Inhibition of human rheumatoid arthritis synovial cell survival by hecogenin and tigogenin is associated with increased apoptosis, p38 mitogen-activated protein kinase activity and upregulation of cyclooxygenase-2

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Abstract. We conducted our study to assess the anti-proliferative and proapoptotic potential of hecogenin and tigogenin, two saponins which are structurally similar to diosgenin. We particularly focused our attention on mitogen-activated protein kinase (MAPK) activation in relation to apoptosis but also with the COX-2 expression and activity. Rheumatoid arthritis (RA) synoviocytes were isolated from fresh synovial biopsies obtained from five RA patients undergoing hip arthroplasty. Measurement of cell proliferation was determined using the MTT assay. Apoptosis was evaluated by studying caspase-8, caspase-9 and caspase-3 activities but also by quantification of DNA fragmentation. Quantification of human phospho-MAPKs was realized by ELISA. COX-2 expression was demonstrated by Western blot analysis and COX-2 activity by assay of endogenous prostaglandin E_2 (PGE_2) production. Tigogenin was more effective than hecogenin in inducing apoptosis in human RA fibroblast-like synoviocytes (FLS) which was caspase dependent but poly(ADP-ribose) polymerase independent and characterized by DNA fragmentation. Our results demonstrated hecogenin- and tigogenin-induced apoptosis through activation of p38 without affecting the JNK and ERK pathways. Indeed, pretreatment with a p38 inhibitor decreased saponin-induced apoptosis with a significant decrease in DNA fragmentation. Furthermore, the rate of apoptosis induced by hecogenin or tigogenin was associated with overexpression of COX-2 correlated with over-production of endogenous PGE_2. These new results provide strong evidence that a family of structurally similar plant steroids is capable of inducing apoptosis in human RA FLS with different rates and different signalling pathways. This study also confirms the discussed appearance of the downregulation or upregulation of COX-2 in cell apoptosis as a function of cell type.

Introduction

Plant steroids have been thoroughly described for their pharmacological properties, including hypocholesterolemic (1), antidiabetic (2) and antioxidant activities (3,4). Particular attention has been given to their potential for cancer chemo-prevention, especially as apoptosis inducers (5-10).

Apoptosis is considered to be one of the mechanisms regulating autoimmune diseases such as rheumatoid arthritis (RA) (11). In the pathogenesis of RA, it is thought that the normal balance between proliferation and apoptosis of synovial fibroblasts is lost, leading to hyperplasia of these fibroblasts (12). Activated synovial cells cause growth of synovium in the articular cavity along with angiogenesis, invade the adjacent bone, promote production of inflammatory mediators by inflammatory cells, and cause cartilage and bone destruction (13). Therefore, it has been shown that stimulation of apoptosis in synovial fibroblasts might be useful for the treatment of RA (14,15).

We showed for the first time that a plant steroid, diosgenin, inhibited the growth of human RA fibroblast-like synoviocytes (FLS) with apoptosis induction characterized by a loss of mitochondrial membrane potential, caspase-3 activation and DNA fragmentation (16). Furthermore, the proapoptotic effect of diosgenin was associated with over-

expression of cyclooxygenase-2 (COX-2) correlated with the overproduction of endogenous prostaglandin E_2 (PGE_2) (16).

Furthermore, we described that diosgenin was the most effective as a cell death inducer compared to two other plant steroids (hecogenin and tigogenin) in the human osteosarcoma 1547 cell line (17). Another study with a series of five saponins structurally similar to diosgenin described the structure-function relationship for saponin effects on cell cycle arrest and apoptosis in osteosarcoma cells using a molecular modelling approach (18).

In this context, we conducted our study to assess the antiproliferative and proapoptotic potential of hecogenin and tigogenin, two saponins which are structurally similar to diosgenin. We particularly focused our attention on mitogen-activated protein kinase (MAPK) activation in relation to apoptosis but also with the COX-2 expression and activity. Our results demonstrate that hecogenin and tigogenin inhibited proliferation and induced apoptosis of human RA FLS but tigogenin was more effective than hecogenin. Saponin-induced apoptosis was caspase dependent but PARP independent and characterized by DNA fragmentation. Our results show hecogenin- and tigogenin-induced apoptosis through p38 activation without an effect on JNK and ERK pathways. Indeed, pretreatment with a p38 inhibitor decreased saponin-induced apoptosis with a significant decrease in DNA fragmentation in human RA FLS.

Furthermore, the rate of apoptosis induced by hecogenin or tigogenin was associated with overexpression of COX-2 correlated with overproduction of endogenous PGE_2. Tigogenin was more effective than hecogenin for the inhibition of proliferation, activation of caspase-3 activity and DNA fragmentation, but we also demonstrated that tigogenin was more effective than hecogenin in stimulating COX-2 expression and activity.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and penicillin-streptomycin were supplied by Gibco BRL (Cergy Pontoise, France). Collagenase was obtained from Worthington Biochemical Corp. (Halls Mill Road, Freehold, NJ, USA). Dispase, hyaluronidase, DNase I, hecogenin (5α-spirostan-3ß-ol-12-one), tigogenin [(25R)-5α-spirostan-3(-ol)], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and monoclonal antibody ß-actin were purchased from Sigma (Saint Quentin Fallavier, France). PARP and COX-2 monoclonal antibodies were supplied by Santa Cruz Biotechnology (TEBU, Le Perray en Yvelines, France). Cell Death Detection ELISA[PLUS] was supplied by Roche Diagnostics (Meylan, France). ELISA kits for PGE_2 were purchased from Cayman Chemical (SpiBio, Massy, France). Recombinant human IL-1ß and Phospho-ERK1/ERK2, Phospho-JNK and Phospho-p38α DuoSet IC ELISA were purchased from R&D Systems (Lille, France). SB203580 was purchased from Calbiochem (VWR, Fontenay-sous-Bois, France).

