Calculation and statistical analysis. The results (mean ± SEM) were expressed as the concentration of AGG, MMP-3, TIMP-1, MIP-1ß, NO_2, and PGE_2 in the culture supernatants (3 to 12 days), CM, and FRM (or the sum of these values for the total production) per µg DNA. A multivariate repeated measures ANOVA model with calculation of contrasts was performed on all the experiments (MANOVA; SAS system, General Linear Models).

RESULTS

Effect of A1S2 on cell viability and DNA content. The DNA content (3.7 ± 0.3 µg/well after 12 days of culture) increased by 38% over the duration of culture, and was not significantly affected by the presence of IL-1ß (10^{-10} M). Cell death, determined by the release of LDH from the cells during the 12 days of culture, ranged between 5% and 8%. IL-1ß, even after 12 days of incubation, did not modify LDH release. At concentrations ranging between 0.625 and 40.0 µg/ml, A1S2 did not significantly affect cell death score or DNA content, even in IL-1ß treated culture. At 10 µg/ml, neither A nor S added separately significantly affected cell viability (data not shown).

Effects of A1S2 on AGG production. AGG production was essentially immobilized in the alginate beads. Seventy percent of the newly synthesized AGG was found in the FRM, 28% in the CM, and only 2% in the supernatant. After 12 days' incubation, the total production of AGG was enhanced by A1S2 in a concentration dependent manner: IC_{50} = 7.86 µg/ml (95% CI 7.55–8.17), $p < 0.001$ (Figure 1A). In basal conditions, AGG production increased in a time dependent manner until the 6th day, and then reached a steady state. In the presence of A1S2 (10 µg/ml), AGG synthesis was not different from the control in excess of the initial 6 days of treatment, but increased thereafter until day 12. The stimulating effect of A1S2 on AGG production was already significant after 9 days of incubation (Figure 1B). When the different compartments (FRM, CM, supernatant) of the culture were individually analyzed for their content of AGG, it appeared that the increase in AGG content occurred primarily in the FRM (Figure 1C). We also compared the effect of A1S2 (10 µg/ml) with effects of avocado (A) or

