Cyclooxygenase-2 and 5-lipoxygenase pathways in diosgenin-induced apoptosis in HT-29 and HCT-116 colon cancer cells

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Received December 14, 2009; Accepted January 29, 2010

DOI: 10.3892/ijo_00000601

Abstract. We studied the relationship between diosgenin-induced apoptosis and arachidonic acid metabolism in two cancer cell lines, the HT-29 and HCT-116. Diosgenin is a steroidal saponin known for its antiproliferative and pro-apoptotic effects on cancer cells. We focused our attention on two enzymes intervening in two different pathways of arachidonic metabolism: cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX). HT-29 and HCT-116 cells express 5-LOX but only HT-29 cells express COX-2 since HCT-116 are deficient. After 40 μM diosgenin treatment, we observed apoptosis hallmarks in both cell lines but HT-29 cells were more resistant with delayed apoptosis. In these cells, COX-2 expression and activity were increased and for the first time we showed that diosgenin also increased 5-LOX expression and enhanced leukotriene B_4 production. Inhibition of 5-LOX by AA-861 significantly reduced apoptosis in both cell lines but COX-2 inhibition by NS-398 strongly sensitized HT-29 cells to diosgenin-induced apoptosis compared to HCT-116 cells. In this study, we showed the implication of COX-2 and 5-LOX in diosgenin-induced apoptosis but these results demonstrate how difficult it is to assess the correlation between the apoptotic signalling pathway of diosgenin and arachidonic acid metabolism with certitude.

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Key words: colon cancer cells, diosgenin, apoptosis, cyclo-oxygenase-2, 5-lipoxygenase

Introduction

Pathogenesis and prevention of colon cancer is presently an important topic of research since it is the second deadliest cancer and the most common cancer for both men and women in the Western population. Apoptosis plays an important role in cancer development and therapy and is regulated by many different signalling pathways. Thus any agent that can selectively induce apoptosis in cancer cells is potentially useful in cancer therapy (1-3).

Diosgenin is a steroidal saponin found in several plants, including *Solanum* and *Dioscorea* species, which has gained importance recently especially in the pharmaceutical industry (4). Diosgenin has been shown to have antitumor effects on cancer cells. It inhibits the growth of cell lines through cell cycle arrest and induction of apoptosis (5-8), including the two colorectal cancer cell lines, HT-29 and HCT-116 (9,10).

Recent studies concerning the relationship between the arachidonic acid (AA) cascade and carcinogenesis revealed novel molecular targets for cancer treatment (11). It has been demonstrated that the metabolism of AA, a polyunsatured fatty acid, by either the cyclooxygenase (COX) or lipoxygenase (LOX) pathway, generates a host of proinflammatory metabolites called eicosanoids including prostaglandins (PGs), thromboxanes and leukotrienes (LTs), respectively. These products are known to modulate diverse physiological and pathological responses and notably, PGs and LTs are involved in angiogenesis, apoptosis, hyperproliferation, immunosuppression and invasiveness (12). Several studies have shown that COX-2 expression is increased in colorectal cancer cells and that activation of this isoenzyme is an early event in colon carcinogenesis (13). PGE_2, the main metabolite of COX-2, mediates tumor survival by several mechanisms. It inhibits tumor cell apoptosis and induces tumor cell proliferation (14). Furthermore, the expression of 5-LOX appears to be occasionally upregulated during neoplastic transformation (15). LTB_4, a terminal product of

the 5-LOX metabolic pathway, inhibits apoptosis and has been shown to be procarcinogenic in several studies (16). It has been reported in several works that diosgenin treatment induced COX-2 overexpression and enhanced PGE_2 production in different cancer cell lines (5,7,17), but its effects on the 5-LOX pathway have not yet been studied.

The two colorectal cancer cell lines selected for this study express 5-LOX but COX-2 is constitutively expressed in HT-29 cells whereas HCT-116 cells are deficient. The aim of the present study was to ascertain a possible relationship between 5-LOX expression and activity during diosgenin-induced apoptosis in human colorectal cancer cells but also in regard to COX-2 expression and activity.

We demonstrated for the first time that diosgenin enhanced 5-LOX activity and its inhibition by AA-861 significantly reduced apoptosis of colorectal cancer cells. Furthermore, COX-2-expressing colorectal cancer cells were less sensitive to diosgenin-induced apoptosis compared to COX-2 deficient colorectal cancer cells. Pre-treatment with a COX-2 selective inhibitor markedly sensitized colon cancer cells expressing COX-2 to diosgenin-induced apoptosis but protected HCT-116 cells.