Preparation of human synovial cells. RA synoviocytes were isolated from fresh synovial biopsies obtained from five RA patients undergoing hip arthroplasty. All patients fulfilled the 1987 American Rheumatism Association criteria for RA (19). The mean age of the patients was 60.9±3.1 years (range, 57-64 years) and the mean disease duration was 7.9±2.9 years. At the time of surgery, the disease activity score (DAS 28) was >3.2. The study protocol was approved by the local institutional review boards, and all subjects gave written informed consent. Synovia were minced and digested with 1.5 mg/ml collagenase-dispase, 1 mg/ml hyaluronidase and 0.15 mg/ml DNase I for 3-4 h at 37°C as previously described (20). After centrifugation, cells were re-suspended in DMEM supplemented with 10% FCS, 4.5 g/l D-glucose, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL) in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C.

After 48 h, non-adherent cells were removed. Adherent cells (macrophage-like and fibroblast-like synoviocytes, FLS) were cultured in complete medium and, at confluence, cells were trypsinized and only FLS were passed. These cells were used between passages 4 and 8 when they morphologically resembled FLS after indirect immunofluorescence study. RA FLS were cultured 45-60 days prior to experimentation. This delay eliminated all possible interactions resulting from a pre-operative treatment (NSAIDs, analgesics, DMARDs or steroids).

Culture of human RA FLS. RA FLS were trypsinized between passages 4 and 8. Cell count and viability were determined, and cells were plated in culture plates or flasks (Sarstedt, Orsay France). Viability, measured by trypan blue dye exclusion at the start and the end of culture, was always >95%. RA FLS (10^5) from RA patients were used for indirect immunofluorescence as previously described (16).

For all experiments, RA FLS were allowed to adhere and grow for 48 h in culture medium prior to exposure to 10 and 40 μM hecogenin or tigogenin. A stock solution of 10^{-2} M hecogenin or tigogenin was prepared in ethanol and diluted in culture medium to give a final concentration of 10 and 40 μM. The same amount of ethanol (<0.5%) was added to control cells. After treatment, culture medium was not changed during the entire study.

Human RA FLS proliferation and light microscopy. Measurement of cell proliferation was determined using the MTT assay. Cells (10^3 cells/well) were plated in 96-well culture plates and grown 48 h before treatment with 10 and 40 μM hecogenin or tigogenin for 24-96 h. MTT was carried out daily as previously described (21) and experiments were performed in six duplicate assays.

For light microscopy, after 24 h of treatment, RA FLS cultured cells were fixed in phosphate-buffered saline (PBS) (pH 7.4) containing 4% paraformaldehyde for 20 min at room temperature and washed in PBS for 15 min. Observations were realized with phase-contrast microscopy.

Caspase-8 and caspase-9 activity assays. Caspase-8 and caspase-9 activities were respectively assayed using caspase-8 and caspase-9 colorimetric assay (R&D Systems). RA FLS cells were cultured in 75 cm^2 flasks and treated or not with 40 μM hecogenin or tigogenin for 30 min, 2 h and 6 h. Cells were first lysed to collect their intracellular contents.

lysate was then tested for protease activity by the of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroalanine (pNA). Cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantified spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction.

Caspase-3 activity assay. Caspase-3 activity was assayed using Quantikine® human active caspase-3 (R&D Systems) as previously described (22). Following the same treatments as those used for caspase-8 and caspase-9 activity detection, human RA FLS were incubated with 10 μM biotin-ZVKD-fmk inhibitor for 1 h at 37°C. Caspase-3 activity was then measured in accordance with the manufacturer's protocol (R&D Systems). Briefly, cells were harvested, washed in PBS and re-suspended in extraction buffer containing protease inhibitors. Standards and sample extracts containing covalently linked active caspase-3-ZVKD-biotin were added to a microplate pre-coated with monoclonal antibody specific for caspase-3. Then, streptavidin conjugated to horseradish peroxidase was added to the wells. The amount of active caspase-3 was quantified by colorimetry at 450 nm after addition of the HRP substrate.

Apoptosis quantification: DNA fragmentation. Human RA FLS were cultured in 6-well culture plates ($2x10^5$ cells/well). Cells were treated without or with 40 μM hecogenin or tigogenin alone for 12 and 24 h, or pre-incubated for 2 h with 10 μM SB203580 (p38 inhibitor) before addition of 40 μM hecogenin or tigogenin for 24 h. Apoptosis was quantified on pooled cells (floating and adherent) using the 'cell death' ELISA (Cell Death Detection ELISA^{PLUS}). Cytosol extracts were obtained according to the manufacturer's protocol and apoptosis was measured as previously described (16).

Quantification of human phospho-ERK1/ERK2, phospho-JNK and phospho-p38α. Human RA FLS were grown in 25 cm^2 tissue culture flasks for 48 h before treatment. After washing with PBS (pH 7.4), cells were incubated at 37°C with 40 μM hecogenin or tigogenin for 20 min, 1 h, 6 h and 24 h or with interleukin (IL)-1ß (1 ng/ml) alone for 20 min as a positive control. After treatment, 10^6 cells were homogenized in lysis buffer in accordance with the manufacturer's protocol (R&D Systems). Before assay, one plate was coated with 100 μl per well of capture antibody (4 μg/ml) overnight at room temperature. The plate was washed three times with 400 μl wash buffer and blocked by adding 300 μl blocking buffer to each well for 1-2 h at room temperature. Before use, cell lysates were centrifuged at 2000 x g for 5 min and supernatants were diluted 6-fold. Phospho-ERK1/ERK2, phospho-JNK or phospho-p38α detection was performed according to the manufacturer's instructions (R&D Systems) as previously described (23).