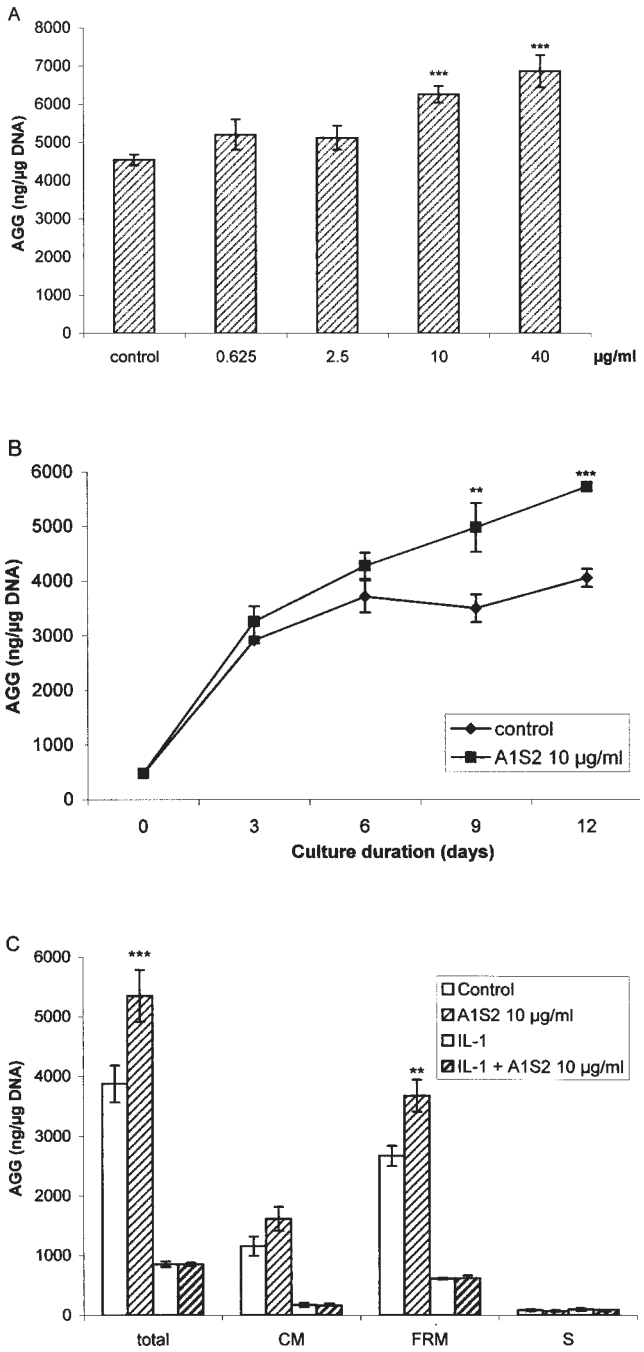

Figure 1. (A) Effect of increased concentrations of A1S2 on basal production of aggrecan (AGG) by human OA chondrocytes. Values correspond to the total amounts (sum of accumulated amounts found in supernatant, CM, and FRM) of AGG produced by chondrocytes during 12 days of culture. (B) Kinetic curves of AGG production in the absence or presence of A1S2 10 µg/ml. (C) Distribution of AGG in the different culture compartments after 12 days of culture. S: supernatant (3 to 12 days), CM: cell-associated matrix, FRM: further removed matrix. Values are means ± SEM (n = 9) of 3 independent cultures performed with cartilage specimens from 3 different donors (a 52-year-old man, grade I; a 38-year-old woman, grade I; and a 35-year-old woman, grade I). Statistical significance in comparison to controls: **p < 0.01, ***p < 0.001.

soybean (S) unsaponifiables added separately in the culture medium at doses of 3.3 and 6.6 µg/ml, respectively. This analysis revealed that in the mixture, A and S had no additive effect (Table 1). At 10 µg/ml, A and S added separately were as efficient as A1S2 on aggrecan production.

IL-1ß (10^{-10} M) drastically decreased total AGG production (sum of AGG found in supernatant, FRM, and CM) by 73% and modified AGG distribution through the different culture compartments. The ratio of AGG found in the supernatants increased from 2% to 8% in the presence of IL-1ß, suggesting that IL-1ß induced AGG degradation. Neither A nor S mixed or added separately were capable of reversing the effect of IL-1 on AGG synthesis (data not shown).

Finally, we tested the effect of A1S2 on the recovery of AGG synthesis after treatment with IL-1ß. In this experiment, chondrocyte cultures were incubated 3 days with IL-1ß, washed, and then cultured for the next 15 days with or without A1S2 (10 µg/ml). As shown in Figure 2, pretreatment with IL-1ß fully blocked AGG synthesis throughout the culture period, whereas A1S2 restored AGG synthesis after 9 days of incubation. After 15 days, AGG production had recovered by 88%.

Effects of A1S2 on MMP-3 and TIMP-1 production. Under basal conditions, A1S2 dose dependently inhibited stromelysin-1 (MMP-3) production by human OA chondrocytes ($0.01 \leq p \leq 0.001$; IC_{50} = 6.36 µg/ml; 95% CI 6.64–6.08) (Figure 3A). By comparison with the control group, the inhibitory effect of 10 µg/ml A1S2 was already significant at day 3 of culture ($p \leq 0.01$) and its magnitude increased with incubation time (Figure 3B). After 12 days, basal MMP-3 synthesis was decreased by 30%. A1S2 (10 µg/ml) and S (6.6 µg/ml) showed a similar effect on this variable, while A (3.3 µg/ml) did not modify MMP-3 synthesis, suggesting that S was the active fraction (Table 1). At 10 µg/ml, A1S2, S, and A inhibited MMP-3 synthesis similarly.

In the presence of IL-1ß, MMP-3 increased markedly over the control values during the first 3 days of culture, reached a maximum after the 3rd day, and then remained stable until the end of the culture. In contrast, in the basal condition, MMP-3 synthesis increased throughout the culture period. A1S2 weakly, but significantly inhibited the IL-1ß induced MMP-3 overproduction observed after 3 days of incubation (Figure 3B).

Under basal conditions, MMP-3 production by OA chondrocytes largely exceeded TIMP-1 production (Table 1).

Table 1. Effect of A1S2, avocado (A), and soybean (S) on basal production of aggrecan (AGG), stromelysin-1 (MMP-3), tissue inhibitor of metalloproteinases-1 (TIMP-1), interleukin 6 (IL-6), IL-8, macrophage inflammatory protein-1ß (MIP-1ß), and nitrite (NO_2) after 12 days of culture. Values are means ± SEM (n = 9) of 3 independent cultures performed with cartilage specimens from 3 different donors.