Materials and methods

Cell lines, cell culture, treatment and light microscopy. The HT-29 cell line was purchased from American Culture Type Collection (LGC Standards, Middlesex, UK) and the HCT-116 cell line was kindly provided by Dr J.M. Barret (Laboratoires Pierre Fabre, Castres, France). Cells were, respectively, seeded at $5x10^6$ and $3x10^6$ cells in 75 cm^2 tissue culture flasks, grown in DMEM and MEM medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 5% fetal calf serum (FCS) (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37˚C. Cells were grown for 36 h in culture medium prior to exposure or not to 40 μM diosgenin (Sigma Aldrich, Saint Quentin Fallavier, France). A stock solution of 10^{-2} M diosgenin was prepared in ethanol, and diluted in culture medium to give the appropriate final concentration. The same amount of vehicle (ethanol 0.1%) was added to control cells. Cell viability was determined by the trypan blue dye exclusion method. For light microscopy, after treatment, cultured cells were examined under phase-contrast microscopy (magnification, x400), and pictures were taken with an image acquisition system (Nikon, Champigny sur Marne, France). When pharmacological inhibitors of COX-2 (NS-398) (Cayman Chemical, SpiBio, Massy, France) or 5-LOX (AA-861) (Sigma, Saint Quentin Fallavier, France) were used, cells were pretreated with 10 μM NS-398 or AA-861 12 h before adding 40 μM diosgenin for 48 h.

Cell proliferation assay and viability assessment. Measurement of cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HT-29 and HCT-116 cells were cultured and plated, respectively, at 10^4 and $6x10^3$ cells/well in 5% FCS medium in 96-well culture plates and grown 36 h before treatment or not (time 0) with diosgenin at 40 μM for 24-72 h. MTT tests were carried out daily as previously described (6) and experiments were performed in three independent assays.

Caspase-3 activity assay. Caspase-3 activity was assayed using Quantikine® human active caspase-3 (R&D Systems) as previously described (18). Colorectal cancer cells were cultured in 75 cm^2 flasks and treated or not with 40 μM diosgenin for 24 h, and then incubated with 10 μM biotin-ZVKD-fmk inhibitor for 1 h at 37˚C. Caspase-3 activity was measured in accordance with the manufacturer's protocol (R&D Systems). Briefly, cells were harvested, washed in PBS and resuspended 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 HRP substrate.

Apoptosis quantification: DNA fragmentation. HT-29 and HCT-116 cells were cultured in 6-well culture plates (respectively $7.2x10^5$ and $4.5x10^5$ cells/well). Cells were treated without or with 40 μM diosgenin for 24 and 48 h. For other experiments, cells were pre-incubated for 12 h with 10 μM NS-398 (COX-2 inhibitor) or 10 μM AA-861 (5-LOX inhibitor) before addition of 40 μM diosgenin for 48 h. Apoptosis was quantified on pooled cells (floating and adherent) using the cell death ELISA (Cell Death Detection $ELISA^{plus}$, Roche Diagnostics). Cytosol extracts were obtained according to the manufacturer's protocol and apoptosis was measured as previously described (19).

Protein expression analysis. After 40 μM diosgenin treatment for 24 and 48 h, cells were washed and lysed in RIPA lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% NP-40, 0.1% SDS, 20 μg/ml aprotinin) containing protease inhibitors (Complete™ Mini, Roche Diagnostics, Meylan, France). Western blot was performed as previously described (5,20). Briefly, proteins (10-100 μg) were separated by electrophoresis on SDS-polyacrylamide gels, transferred to PVDF membranes (Amersham Pharmacia Biotech, Saclay, France) and probed with respective antibodies against poly(ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology; TEBU, Le Perray en Yvelines, France), COX-2 and 5-LOX (Cayman Chemical). After incubation with secondary antibodies (Dako), blots were developed using enhanced chemiluminescence reagents (Amersham). Membranes were then reblotted with anti-ß-actin monoclonal antibody (Sigma). Western blots were analyzed by densitometry (GBOX, Ozyme, France) and protein expression was normalized to ß-actin.

Assay of PGE_2 and LTB_4 production. PGE_2 levels in culture medium were measured by enzyme immunoassay. Measurements were quantified on supernatants from treated (40 μM diosgenin) or not (control) HT-29 cells. The PGE_2 concentration in the medium was measured using an ELISA kit according to the manufacturer's instructions (Cayman Chemical) and was normalized with respect to the number of