COX-2 expression analysis. Human RA FLS were cultured in 150 cm^2 tissue culture flasks. After a 40 μM hecogenin or tigogenin treatment for 30 min, 2 h and 24 h, adherent cells were trypsinized and pooled with the floating cell fraction. Western blot analysis was performed as previously described
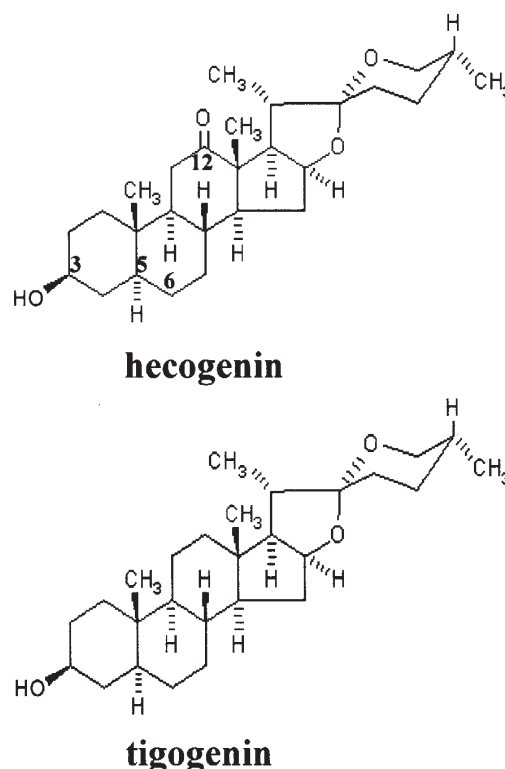

hecogenin

tigogenin

Figure 1. Molecular structure of the hecogenin and tigogenin molecules.

(24) using the primary monoclonal antibodies ß-actin (mouse anti-human ß-actin, 1:5000; Sigma), PARP (mouse anti-human PARP, 1:100; Santa Cruz Biotechnology), COX-2 (mouse anti-human COX-2, 1:100; Santa Cruz Biotechnology) and secondary polyclonal antibody conjugated with peroxidase (Dako). Blots were visualized using enhanced chemiluminescence reagents (Amersham Biosciences) and immediately exposed to X-ray film.

Assay of PGE$_2$ production. Human RA FLS were grown in 25 cm^2 tissue culture flasks for 48 h before treatment. After washing with PBS (pH 7.4), cells were treated for 2 and 24 h at 37°C with 40 μM hecogenin or tigogenin in DMEM containing 10% (v/v) FCS in a 5% CO_2 atmosphere. The PGE$_2$ concentration in the medium was measured using an ELISA kit according to the instructions of the manufacturer (Cayman Chemical) and was normalized with respect to the number of viable cells present in the particular culture at the time of sampling.

Statistical analysis. The median and standard deviation (SD) were calculated using Excel (Microsoft Office, version 98). Statistical analysis of differences was carried out by analysis of variance (ANOVA) using StatView version 5.0 (SAS Institute Inc, Cary, NC, USA). A p-value <0.05 [Fisher's Protected Least Significant Difference (PLSD) test] was considered to indicate significant differences.

Results

The effect of hecogenin and tigogenin on human RA FLS proliferation and morphological modifications. Cells were
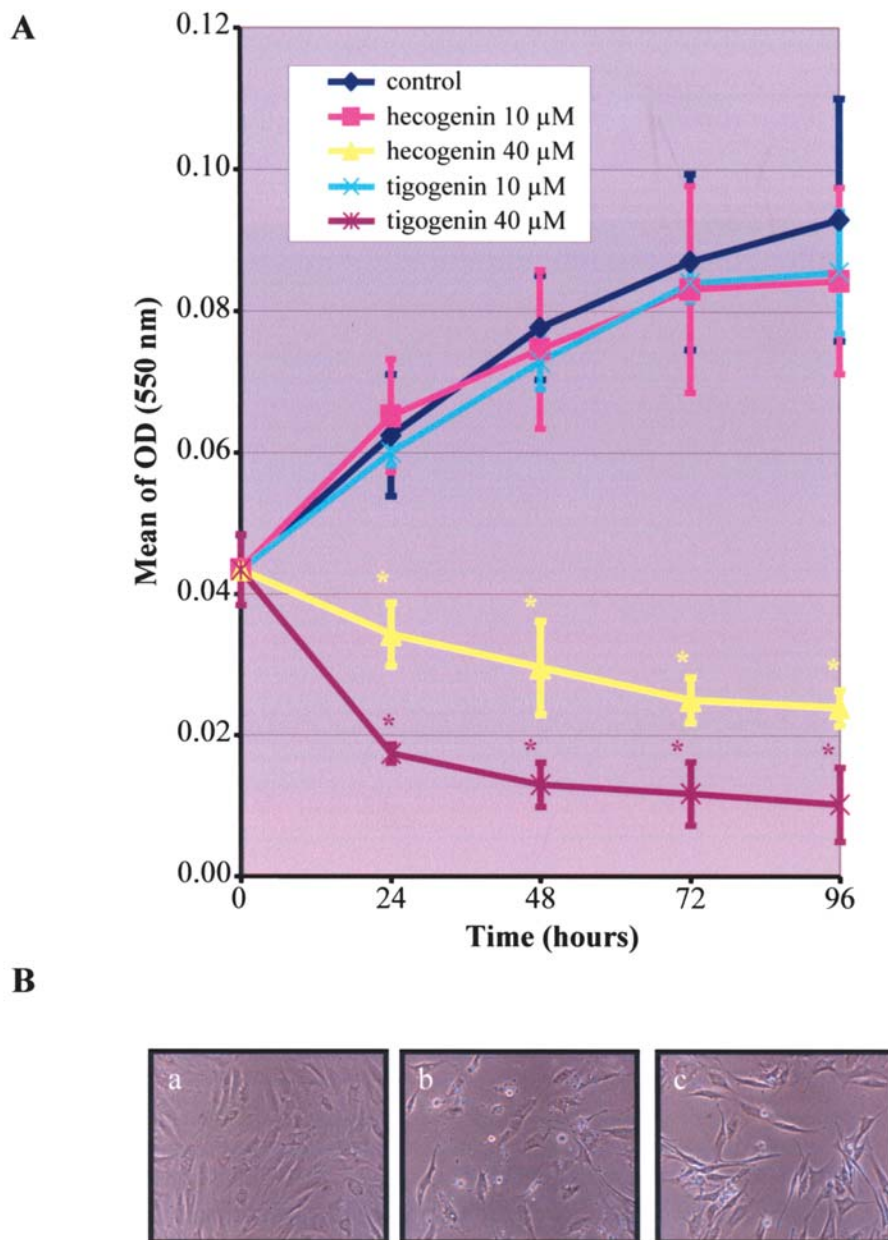
Figure 2. (A) Effect of hecogenin and tigogenin on proliferation of human RA FLS. Cells were cultured in 10% FCS medium for 48 h and then incubated (time 0) for 24-96 h with saponin at 10 and 40 μM. RA FLS proliferation was evaluated by the MTT test. Measurements were made on FLS from five different patients. Representative results from six independent experiments are shown; values are the mean ± SD from triplicate cultures. *p<0.05 (Fisher's PLSD test) was considered to indicate significant differences compared to the control. (B) Morphologic changes in human RA FLS. Cells were incubated for 24 h without (a) or with 40 μM hecogenin (b) or tigogenin (c). Original magnification, x400.