	Controls	A1S2, 10 µg/ml	A, 3.33 µg/ml	A, 10 µg/ml	S, 6.6 µg/ml	S, 10 µg/ml
AGG, ng/µg DNA	4542 ± 143	6261 ± 217*	5242 ± 168*†	5728 ± 505*	5641 ± 156*	5752 ± 208*
MMP-3, ng/µg DNA	6233 ± 185	4246 ± 286*	6803 ± 326†	4463 ± 308*	4147 ± 88*	4225 ± 434*
TIMP-1, ng/µg DNA	307.8 ± 11.7	295.1 ± 26.1	274.6 ± 23.6	272.6 ± 10.2	354.6 ± 15.5*††	378.0 ± 17*††
IL-6, pg/µg DNA	5162 ± 139	734 ± 113*	3287 ± 57*†	1718 ± 53*†	607 ± 19*	625 ± 24*
IL-8, pg/µg DNA	3234 ± 103	893 ± 83*	2636 ± 112*†	1719 ± 49*†	1162 ± 68*††	1287 ± 33*††
MIP-1ß, pg/µg DNA	54.77 ± 0.15	25.54 ± 0.60*	53.70 ± 2.10†	39.28 ± 1.34*†	28.10 ± 1.90*	23.7 ± 1.74*
NO_2, nmol/µg DNA	4.03 ± 0.49	0.75 ± 0.10*	3.90 ± 0.41†	1.29 ± 0.01*†	1.38 ± 0.04*††	1.32 ± 0.5*††

* Significant difference, $p < 0.05$ between treated groups and controls. † Significant difference between A treated group and A1S2 treated group. †† Significant difference between S treated group and A1S2 treated group.

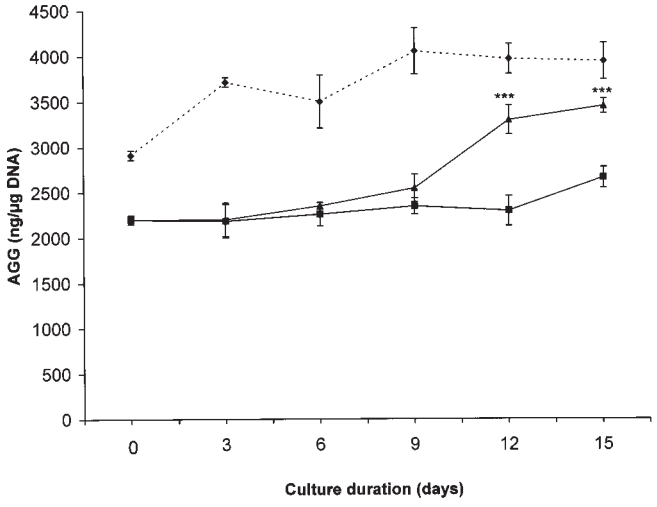

Figure 2. Effect of A1S2 (10 µg/ml) on AGG synthesis recovery after 3 day treatment with IL-1ß (10^{-10} M). Curves represent total production, which correspond to the sum of AGG found in supernatant, CM, and FRM. In these experiments, chondrocyte cultures were pretreated or not (◆) for 3 days with 10^{-10} M IL-1ß, washed, and then cultured for 15 days (day 0 to day 15) in the absence (■) or presence (▲) of A1S2 (10 µg/ml). Values are means ± SEM (n = 9) of 3 independent cultures performed with cartilage specimens from 3 different donors (a 51-year-old man, grade III; a 55-year-old man, grade I; and a 44-year-old woman, grade II). Statistical significance in comparison to controls (treated with IL-1ß): ***$p < 0.001$.