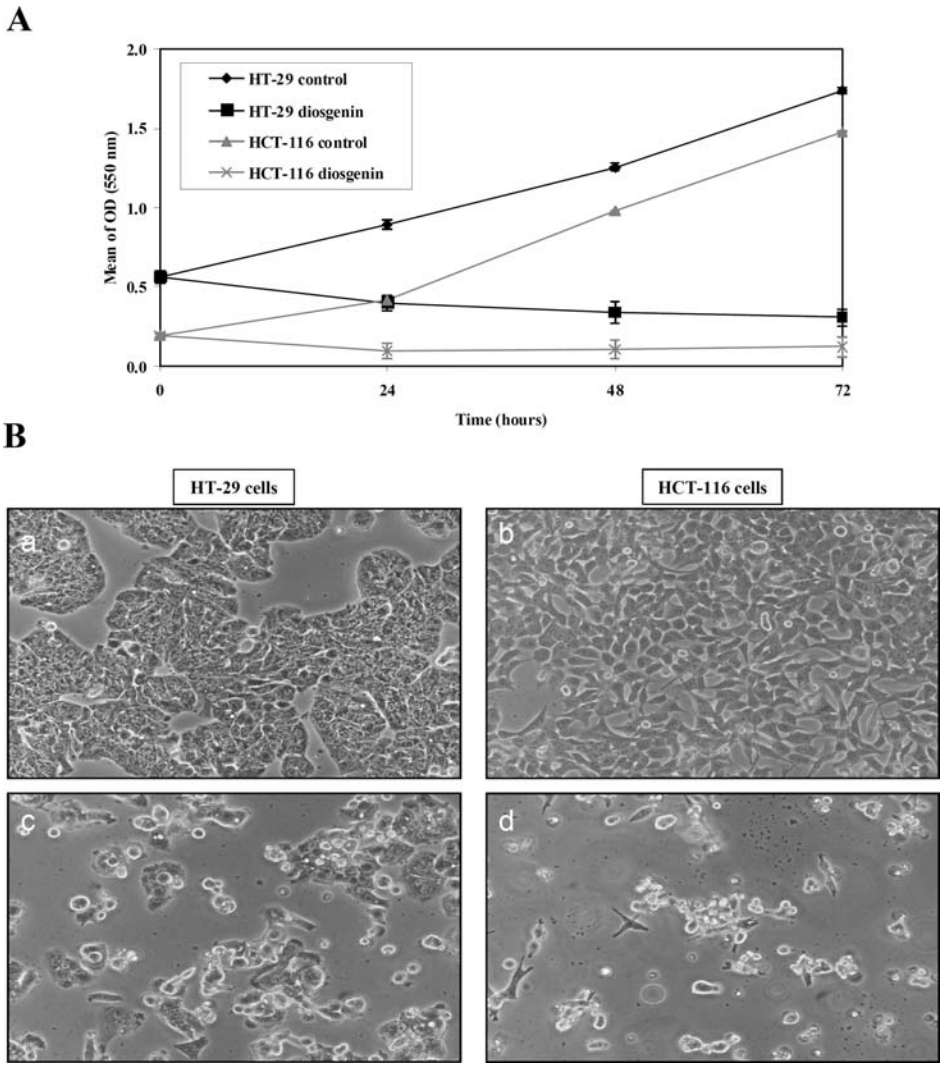

Figure 1. (A) Effect of diosgenin on proliferation of HT-29 and HCT-116 cells. Cells were cultured in 5% FCS medium for 36 h and then incubated (time 0) for 24-72 h with 40 μM diosgenin. Cell proliferation was evaluated by the MTT test. Representative results from six independent experiments are shown; values are the mean ± SD from triplicate cultures. $^{*}p<0.05$ was considered to indicate significance compared to control (Fisher's protected-least-significant-difference test). (B) Morphologic changes in colorectal cancer cells. HT-29 and HCT-116 cells were incubated for 48 h without (a, b) or with 40 μM diosgenin (c, d). Original magnification, x400.

viable cells present in the particular culture at the time of sampling.

LTB_4 levels in culture medium were measured by enzyme immunoassay. Measurements were made on supernatants from treated (40 μM diosgenin) or not (control) HT-29 and HCT-116 cells stimulated at 37˚C by 5 μM $CaCl_2$ and 1.25 μM $MgCl_2$ for 5 min, and then by the addition of 1 μl of a solution (0.5 mg/ml) of calcium ionophore A23187 and 5 μl of exogenous arachidonic acid (10 mg/ml) for 15 min. The LTB_4 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 medians and standard deviations (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). Fisher's protected-least-significant-difference (PLSD) test was used, and $p<0.05$ was considered to indicate statistical significance.

Results

Diosgenin effect on HT-29 and HCT-116 cell proliferation and morphological modifications. Cells were cultured in 5% FCS-medium with or without 40 μM diosgenin for 24-72 h and cell proliferation was evaluated by the MTT test (Fig. 1A). Under our experimental conditions, a decrease in proliferation was observed as early as 24 h after diosgenin treatment, where the percentage of inhibition was, respectively, -55% ($p<0.05$) and -76% ($p<0.05$) for HT-29 and HCT-116 cells (Fig. 1A). Proliferation of human colorectal cancer cells decreased until 72 h (-82 and -92% for HT-29 and HCT-116 cells, respectively, $p<0.05$) in the presence of 40 μM diosgenin. We chose 40 μM diosgenin for these experiments and the following ones based on results obtained with various cancer cell types (5-7,9).