cultured in 10% FCS-medium with or without 10 and 40 μM hecogenin or tigogenin (Fig. 1) for 24-96 h, and cell proliferation was evaluated by the MTT test (Fig. 2A). Under our experimental conditions, a decrease in proliferation was observed up to 24 h after hecogenin and tigogenin treatment (40 μM), especially at 24 h for tigogenin when the percentage of inhibition was -69% (p<0.05) compared to -46% (p<0.05) for hecogenin (Fig. 2A). Proliferation of human RA FLS decreased until 96 h (-75% and -89%, p<0.05) in the presence of 40 μM hecogenin or tigogenin respectively. An antiproliferative effect was not observed for these two saponins at 10 μM compared to the control (Fig. 2A). For this reason, we chose 40 μM for treatment in subsequent experiments.

Direct observation with phase-contrast microscopy demonstrated that human RA FLS treated with 40 μM hecogenin or tigogenin for 24 h (Fig. 2B, b and c) showed numerous morphological differences compared to control cells (Fig. 2B, a). Indeed, cell shrinkage, cytoplasm condensation and formation of cytoplasmic filaments appeared after 40 μM of saponin treatment for 24 h.

Apoptosis induced by hecogenin or tigogenin was caspase dependent and PARP independent. Induction of apoptosis requires activation of specific proteins. It is well known that apoptosis is characterized by chromatin condensation and DNA fragmentation, and is mediated by the cysteine protease family called caspases, such as caspase-3, which is the major
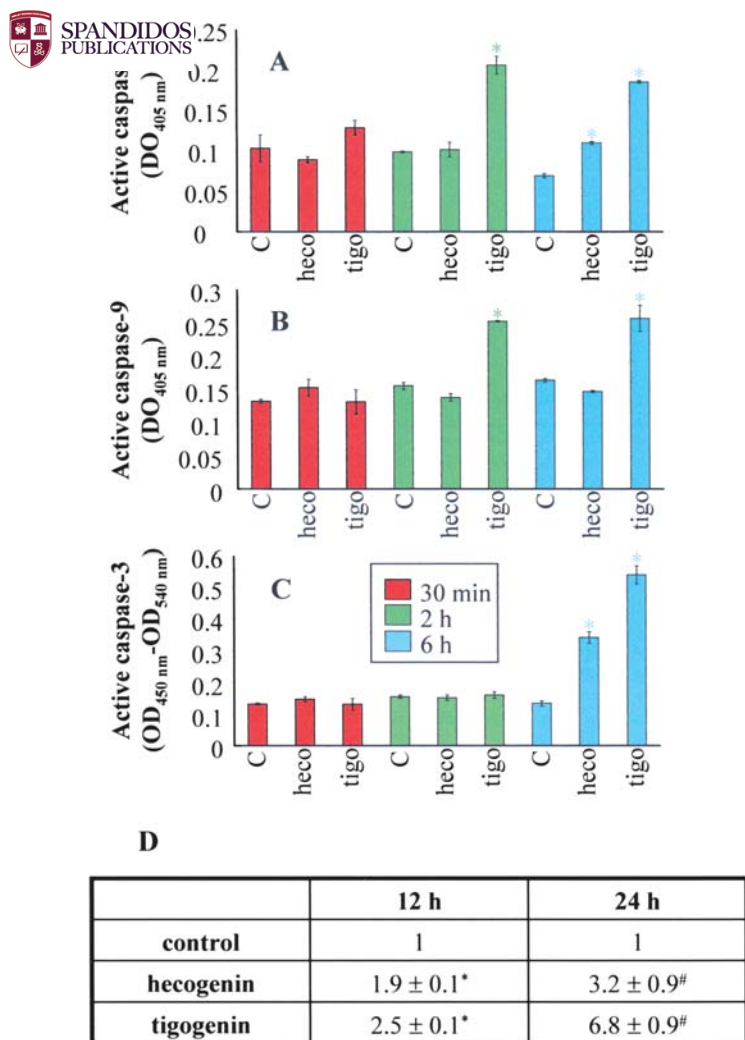
Figure 3. Effect of hecogenin and tigogenin on initiator caspase-8 and caspase-9 activities, central effector caspase-3 activity and DNA fragmentation in human RA FLS. RA FLS cells were cultured in 75 cm² flasks and treated or not with 40 μM hecogenin (heco) or tigogenin (tigo) for 30 min, 2 h and 6 h. Caspase-8 (A) and caspase-9 (B) activities were respectively assayed using caspase-8 and caspase-9 colorimetric assay (R&D Systems) and quantitated spectrophotometrically at a wavelength of 405 nm. Caspase-3 activity (C) was assayed using Quantikine® human active caspase-3 (R&D Systems). Following the same treatments as those used for caspase-8 and caspase-9 activity detection, human RA FLS were incubated with 10 μM biotin-ZVKD-fmk inhibitor for 1 h at 37˚C. The amount of active caspase-3 was quantified by colorimetry at 450 nm. Values are expressed as the mean ± SD and one representative of three independent experiments is shown. Apoptosis was quantified on floating and adherent cells using 'cell death' ELISA based on DNA fragmentation (D). The fold induction of DNA fragmentation is shown relative to the value for the control (C) culture, which was equated to 1. Data are expressed as the mean ± SD of three experiments from three different patients. *,#p<0.05 (Fisher's PLSD test) was considered to indicate significant differences compared to controls.

executioner of apoptosis. PARP is one of the best known caspase substrates and its inactivation by cleavage is now an apoptosis hallmark. DNA fragmentation occurs simultaneously with this phenomenon and is now considered as a major marker of apoptotic cells.