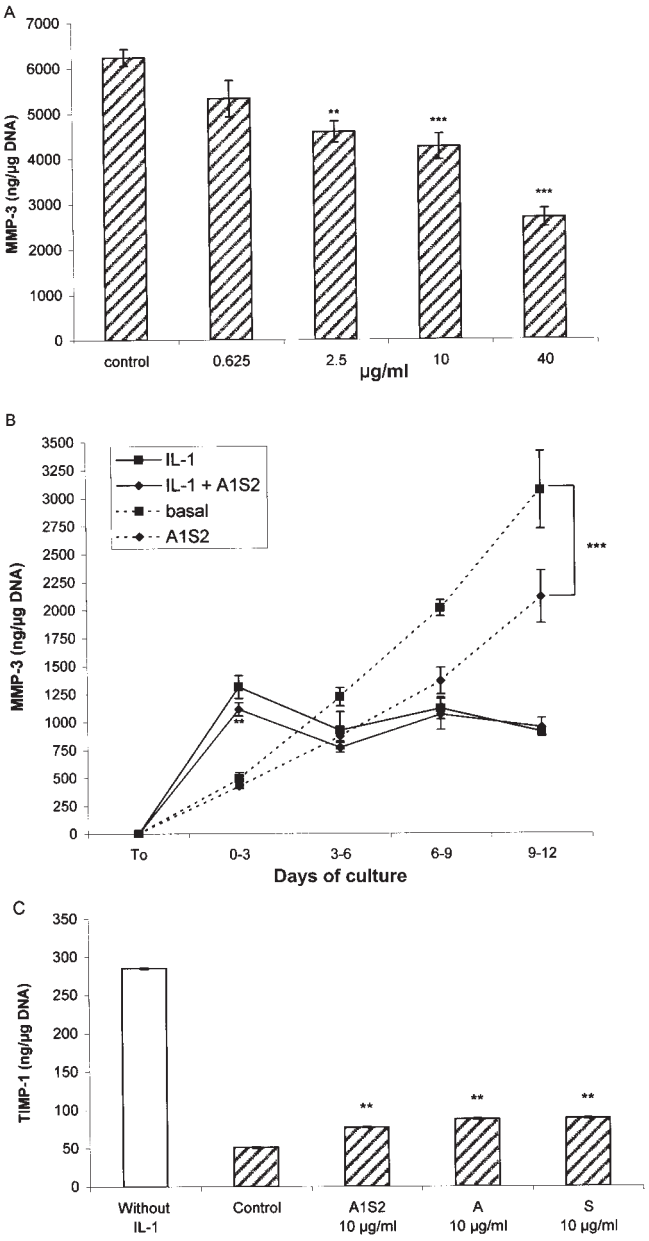

Figure 3. Effect of A1S2 on stromelysin-1 (MMP-3) and TIMP-1 synthesis by human OA chondrocytes. (A) Dose effect of A1S2 on basal production of MMP-3. Values correspond to amounts of MMP-3 released in the culture supernatant by chondrocytes during a 3 day culture period (Time 0 to 3, 4 to 6, 7 to 9, and 10 to 12). (B) Kinetic curves of MMP-3 synthesis in the absence (broken lines) or presence of 10^{-10} M IL-1ß (solid line) and without (■) or with (◆) A1S2 (10 µg/ml). (C) Effect of A1S2, avocado, and soybean 10 µg/ml on the inhibitory effect of IL-1ß on TIMP-1 production. Values correspond to the total amounts (sum of accumulated amounts found in supernatant, CM, and FRM) of MMP-3 produced by chondrocytes during 12 days of culture. Values are represented by means ± SEM (n = 9) of 3 independent cultures performed with cartilage specimens from 3 different donors (a 52-year-old man, grade I; a 38-year-old woman, grade I; and a 35-year-old woman, grade I). Statistical significance in comparison to controls: **p < 0.01, ***p < 0.001 (MANOVA test).

After 12 days of culture, the ratio MMP-3/TIMP-1 was equal to 22. At 10 µg/ml, A1S2 and A did not significantly modify basal TIMP-1 production, whereas S increased it by 23%. As primarily a consequence of the MMP-3 decrease, the ratio MMP-3/TIMP-1 was reduced by half in the presence of A1S2 (10 µg/ml). In the presence of IL-1ß (10^{-10} M), the production of TIMP-1 was decreased by 82%. This IL-1ß inhibitory effect was weakly reversed by A1S2, A, and S at 10 µg/ml (Figure 3C).

In our experimental conditions, avocado/soybean unsaponifiables decreased MMP-3/TIMP-1 ratio even in the presence of IL-1ß, indicating that A1S2 administered therapeutically could inhibit enzymatic degradation of cartilage in OA.

Effect of avocado/soybean unsaponifiables on MIP-1ß, IL-6, IL-8, NO•, and PGE_2 production. Under basal conditions, A1S2 inhibited, in a dose and time dependent manner, IL-6 (IC_{50} = 0.50 µg/ml, 95% CI 0.46–0.54), IL-8 (IC_{50} = 0.89 µg/ml, 95% CI 0.80–0.98), MIP-1ß (IC_{50} = 3.22 µg/ml, 95% CI 3.12–3.32), and NO• (IC_{50} = 0.69, 95% CI 0.54–0.84) production by OA chondrocytes (Figures 4 and 5), whereas PGE_2 was found to be below the detectable level (Figure 6).

A1S2 (10 µg/ml) and soybean (6.6 µg/ml) showed a similar effect on MIP-1ß production, while avocado (3.3 µg/ml) had no effect. On the other hand, A1S2 had a greater effect on NO• production than S (6.6 µg/ml), while A (3.3 µg/ml) had no effect, suggesting that A and S acted in synergy on this variable. A (3.3 µg/ml) and S (6.6 µg/ml) also synergized to inhibit IL-8 production (Table 1). IL-1ß markedly increased IL-6, IL-8, MIP-1ß, and NO• by 20, 12, 40, and 2 times, respectively (data not shown). A1S2 did not significantly modify the stimulating effect of IL-1ß on MIP-1ß and NO•, but strongly inhibited IL-1ß stimulated PGE_2 synthesis (Table 2). At 10 µg/ml, A1S2, A, and S had a similar effect on IL-1ß stimulated PGE_2 production (Figure 6).

Avocado/soybean unsaponifiables were also efficient at reducing chondrocyte production of some proinflammatory mediators involved in the onset and development of pain and synovial inflammation in OA. These observations support the symptomatic effects of A1S2 observed in clinical studies.