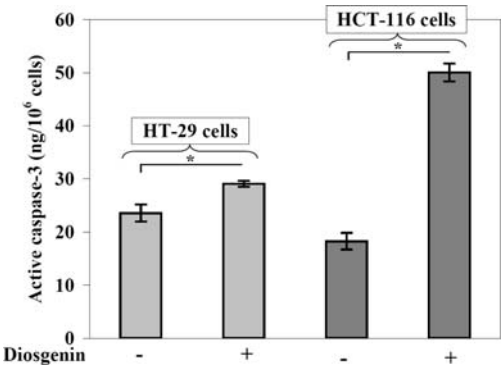

Figure 2. Effect of diosgenin on central effector caspase-3 activity in HT-29 and HCT-116 cells. Cells were cultured in 75 cm^2 flasks and treated or not with 40 μM diosgenin for 24 h. Caspase-3 activity was assayed using Quantikine human active caspase-3 (R&D Systems). Following the treatment, cells 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 mean ± SD of three experiments. *p<0.05 (Fisher's PLSD test) was considered to indicate significance compared to relevant controls.

Direct observation with phase-contrast microscopy demonstrated that cells treated with 40 μM diosgenin for 48 h (Fig. 1Bc and d) had numerous morphological differences compared to control cells (Fig. 1Ba and b). Cell shrinkage, cytoplasm condensation and formation of cytoplasmic filaments appeared 48 h after 40 μM diosgenin treatment.

Diosgenin induced apoptosis with caspase activation and with or without PARP cleavage. Apoptosis induction 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 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.

We showed that diosgenin induced executive caspase-3 activity after 24 h treatment (Fig. 2). Caspase-3 activation by diosgenin in HT-29 cells was significant, but weak (+1.3-fold versus control, p<0.05). However, caspase-3 activation was greater in HCT-116 cells (+2.7-fold versus control, p<0.05).

The slight activation of caspase-3 in HT-29 cells was not followed by cleavage of PARP after treatment with diosgenin. Western blot detected the native form (112 kDa) of PARP but not the significant cleaved fragment at 85-kDa in treated HT-29 cells (Fig. 3A). In contrast, cleaved fragment of PARP was present in HCT-116 cells starting at 24 h diosgenin treatment and was strongly expressed after 48 h treatment (Fig. 3A).

DNA fragmentation, considered as a major marker of apoptotic cells, was observed in both cell lines after diosgenin treatment. Quantitative determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) was performed by ELISA in our study. Results showed that DNA fragmentation was induced in HT-29 cells after 48 h

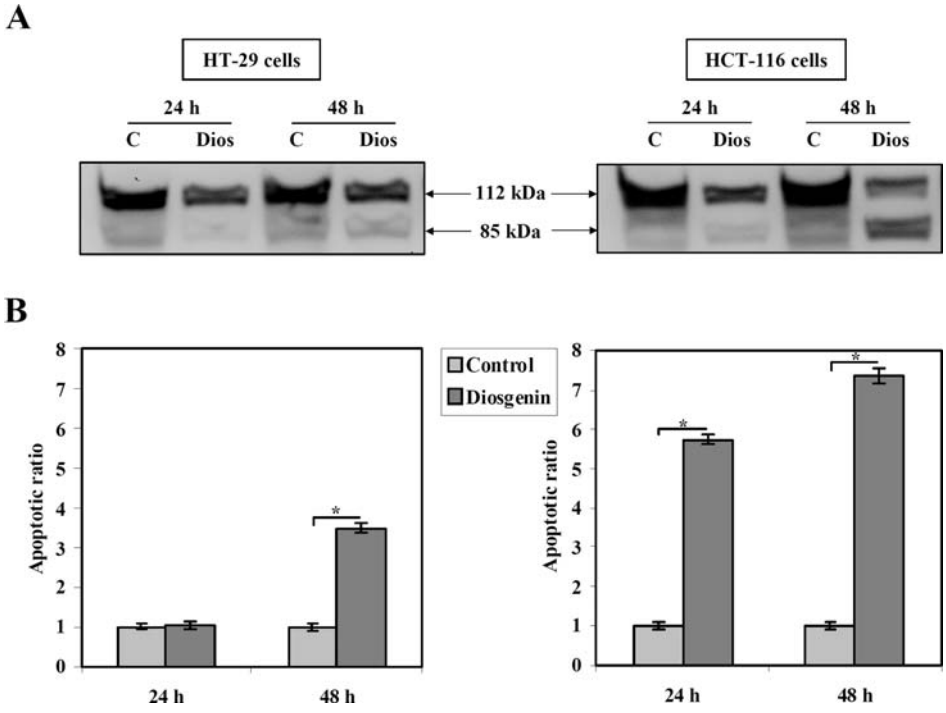

Figure 3. Effect of pro-apoptotic dose of diosgenin on PARP cleavage and related DNA fragmentation. PARP cleavage was analysed by Western blotting (A). Total proteins were extracted from cells treated or not (C, control) with 40 μM diosgenin (Dios). PARP cleavage was assessed by detection of a 85 kDa band after probing with a mouse anti-human PARP antibody. One representative of three independent experiments is shown. Apoptosis was quantified on floating and adherent cells using cell death ELISA based on DNA fragmentation (B). The fold induction (apoptotic ratio) of DNA fragmentation is shown relative to the value for the control culture, which is taken as 1. Data are expressed as mean ± SD of three independent experiments. *p<0.05 (Fisher's PLSD test) was considered to indicate significance compared to relevant controls.