First, we studied caspase-8 activity, the central initiator caspase in the extrinsic (death receptor) pathway, and caspase-9 activity, the central initiator caspase in the intrinsic

(mitochondrial) pathway. Our results showed that only tigogenin induced both caspase-8 and caspase-9 activities after 2 h of treatment (2.1- and 1.6-fold increases respectively versus control, p<0.05) (Fig. 3A and B). After 6 h of treatment, hecogenin activated caspase-8 (1.6-fold increase versus control, p<0.05) but tigogenin treatment provoked a more pronounced effect (2.6-fold versus control, p<0.05) (Fig. 3A). Furthermore, the activation of caspase-9 by tigogenin was always significant after 6 h of treatment (1.5-fold increase versus control, p<0.05) whereas the effect of hecogenin was caspase-9 independent over time in human RA FLS (Fig. 3B).

Following activation of both initiator caspases, we showed that both saponins (hecogenin and tigogenin) induced executive caspase-3 activity after 6 h of treatment (Fig. 3C). Activation of caspase-3 by hecogenin was increased 2.5-fold versus control (p<0.05). Caspase-3 activation was greater in the presence of tigogenin (4.0-fold versus control, p<0.05).

Surprisingly, the activation of caspase-3 was not followed by cleavage of PARP after treatment with hecogenin or tigogenin. Western blotting detected the native form (112 kDa) of PARP but not the cleaved fragment at 85 kDa in untreated or treated human RA FLS (data not shown).

However, DNA fragmentation, considered as a major marker of apoptotic cells, has been observed in human RA FLS after hecogenin or tigogenin treatment. Quantitative determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) was performed with ELISA in our study. Results showed that DNA fragmentation was induced in cells as early as 12 h after hecogenin or tigogenin treatment (1.9-and 2.5-fold increases respectively versus control, p<0.05) but DNA fragmentation was markedly increased at 24 h (3.2-fold for hecogenin and 6.8-fold for tigogenin versus control, p<0.05) (Fig. 3D).

Involvement of MAPKs in hecogenin- or tigogenin-induced apoptosis in human RA FLS. MAPKs represent an attractive target for RA treatment because they can regulate cell proliferation and apoptosis. It has been reported that JNK/p38 MAPK and ERK play opposite roles in apoptosis induction in such a way that the former promotes apoptosis induction whereas the latter inhibits it. In order to elucidate potentially different actions of the three groups of MAPKs in human RA FLS, we studied saponin-triggered MAPK signal transduction cascades in relation to apoptosis induction. We showed that 40 μM hecogenin activated p38 as early as 1 h in human RA FLS (1.4-fold increase versus control, p<0.05) with a maximum at 6 h (2.0-fold versus control, p<0.05) and 1.5-fold at 24 h versus control (p<0.05) (Fig. 4A, a). Furthermore, 40 μM tigogenin more strongly activated p38 as early as 1 h (2.0-fold increase versus control, p<0.05) and at 6 h (1.9-fold versus control, p<0.05) with a maximum at 24 h (2.3-fold versus control, p<0.05 (Fig. 4A, a). However, both saponins had no effect on JNK and ERK phosphorylation over time in human RA FLS (Fig. 4A, b and c respectively). IL-1ß treatment for 20 min served as a positive control of MAPK activation.