DISCUSSION

We investigated the effects of A1S2 on human OA chondrocytes cultured in alginate. Until now, the effects of A1S2 were primarily studied in animal cells cultured in monolayers for a 3 day period. Further, our study describes for the first time the effects of A1S2 on AGG, MIP-1ß, and NO• production.

Drugs for the treatment of OA have been distinguished according to their symptom and/or structure-modifying effects. A1S2 has showed a delayed and persistent symptomatic effect in the treatment of knee and hip OA[21]. Nevertheless, we do not yet know whether A1S2 also has

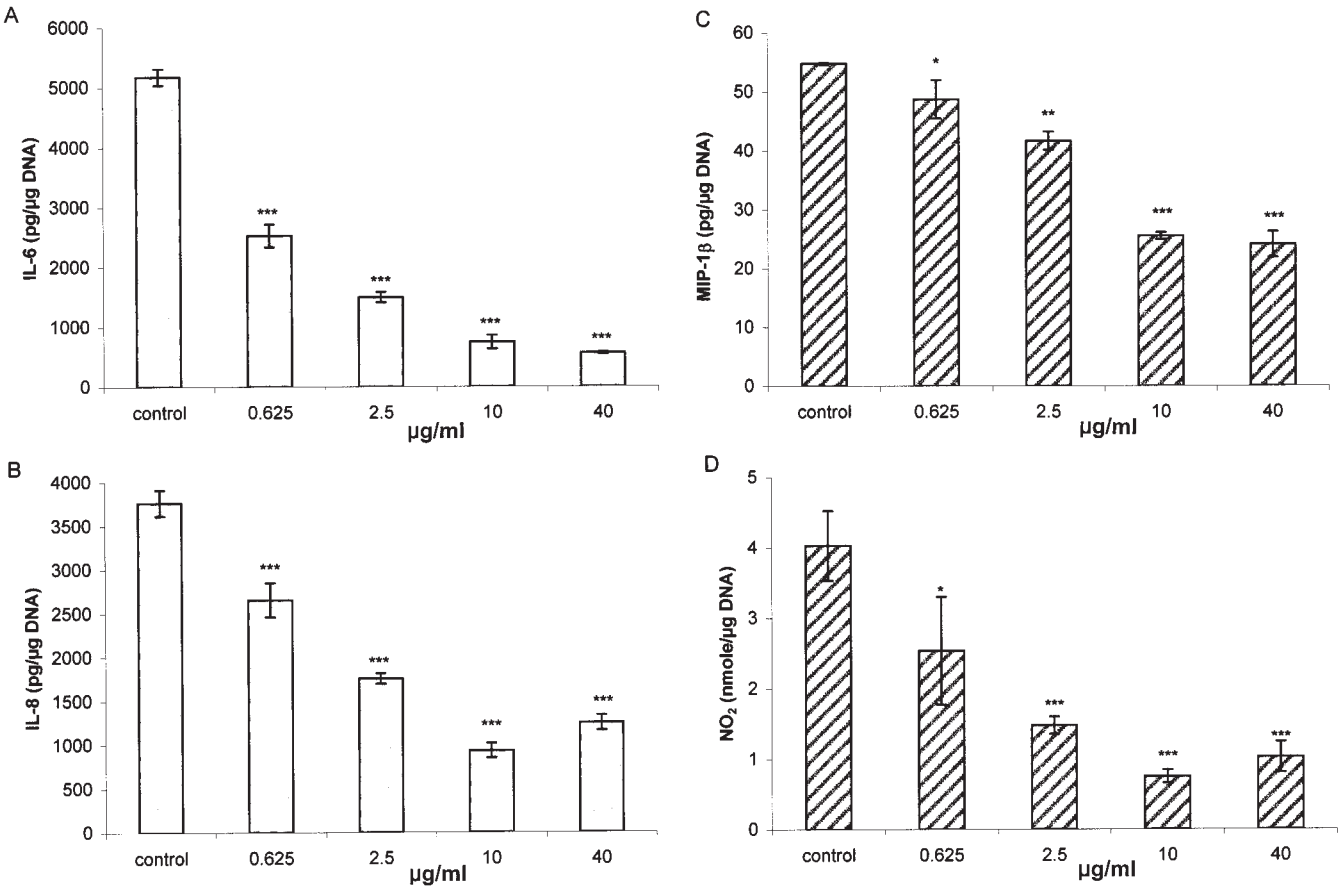

Figure 4. Effects of increased doses of A1S2 on basal IL-6 (A), IL-8 (B), MIP-1ß (C), and NO• (D) production by human OA chondrocytes cultured for 12 days in alginate beads. Values are means ± SEM (n = 9) of 3 independent cultures performed with cartilage specimens from 3 different donors (a 52-year-old man, grade I; a 38-year-old woman, grade I; and a 35-year-old woman, grade I). Statistical significance in comparison to controls: *p < 0.05, **p < 0.01, ***p < 0.001.