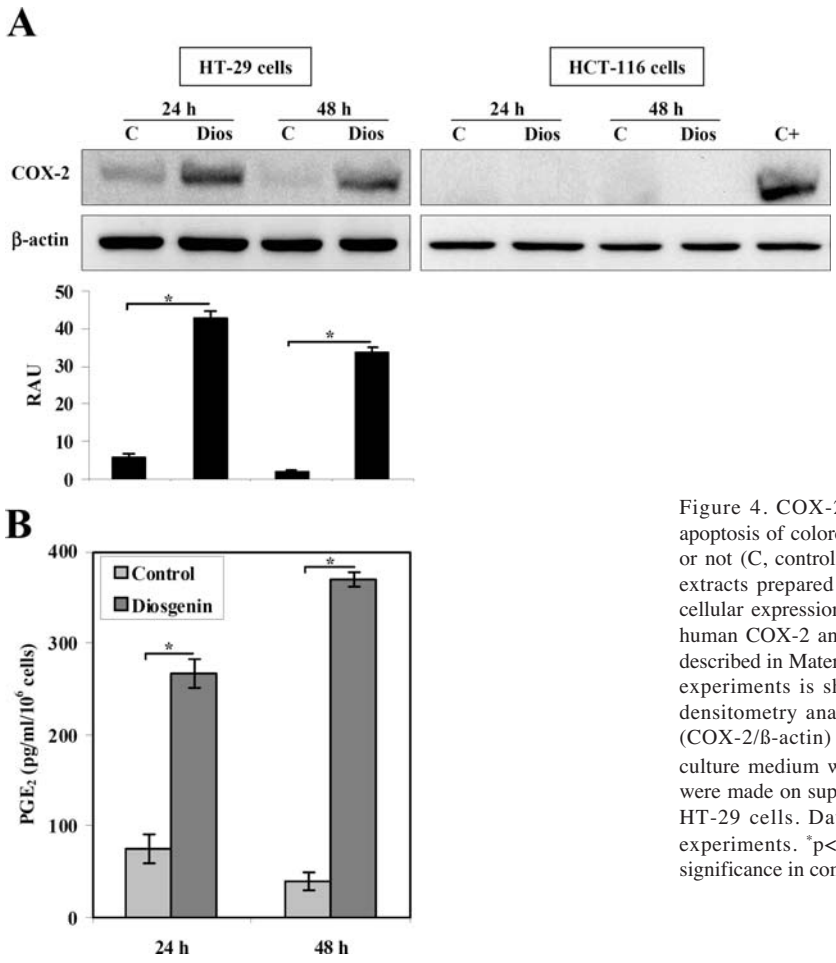

Figure 4. COX-2 expression and activity during diosgenin-induced apoptosis of colorectal cancer cells. HT-29 and HCT-116 cells were treated or not (C, control) with 40 μM diosgenin (Dios) for 24 and 48 h. Protein extracts prepared from the cells were subjected to Western blotting and cellular expression of COX-2 and ß-actin was analyzed using mouse anti-human COX-2 and mouse anti-human ß-actin antibodies, respectively, as described in Materials and methods. One representative of three independent experiments is shown. Quantification of each band was performed by densitometry analysis software and results were expressed as the ratio (COX-2/ß-actin) in relative arbitrary units (RAU). (B) PGE_2 levels in culture medium were measured by enzyme immunoassay. Measurements were made on supernatants from treated (40 μM diosgenin) or not (control) HT-29 cells. Data are expressed as mean ± SD of four independent experiments. *p<0.05 (Fisher's PLSD test) was considered to indicate significance in comparison with the relevant controls.

diosgenin treatment (+3.5-fold versus control, p<0.05) whereas DNA fragmentation was markedly enhanced after 24 h diosgenin treatment for HCT-116 cells (+5.7-fold versus control, p<0.05) and +7.4-fold versus control (p<0.05) after 48 h (Fig. 3B).

Upregulation of COX-2 expression and activity in diosgenin-induced HT-29 cell death. Numerous studies have demonstrated that COX-2 expression prevented apoptosis in cancer cells, especially in colon cancer. Here, we demonstrated that diosgenin-induced COX-2 overexpression in HT-29 cells (Fig. 4A). This overexpression was correlated with COX-2 activity. Indeed, PGE_2 production increased over time after diosgenin treatment: +3.7- and +9.4-fold (p<0.05) at 24 and 48 h, respectively, compared to controls (Fig. 4B). Furthermore, as previously described in the literature (21), we showed the absence of COX-2 expression in HCT-116 cells (Fig. 4A).