In the present study, we investigated whether or not p38 was implicated in the apoptotic signalling pathway of
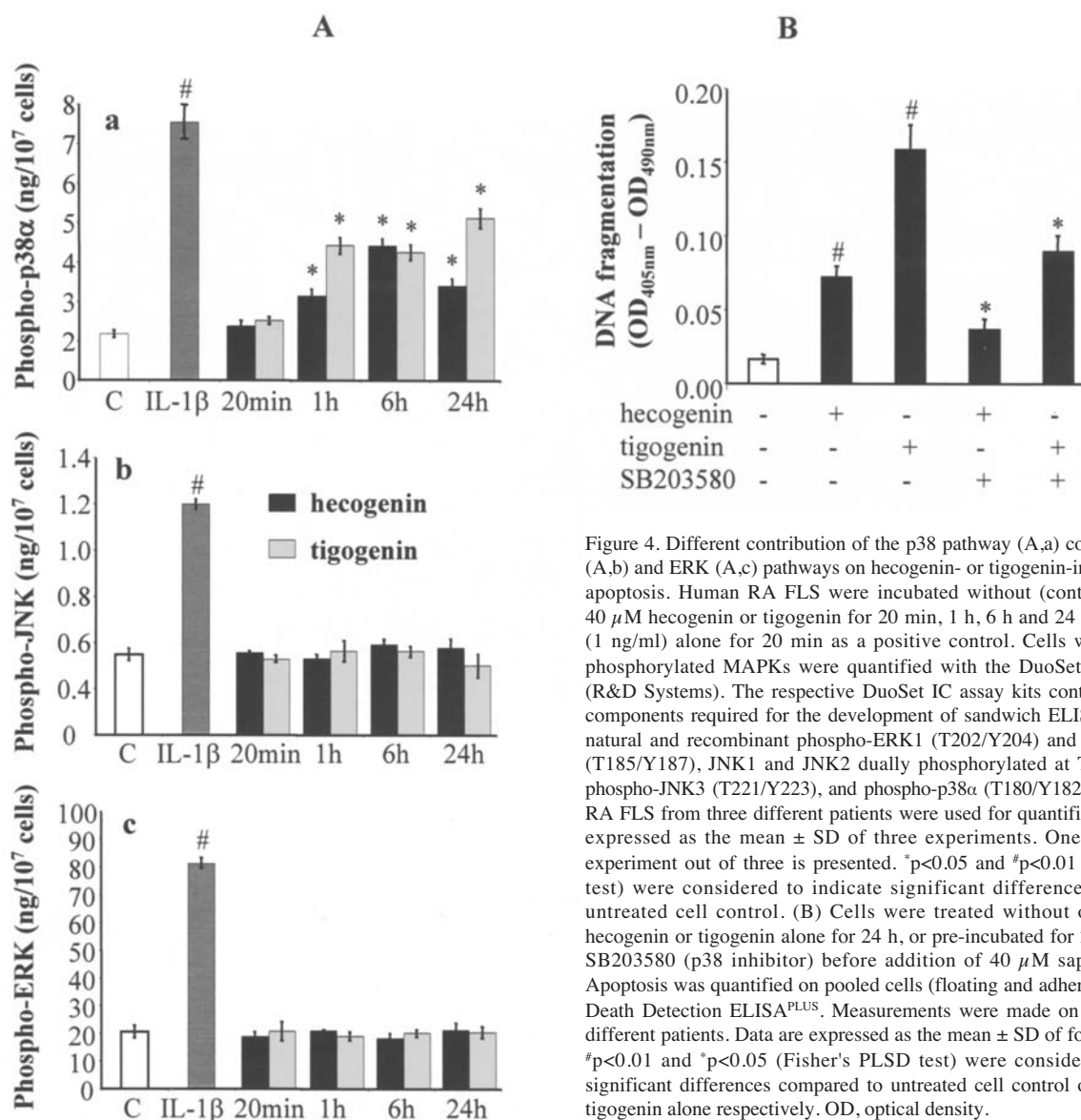
Figure 4. Different contribution of the p38 pathway (A,a) compared to JNK (A,b) and ERK (A,c) pathways on hecogenin- or tigogenin-induced RA FLS apoptosis. Human RA FLS were incubated without (control, C) or with 40 μM hecogenin or tigogenin for 20 min, 1 h, 6 h and 24 h or with IL-1ß (1 ng/ml) alone for 20 min as a positive control. Cells were lysed, and phosphorylated MAPKs were quantified with the DuoSet® IC assay kits (R&D Systems). The respective DuoSet IC assay kits contained the basic components required for the development of sandwich ELISAs to measure natural and recombinant phospho-ERK1 (T202/Y204) and phospho-ERK2 (T185/Y187), JNK1 and JNK2 dually phosphorylated at T183/Y185 and phospho-JNK3 (T221/Y223), and phospho-p38α (T180/Y182) in cell lysates. RA FLS from three different patients were used for quantification. Data are expressed as the mean ± SD of three experiments. One representative experiment out of three is presented. *p<0.05 and $^#$p<0.01 (Fisher's PLSD test) were considered to indicate significant differences compared to untreated cell control. (B) Cells were treated without or with 40 μM hecogenin or tigogenin alone for 24 h, or pre-incubated for 2 h with 10 μM SB203580 (p38 inhibitor) before addition of 40 μM saponin for 24 h. Apoptosis was quantified on pooled cells (floating and adherent) by the Cell Death Detection ELISA[PLUS]. Measurements were made on FLS from four different patients. Data are expressed as the mean ± SD of four experiments. $^#$p<0.01 and *p<0.05 (Fisher's PLSD test) were considered to indicate significant differences compared to untreated cell control or hecogenin or tigogenin alone respectively. OD, optical density.

hecogenin and tigogenin in human RA FLS. We chose to study DNA fragmentation, a final step of apoptosis. Results showed that DNA fragmentation was reduced in human RA FLS pretreated with a specific inhibitor of transducing apoptotic factor p38 (Fig. 4B). Indeed, 10 μM of SB203580 pretreatment for 2 h before addition of 40 μM hecogenin or tigogenin decreased DNA fragmentation of human RA FLS (-48 and -44% respectively versus hecogenin or tigogenin alone) (Fig. 4B).

Upregulation of COX-2 expression and activity in saponin-induced RA FLS death. Numerous current studies have demonstrated that COX-2 expression prevents apoptosis in cancer cells, especially in colon cancer. In contrast to other cell types, the effects of COX-2 and related PGE$_2$ in the regulation of apoptosis could very well be cell type specific. Here, we demonstrated that hecogenin or tigogenin induced COX-2 overexpression over time (Fig. 5A). Indeed, our results showed induction of COX-2 expression as early as 2 h after treatment with 40 μM hecogenin or tigogenin and the expression of COX-2 was markedly enhanced at 24 h after

saponin treatment with a large difference in COX-2 over-expression between hecogenin and tigogenin (Fig. 5A).

This overexpression was correlated with COX-2 activity. PGE$_2$ production was increased over time after saponin treatment; 2.0- and 2.7-fold for hecogenin and tigogenin respectively (p<0.05) at 2 h and 3.3- and 5.3-fold respectively (p<0.05) at 24 h compared to the control (Fig. 5B).