structure-modifying properties, as suggested in a subgroup of patients with advanced joint space narrowing of the hip (≤ 2.45 mm at baseline) in a radiological pilot study with a 2 year followup[23]. Our study supports the hypothesis that A1S2 could interfere with the structural changes of the disease. Indeed, A1S2 was shown to promote cartilage matrix formation and to restore aggrecan synthesis after IL-1 treatment. As well, A1S2 decreased MMP-3 production by OA chondrocytes and partially reversed the inhibitory effect of IL-1ß on TIMP-1 synthesis. MMP-3 is particularly effective in cartilage degradation, since it cleaves a wide variety of matrix macromolecules, e.g., proteoglycans, fibronectin, and several collagens[32,33]. Further, MMP-3 has been found to be capable of activating other metalloproteinases (collagenase-1, collagenase-3, and 92 kDa gelatinase)[34]. From these findings, we can conclude that A1S2 downregulates synthesis by chondrocytes of some catabolic factors that contribute to the onset and development of cartilage lesions in OA. In parallel, A1S2 promotes the production of aggrecan, one of the major matrix components, and restores its synthesis after IL-1ß treatment. Taken together, these findings suggest that A1S2 could be helpful for promoting cartilage repair in OA. Nevertheless, these *in vitro* observations must be extrapolated with caution to clinical situations since *in vitro*/*in vivo* conformity has not yet been confirmed.

Interestingly, the effect of A1S2 on AGG was delayed and became significant only after 6 to 9 days of incubation. This observation suggests that the effect of A1S2 could be secondary and mediated by regulatory peptides secreted by chondrocytes themselves. Transforming growth factors (TGF) are potential candidates, since TGF-ß1 and -ß2 expression has been reported to be upregulated by A1S2 in bovine chondrocytes cultured in monolayers[25]. Interestingly, TGF-ß1 and -ß2 exhibit similar effects on aggrecan compared to those described for A1S2[35].

Another important feature in OA is inflammation of the synovium. Inflammatory synovium changes in OA are visible at areas near the cartilage lesions[36]. Among these factors, cytokines released by chondrocytes play an important role. In this study, we demonstrated that OA chondrocytes produced concentrations of MIP-1ß similar to those found in synovial fluid of patients with OA[37,38] and that A1S2 was a potent inhibitor of this production. Further, and confirming a previous study[26], we have shown that A1S2