Effect of diosgenin on 5-LOX expression and activity in human colorectal cancer cells. COX-2 and 5-LOX have been reported to be simultaneously up-regulated in colorectal cancer (22,23). Recently, it was shown that 5-LOX expression is up-regulated in human colorectal cancer specimens, and correlated with tumor size, depth and vessel invasion (24). We showed 5-LOX expression in HT-29 and HCT-116 cells (Fig. 5A). Diosgenin markedly increased 5-LOX expression after 48 h only in HT-29 cells. 5-LOX activity, as defined by LTB_4 production, was also present in HT-29 and HCT-116 cells. LTB_4 synthesis increased after diosgenin treatment in both cell lines (Fig. 5B): +2.0- and +3.2-fold (p<0.05) at 24 and 48 h, respectively, compared to controls in HT-29 cells, and +2.4- and +4.3-fold (p<0.05) at 24 and 48 h, respectively, compared to controls in HCT-116 cells.

Effect of specific COX-2 and 5-LOX inhibitors on diosgenin-induced apoptosis in human colorectal cancer cells. To extend the results presented above, we wanted to know if COX-2 and 5-LOX were directly associated with diosgenin-induced human HT-29 and HCT-116 apoptosis. In order to clarify this point, we used a specific COX-2 inhibitor (NS-398) and a specific 5-LOX inhibitor (AA-861) to verify DNA fragmentation after diosgenin treatment.

Cells were pre-incubated for 12 h with 10 μM NS-398 or 10 μM AA-861 before adding 40 μM diosgenin for 48 h. We observed a large increase in DNA fragmentation in HT-29 cells with NS-398 pre-treatment before addition of diosgenin (+3.2-fold, p<0.05) versus diosgenin alone (Fig. 6). However, pre-treatment with AA-861 before diosgenin treatment decreased DNA fragmentation (-1.6-fold, p<0.05) versus diosgenin alone. In HCT-116 cells NS-398 or AA-861 pre-treatment before diosgenin treatment decreased DNA fragmentation (-1.4 and -2.3-fold, respectively, p<0.05) versus diosgenin alone (Fig. 6).

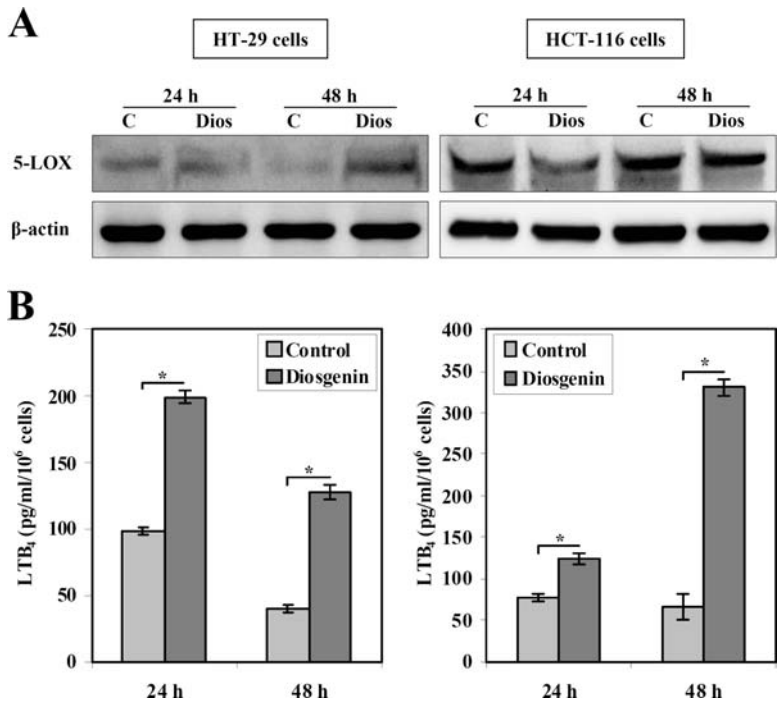

Figure 5. 5-LOX expression and activity during diosgenin-induced apoptosis of colorectal cancer cells. HT-29 and HCT-116 cells were treated or not (C, control) with 40 μM diosgenin (Dios) for 24 and 48 h. Protein extracts prepared from the cells were subjected to Western blotting and cellular expression of 5-LOX and ß-actin was analyzed using rabbit anti-human 5-LOX and mouse anti-human ß-actin antibodies, respectively, as described in Materials and methods. One representative of three independent experiments is shown. (B) LTB_4 levels in culture medium were measured by enzyme immunoassay. Measurements were made on supernatants from treated (40 μM diosgenin) or not (control) cultured cells stimulated at 37˚C by 5 μM $CaCl_2$ and 1.25 μM $MgCl_2$ for 5 min, then by the addition of 1 μl of 0.5 mg/ml calcium ionophore A23187 and 5 μl of exogenous arachidonic acid (10 mg/ml) for 15 min. Data are expressed as mean ± SD of four independent experiments. *p<0.05 (Fisher's PLSD test) was considered to indicate significance in comparison with the relevant controls.