Discussion

Saponins have drawn scientific attention due to their structural diversity and the significance of their biological activities. Some studies have revealed that differences in saponin structure including the type and number of sugar moieties attached by a glycosidic bond at C-3 (Fig. 1) influence biological responses (25,26). Wang *et al* (27) compared dioscin, a spirostenol (diosgenin-3-*O*-(α-L-rhamnopyranosyl (1->2))(α-L-rhamnopyranosyl(1->4))), and methyl protodioscin, a furostanol with 26-*O*-glycopyranoside. These authors showed that only dioscin induces apoptosis although these
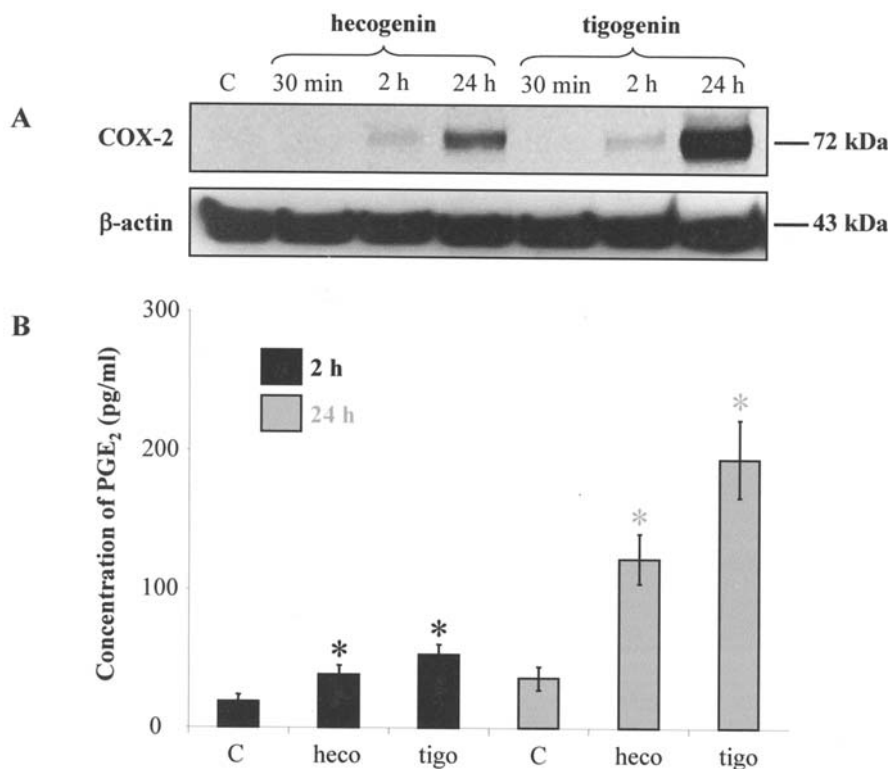
Figure 5. COX-2 expression (A) and activity (B) in hecogenin- or tigogenin-induced RA FLS apoptosis. Cells were cultured without saponin (C, control) or were incubated with 40 μM hecogenin (heco) or tigogenin (tigo) for 30 min, 2 h and 24 h. Protein extracts prepared from cells were subjected to Western blotting and cellular expressions of COX-2 and ß-actin were estimated using mouse anti-human COX-2 and ß-actin antibodies respectively as described in Materials and methods. Quantification of each band was performed by densitometry analysis software. One of three representative experiments is shown from three different patients. The PGE$_2$ levels in the culture medium were measured by enzyme immunoassay. Cells were cultured in 10% FCS medium for 48 h and then treated or not (C, control) with 40 μM hecogenin or tigogenin for 2 and 24 h. Measurements were made on FLS from four different patients. Data are expressed as the mean ± SD of four experiments. *$p < 0.05$ (Fisher's PLSD test) was considered to indicate significant differences compared to the controls.

two compounds are structurally similar. They suggested that the structure of the spirostenol must play a critical role in the activity of dioscin.

From our previous studies (17,18), we already speculated that the 5,6-double bond probably plays a significant role in biological effects. In the present study, we decided to focus on this structural characteristic to shed light on the reactivity of this site in apoptosis, MAPK pathways and COX-2 expression. Recently, we described, for the first time, that diosgenin induced apoptosis in human RA FLS with COX-2 expression (16) but also inhibited the activation of survival transducing factors such as ERK while activating apoptotic transducers such as p38 and JNK (28). The aim of our study was to compare hecogenin and tigogenin [lacking the 5,6-double bond compared to diosgenin (18)] with our previous results concerning diosgenin (16,28). Hecogenin and tigogenin only differ in the presence or absence respectively of a keto group at C-12 (Fig. 1).

Hecogenin and tigogenin inhibited human RA FLS proliferation and this effect was more pronounced for tigogenin. Our study also showed that cells treated with both saponins became rounder, exhibited shrinkage, and separated from adjacent cells. Plant steroids have been thoroughly described for their pharmacological properties, including hypocholesterolemic, antidiabetic and antioxidant activities. Particular attention has been given to their potential for cancer chemoprevention, especially as apoptosis inducers (29). It is

of great importance to understand the mechanisms of apoptosis in RA, as impaired apoptosis in synovial cells is closely associated with hyperplasia of synovial tissues found in patients with RA (30).

Apoptosis is a highly orchestrated and controlled form of cell death, distinct from the pathologic process of necrosis that occurs as a result of cellular damage. Apoptosis involves specific initiating stimuli and intracellular signals, and requires expression of a well-defined set of genes that accomplish the cellular program. In general, apoptosis involves sequential activation of a proteolytic cascade of enzymes named caspases (31). Caspase-3 activation is considered as a convenient marker of apoptosis and is regarded as the point of no return in the proapoptotic signalling cascade (32). In our study, we showed that only tigogenin induced both caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) activities. Hecogenin only induced caspase-8 activity. Consequently, RA FLS death was clearly related to the activation of the caspase cascade, as both saponins increased caspase-3 activity. We showed that caspase-3 activity was greater after tigogenin treatment in relationship with synergistic activation of upstream cysteine protease caspase-8 and -9 than after hecogenin treatment. However, caspase-3 activation by both saponins was not followed by cleavage of its substrate PARP. These results suggested that hecogenin and tigogenin induced cell death in human RA FLS via caspase-3-dependent and PARP-

independent mechanisms. Furthermore, RA FLS apoptosis induced by hecogenin or tigogenin was quantified by determination of cytoplasmic histone-associated DNA fragments. The apoptotic ratio, determined by ELISA, significantly increased over time for cells treated with saponins. A correlation was shown between the level of caspase-3 activity and DNA fragmentation as a function of the saponin used.