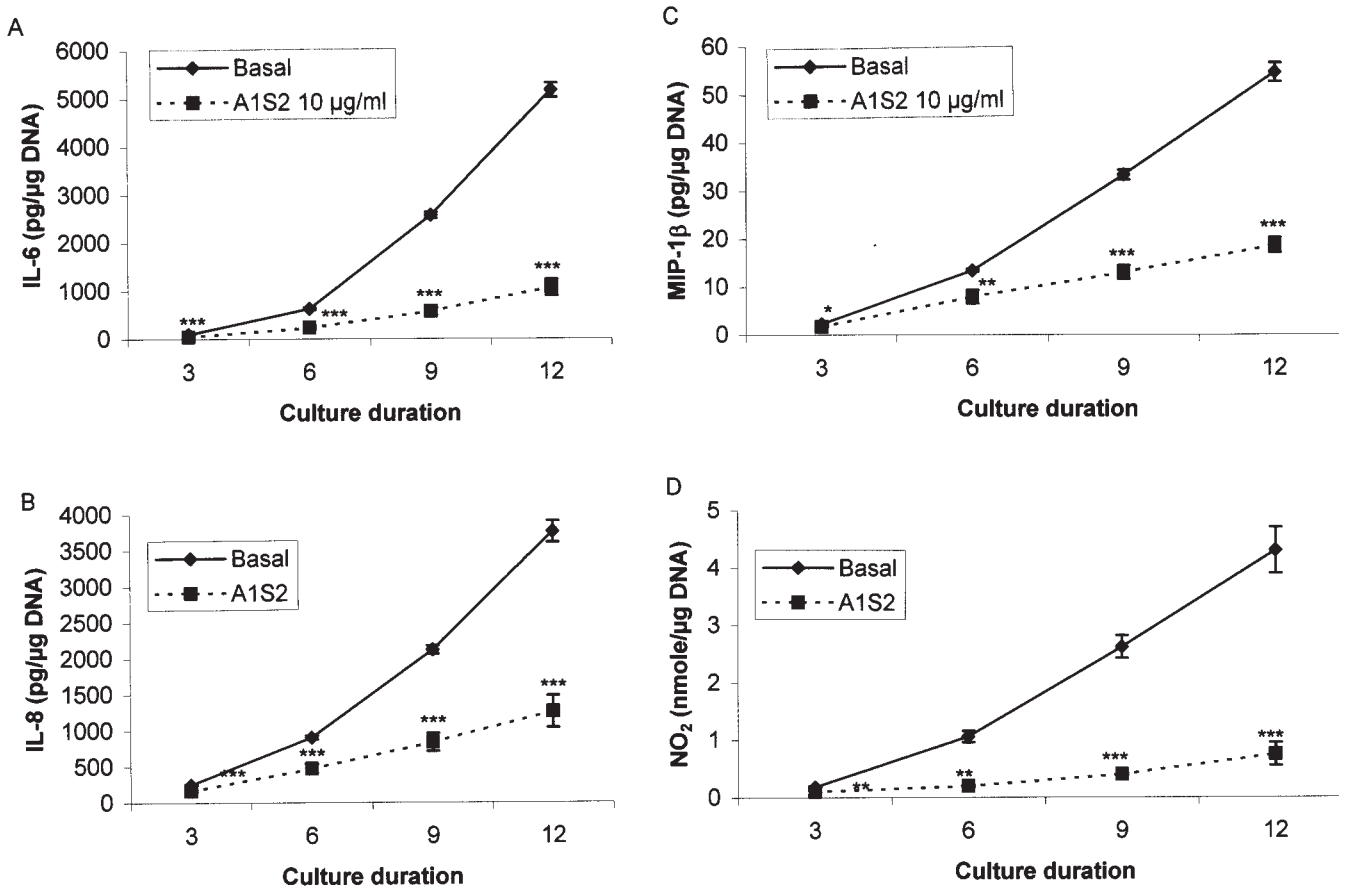

Figure 5. Time effect of A1S2 (10 µg/ml) on basal IL-6 (A), IL-8 (B), MIP-1ß (C), and NO• (D) production by human OA chondrocytes cultured for 12 days in alginate beads. Values are means ± SEM (n = 9) of 3 independent cultures performed with cartilage specimens from 3 different donors (a 52-year-old man, grade I; a 38-year-old woman, grade I; and a 35-year-old woman, grade I). Statistical significance in comparison to controls: *p < 0.05, **p < 0.01, ***p < 0.001.

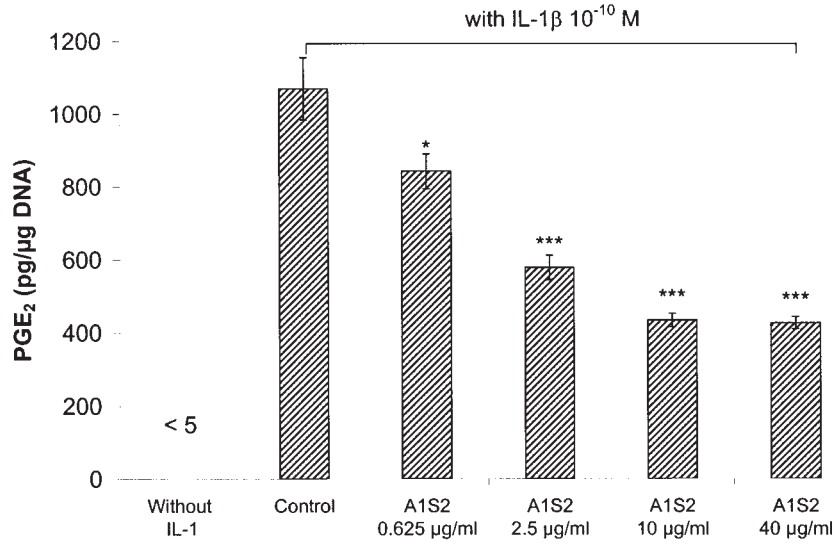

Figure 6. Effects of increased doses of A1S2 on IL-1ß stimulated PGE_2 production by human OA chondrocytes cultured for 12 days in alginate beads. Values are means ± SEM (n = 9) of 3 independent cultures performed with cartilage specimens from 3 different donors (a 52-year-old man, grade I; a 38-year-old woman, grade I; and a 35-year-old woman, grade I). Statistical significance in comparison to controls (IL-1ß treated group): *p < 0.05; ***p < 0.001.

Table 2. Summary of *in vitro* effects of A1S2 (10 µg/ml) on human OA chondrocytes cultured over 12 days in the absence or the presence of IL-1ß 10^{-10} M. IC_{50} values are expressed as µg/ml of A1S2.