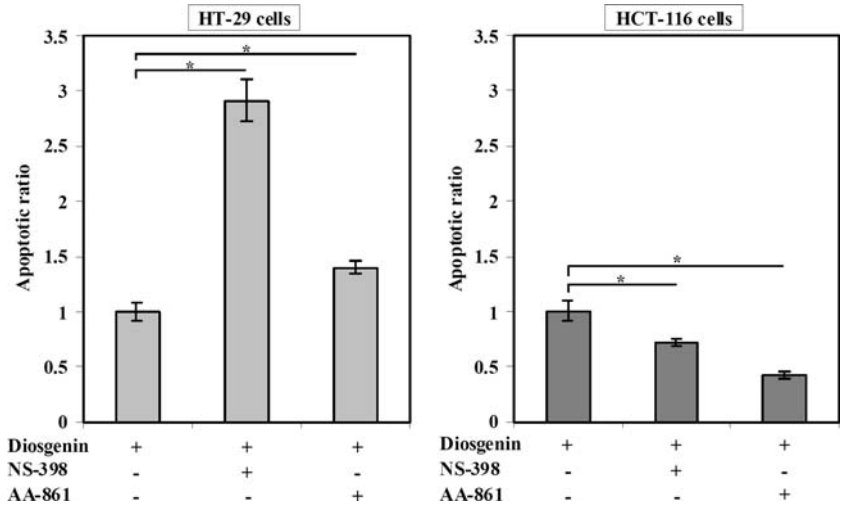

Figure 6. Effect of COX-2- and 5-LOX-specific inhibitors on DNA fragmentation induced by diosgenin. Cells were treated with 40 μM diosgenin alone for 48 h, or pre-incubated for 12 h with 10 μM NS-398 (COX-2 inhibitor) or 10 μM AA-861 (5-LOX inhibitor) before addition of 40 μM diosgenin for 48 h. Apoptosis was quantified on pooled cells (floating and adherent) by the Cell Death Detection ELISA. Measurements were made on cells from four different experiments. Data are expressed as mean ± SD of four independent experiments. *p<0.05 (Fisher's PLSD test) was considered to indicate significance in comparison with the diosgenin group.

Discussion

It is important to understand the mechanisms of apoptosis in cancer cells, as apoptosis is believed to be one of the major consequences of anticancer drug treatment against malignancies. Moreover, an aberrant arachidonic acid (AA) metabolism has been related to carcinogenic processes and its modulation has been suggested to be an important strategy

for cancer prevention and treatment. In this regard, we examined whether diosgenin was able to affect the AA cascade in relation to HT-29 and HCT-116 apoptosis induction for the first time.

In the first part of our study, it was necessary to show diosgenin-induced apoptosis in human colorectal cancer cells as recently described by Raju *et al* (9,10). Diosgenin (40 μM) was used because our previous studies on different cancer cell lines had shown that this concentration induced apoptosis (5-7,25,26).

After 24 h of diosgenin treatment, proliferation of HCT-116 and HT-29 cells was inhibited by -76 and -55%, respectively, showing that HCT-116 cells are sensitive to treatment earlier than HT-29 cells. Decreased cell viability was associated with apoptosis induction. When HT-29 and HCT-116 cells were exposed to diosgenin, they displayed the typical hallmarks of apoptotic death, such as cell shrinkage, cytoplasm condensation, caspase-3 activation, PARP cleavage (only in HCT-116 cells) and DNA fragmentation. Caspase activation leads to proteolytic cleavage of specific cell substrates, including PARP (27), lamins, histone H1, as well as proteins involved in cell growth, survival and death (28). Many natural components of plant extracts induce apoptosis in HT-29 cells with caspase-3 activation and PARP cleavage (29,30). However, diosgenin-induced apoptosis in HT-29 cells was PARP-independent in comparison with HCT-116 cells where PARP cleavage was present as previously described (10). This could be explained by the fact that diosgenin induced only slight caspase-3 activation in HT-29 (9) compared to that observed in HCT-116 cells. Glinghammar *et al* (31) previously described that deoxycholic acid, an anticancer agent, rapidly activated caspase-3 in HCT-116 cells, whereas in HT-29 cells, caspase-3 activation was delayed. In our study, diosgenin induced DNA fragmentation in HT-29 and HCT-116 cells, which was delayed in HT-29 cells. It was correlated with caspase-3 activation levels with or without PARP cleavage.

The main goal of the present study was to evaluate the effect of diosgenin on 5-LOX expression and activity in relation to diosgenin-induced apoptosis in human colorectal cancer cells (HT-29 and HCT-116). These two cell lines express 5-LOX and, we also chose to study the effect of diosgenin on COX-2 expression and activity because HT-29 cells express COX-2 constitutively in contrast to HCT-116 cells which lack COX-2 expression.