It has been reported that JNK/p38 MAPK and ERK play opposite roles in apoptosis induction in such a way that the former promotes apoptosis induction whereas the latter inhibits it (33). Based on these data and our previous results (18), we studied the effect of hecogenin and tigogenin on the activation of survival (ERK) or proapoptotic (p38, JNK) transducing factors in relation to apoptosis in human RA FLS *in vitro*. MAPKs represent an attractive target for RA treatment because they can regulate cell proliferation, apoptosis (34), cytokine expression (35,36) and metallo-proteinase production (37). These MAPKs are expressed in synovial tissue and in cultured FLS (38,39). Treatment of cells with 40 μM hecogenin or tigogenin caused p38 activation but had no effect on JNK and ERK phos-phorylation. IL-1ß treatment, which is known to induce MAPK phosphorylation (40), served as a positive control. Recently, Yen *et al* (41) reported that diosgenin promoted angiogenesis in preosteoblast-like cells by a hypoxia-inducible factor-1α-dependent mechanism involving the activation of p38 MAPK. Our recent study showed that, diosgenin (40 μM) inhibited ERK activation but activated p38 and JNK in human erythroleukemia (HEL) cells (22) but also in human RA FLS (28).

At this stage of our study, we showed that three structurally similar saponins caused apoptosis of synovial cells but with different signalling pathways compared with results previously described for diosgenin (28) where three MAPK pathways were implicated; hecogenin or tigogenin induced apoptosis only by the p38 pathway. To confirm the implication of the p38 kinase signalling pathway in hecogenin- or tigogenin-induced apoptosis in human RA FLS, we used a specific inhibitor for pretreatment before addition of saponins. After quantitative determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) by ELISA, our results showed that SB203580 reduced hecogenin- or tigogenin-induced DNA fragmentation. These findings provide evidence that hecogenin and tigogenin (40 μM) promote apoptosis in human RA FLS by a mechanism involving p38 activation.

RA is characterized by the proliferation of synoviocytes which also produce prostanoids. Eicosanoids and prostanoids are important lipid mediators that are produced at elevated levels in inflamed tissues including rheumatoid synovium and in cultured human RA FLS (20,42-44). Curiously, we showed that the level of apoptosis in RA FLS treated with diosgenin seemed to be associated with upregulation of COX-2 (16,18) in contrast with the majority of data from other cell types such as cancer cells, in which COX-2 expression was shown to prevent apoptosis. Indeed, overexpression of COX-2 in several pathological conditions, such as colon carcinoma, point to a causative role of COX-2 in tumor initiation and/or promotion (45,46). But, in the literature, this phenomenon

has begun to be discussed with regards to cancer type because recently Xu *et al* (47) demonstrated that over-expression of COX-2 in human osteosarcoma cells decreased proliferation and increased apoptosis. In addition, increased PGE$_2$ was shown to be related to the induction of apoptosis in growth plate chondrocytes (48) and, more recently, Pelletier *et al* (49) found that the *in situ* increase in chondrocyte death/apoptosis in experimental osteoarthritis was mainly caspase dependent and was influenced by upregulation of the level of COX-2. Concerning the studies on synoviocytes, it was shown that nitric oxide induced syno-viocyte death through COX-2 expression and PGE$_2$ synthesis with a significant change in mitochondrial membrane potential associated with the activation of caspase-3 (50). These results concerning the effects of nitric oxide are in harmony with our previous study on the effects of diosgenin on RA FLS (16). Previously, we described that diosgenin-induced apoptosis in human cancer cells was associated with PGE$_2$ increased production (24,51). All these studies demonstrated that the effects of COX-2 and related PGE$_2$ in the regulation of apoptosis reflected differences in cellular responses. For this reason, our study focused on COX-2 in order to determine if hecogenin- and tigogenin-induced apoptosis in human RA FLS was also accompanied by upregulation of COX-2 as a function of molecular structure of these saponins.

Under our conditions, hecogenin and tigogenin induced COX-2 expression over time. Increased COX-2 expression was correlated with PGE$_2$ production. We showed that tigogenin was a more potent inducer of apoptosis than hecogenin especially after 24 h of treatment. We demon-strated that, at this time in treatment, tigogenin had a greater effect on COX-2 expression and activity than hecogenin.

In conclusion, we showed for the first time that hecogenin and tigogenin induced apoptosis in human RA FLS *in vitro*. However, we characterized that these two saponins, as a function of molecular structure, induced cell death with different rates of apoptosis. Tigogenin which lacks the 5,6-double bond compared to diosgenin (18) was more effective than hecogenin which lacks the 5,6-double bond but has the additional keto group at C-12, for inhibition of proliferation, for induction of caspase activities, and for DNA fragmentation. Moreover, the effect of these two molecules on apoptosis in human RA FLS was PARP independent.

Based on our previous investigations with diosgenin-induced apoptosis, we confirmed that structurally similar plant steroids (hecogenin and tigogenin) induced COX-2 expression and activity in apoptotic conditions. Diosgenin induced apoptosis in human RA FLS by inhibition of ERK activation but also by activation of p38 and JNK (28). However, in the absence of the 5,6-double bond in their structures, we demonstrated that only the p38 pathway was implicated in hecogenin- and tigogenin-induced apoptosis. Indeed, this result was confirmed by using an inhibitory strategy on p38 signalling. Pretreatment with SB203580 before saponin addition reduced saponin-induced apoptosis.

Taken together, these new results provide strong evidence that a family of structurally similar plant steroids is capable of inducing apoptosis in human RA FLS with different rates

rent signalling pathways. This study also confirms ...issed appearance of the downregulation or up-regulation of COX-2 in cell apoptosis as a function of cell type.

Acknowledgements

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