	No IL-1ß	With IL-1ß
AGG	↑ IC_{50} = 7.86	↔
MMP-3	↓ IC_{50} = 6.36	↓ IC_{50} > 40
TIMP-1	↔	↑ IC_{50} = 9.89
IL-6	↓ IC_{50} = 0.50	↓ IC_{50} > 40
IL-8	↓ IC_{50} = 0.89	↓ IC_{50} > 40
MIP-1ß	↓ IC_{50} = 3.22	↔
NO	↓ IC_{50} = 0.69	↔
PGE^2	Not detectable	↓ IC_{50} = 1.09

inhibited IL-6 and IL-8 production, 2 cytokines that are recognized to promote synovial inflammation. These findings contribute to explaining the antiinflammatory action of A1S2 observed in connective tissue diseases[39]. Indeed, all these cytokines possess proinflammatory properties. As well, these cytokines have been implicated in the local destructive processes that accompany arthritis[40-43]. Taken together, these data suggest that the inhibitory effect of A1S2 on IL-6, IL-8, and MIP-1ß synthesis could have beneficial effects on synovitis and cartilage degradation in OA. Further, inhibition of these cytokines could be responsible for the inhibition of MMP-3 by A1S2.

IL-1 plays a key role in cartilage degradation and is found to be elevated in OA tissues. Surprisingly, in our experimental model, IL-1ß increased MMP-3 synthesis during the first 3 days of incubation, and thereafter failed to increase MMP-3 synthesis. This finding can be related to the effect of IL-1ß on NO• synthesis. Indeed, IL-1ß stimulated NO• production during the first 6 days of incubation, and then this stimulating effect disappeared (data not shown). Considering that NO• is a mediator involved in the stimulating effect of IL-1ß on MMP-3 synthesis[44], we can hypothesize that the absence of NO• overproduction during the 6 last days of culture is responsible for the disappearance of the IL-1ß stimulating effect on MMP-3. A1S2 partially reversed some of the effects of IL-1ß, such as the inhibition of TIMP-1 synthesis or the stimulation of MMP-3, PGE_2, IL-6, or IL-8 production. These data indicate that A1S2 can also be efficient in the presence of IL-1 and can counteract some of its deleterious effects. When A1S2 and IL-1 were added simultaneously, A1S2 had no significant effect on the IL-1ß induced inhibition of AGG synthesis, but it promoted recovery of AGG synthesis when IL-1ß was withdrawn from the medium. This observation suggests that to suppress inflammation before A1S2 administration could be beneficial for the stimulating action of A1S2 on cartilage matrix synthesis.

To our knowledge, the effective components in the A1S2 mixture are not yet clearly identified. Previously we showed that A1S2 had a significantly more marked effect on IL-6 and IL-8 production than A or S added alone at the same concentration as in the A1S2 mixture[26]. In this report, we compared the effects of A1S2 administered at the recommended and currently used *in vitro* concentration of 10 µg/ml, with A and S added separately at the same concentration as found in the mixture. A1S2 has a superior effect on IL-8 and NO• synthesis compared to that observed with A and S added alone. On the other hand, when it was added alone at the concentration found in the mixture, the S fraction was more efficient in TIMP-1 production than A1S2, suggesting that A counteracted the effect of S on this variable. Together, these observations add support to the use of avocado/soybean mixture to treat OA, although we are aware that our study fails to confirm that the net result of A1S2 treatment on cartilage degradation is superior to that of A or S used alone. Further, the superiority of this formulation must be demonstrated *in vivo*.

A1S2 stimulates aggrecan synthesis and accumulation in extracellular matrix and inhibits stromelysin-1 synthesis by OA chondrocytes, suggesting that A1S2 may correct the imbalance between catabolic and anabolic processes in OA. A1S2 counteracts some deleterious effects of IL-1ß and inhibits proinflammatory cytokine synthesis by chondrocytes, indicating that these drugs may also control synovitis in OA (Table 2). These findings lend further support to the rationale for the use of A1S2 in OA.

APPENDIX

Description of A1S2. After concentration by molecular distillation and saponification by hydroxide in ethanol, the unsaponifiable part of avocado and soybean oils is extracted on a counter-current column using ethylene dichloride. Eight of the 9 components identified in the main fraction of avocado oil unsaponifiables (the H or A fraction), accounting for up to 50%, have been characterized as having a homogeneous stucture. A furyl nucleus is substituted in position 2 by an aliphatic, mono- or polyunsaturated chain. The chain length varies from 13 to 17 carbon atoms, always an odd number. This fraction is highly specific for avocado oil unsaponifiables and can be linked with its pharmacological activities. Squalen (2%), long-chain saturated hydrocarbons (up to 5%), polyols (up to 15%), tocopherols (trace amount), and sterols (4–20%), mainly as ß-sitosterols, are the other components of the avocado unsaponifiables. The soybean oil unsaponifiables, almost totally identified, include saturated hydrocarbons (about 1%), squalen (about 4%), tocopherols (around 10%), sterols (40–65%), terpene alcohols (about 1–10%), aliphatic alcohols (< 1%), and methyl sterols (< 5%) [Baillet A. Pharmaceutical expert report. Courbevoie, France: Pharmascience; 1995 (unpublished data)].

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