Recently, involvement of 5-LOX enzyme and its products, in particular LTB_4, in the development and progression of colon cancer, has emerged (16,24). Similarly to COX-2, 5-LOX expression and activity have been found to be up-regulated during colon carcinogenesis (23) and closely related to tumor size, depth and vessel invasion (24). Even if the exact mechanism linking 5-LOX to cancer aetiology remains to be fully elucidated, it seems likely that COX-2 and 5-LOX may represent an integrated system that regulates the proliferative, metastatic and proangiogenic potential of cancer cells. Both enzymes, induce cell cycle progression and block apoptosis, enhance cancer cell chemoresistance and stimulate angiogenesis, with a convergent targeting on vascular endothelial growth factor expression and release (32). In our laboratory, we observed 5-LOX metabolism despite the lack of five-lipoxygenase-activating protein (FLAP) in HT-29 cl. 19A cells (33). Furthermore, we demonstrated COX-2 up-regulation after FLAP transfection in these cells suggesting a possible interaction between the two major pathways of AA metabolism (34).

In this study, we showed that diosgenin increased 5-LOX expression only in HT-29 cells after 48 h treatment. However, its activity was observed as early as 24 h in HT-29 and HCT-116 cells as shown by a large production of LTB_4. It is possible that diosgenin acts only on 5-LOX activity. In another model, we showed dynamic changes in human 12-, 15- and 5-LOX activities in HEL cells during diosgenin-induced differentiation (35). On the other hand, interaction between 5-LOX and COX-2 pathways in HT-29 cells could be possible after diosgenin treatment because we showed COX-2 overexpression but this hypothesis is not possible for HCT-116 cells which lack COX-2 expression.

To understand the possible involvement of 5-LOX and COX-2 in diosgenin-induced apoptosis in HT-29 and HCT-116 cells, we used an inhibitory strategy of these two pathways of AA metabolism. We used DNA fragmentation as a marker to investigate the effect of AA-861 (5-LOX selective inhibitor) and NS-398 (COX-2 selective inhibitor) on diosgenin-induced apoptosis. Pre-treatment with AA-861 before diosgenin addition significantly reduced DNA fragmentation compared to diosgenin alone in HT-29 and HCT-116 cells. In some studies, leukotrienes are able to enhance proliferation, increase survival rate and suppress apoptosis of human cells (16,23,26). Recently, we showed that AA-861 abolished leukotriene synthesis in human T lymphoblastoid cell lines (MOLT4 and Jurkat) (37). It is known that AA-861 inhibited proliferation and induced apoptosis in esophageal cancer cells (38), Jurkat T-cell (39), neuroblastoma cells (36), but also in colon cancer cells (16). However, in our study, AA-861 reduced diosgenin-induced apoptosis in HT-29 and HCT-116 cells. It has been shown that AA-861 inhibited apoptosis induced by the protein synthesis inhibitor PMC in a human astrocytoma cell line (40), and inhibited ATP-induced apoptosis in macrophages (41).

Various studies on selective COX-2 inhibitors revealed their ability to induce apoptosis in a variety of cancer cell lines. For example, we described that NS-398 induced apoptosis in osteosarcoma cells (42). But the potential molecular mechanism of the COX-2 selective inhibitor effect on proliferation and apoptosis of colon cancer cells is unclear. NS-398 may inhibit proliferation and induce apoptosis in colon cancer cells through the COX-2-dependent pathway (HT-29) and the activity of the COX-2-independent pathway (SW480) (43). For the first time, we demonstrated that pre-treatment with NS-398 strongly sensitized HT-29 cells (expressing COX-2) to diosgenin-induced apoptosis but protected HCT-116 cells (lacking COX-2). It has been reported in the literature that NS-398 enhanced radiosensitivity in NCI-H460 human lung cancer cells, which express COX-2 constitutively, but protected HCT-116 cells from the effects of radiation (21). On the other hand, low dose NS-398 effectively increased 5-fluorouracil-sensitivity in high COX-2 protein-expressing HCA-7 colon cancer cells (44).

In conclusion, diosgenin induces apoptosis in HT-29 and HCT-116 cells, and cell death is delayed in HT-29 cells, which correlates with delayed activation of DNA fragmentation. We demonstrated for the first time that diosgenin enhanced LTB_4 production and its inhibition by AA-861 significantly reduced apoptosis of colorectal cancer cells. In addition, we showed that diosgenin increased COX-2 expression and activity in colorectal cancer cells. Furthermore, we demonstrated that its inhibition by NS-398 markedly sensitized HT-29 cells which express COX-2 to diosgenin-induced apoptosis compared to HCT-116 cells which are COX-2 deficient. These results confirm how difficult it is to assess with certainty the correlation between the apoptotic signalling pathway of diosgenin and AA metabolism even on two colorectal cancer cell lines.

Acknowledgments

We acknowledge Dr D.Y. Léger and Dr C. Cailleteau for corrections in the preparation of this manuscript. This work was supported in part by the Ministère de l'Education Nationale, de la Recherche et de la Technologie, the Conseil Régional du Limousin and by the Comité d'Orientation Recherche Cancer (CORC).

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