The anti-apoptotic effect of leukotriene D₄ involves the prevention of caspase 8 activation and Bid cleavage

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We have shown in a previous study that leukotriene D₄ (LTD₄) signalling increases cell survival and proliferation in intestinal epithelial cells [Öhd, Wikström and Sjölander (2000) *Gastroenterology* 119, 1007–1018]. This is highly interesting since inflammatory conditions of the bowel are associated with an increased risk of developing colon cancer. The enzyme cyclo-oxygenase 2 (COX-2) is important in this context since it is up-regulated in colon cancer tissues and in tumour cell lines. Treatment with the COX-2-specific inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl)methane sulphonamide has been shown previously to cause apoptosis in intestinal epithelial cells. In the present study, we attempted to elucidate the underlying mechanisms and we can now show that a mitochondrial pathway is employed. Inhibition of COX-2 causes release of cytochrome *c*, as shown by both Western-blot and microscopy studies, and as with apoptosis, this is significantly decreased by LTD₄. Since previous studies showed increased Bcl-2 levels on LTD₄ stimulation, we further studied apoptotic regulation at the mitochondrial level.

From this we could exclude the involvement of the anti-apoptotic protein Bcl-X_L as well as its pro-apoptotic counterpart Bax, since they are not expressed. Furthermore, the activity of the pro-apoptotic protein Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death) was completely unaffected. However, inhibition of COX-2 caused cleavage of caspase 8 into a 41 kDa fragment associated with activation and caused the appearance of an activated 15 kDa fragment of Bid. This indicates that *N*-(2-cyclohexyloxy-4-nitrophenyl)methane sulphonamide-induced apoptosis is mediated by the activation of caspase 8, via generation of truncated Bid, and thereafter release of cytochrome *c*. Interestingly, LTD₄ not only reverses the effects induced by inhibition of COX-2 but also reduces the apoptotic potential by lowering the basal level of caspase 8 activation and truncated Bid generation.

Key words: Bad, Bcl-X_L, cyclo-oxygenase 2, cytochrome *c*, epithelial cell.

INTRODUCTION

The eicosanoid leukotriene D₄ (LTD₄) is a powerful inflammatory mediator, derived from arachidonic acid via the 5-lipoxygenase pathway [1]. Similar to the other cysteinyl leukotrienes (leukotriene C₄ and E₄), it exerts its effects through specific cell-surface receptors of the G-protein-coupled, seven-transmembrane type. Two such receptors have been cloned, CysLT₁ and CysLT₂ [2,3]. In binding studies, the CysLT₁ receptor has been shown to have highest affinity for LTD₄, whereas CysLT₂ showed equal binding capacity for LTC₄ and LTD₄ [2,3]. CysLT₁ is generally believed to be most important for LTD₄-induced signalling and we have shown previously that this receptor signals via two different G-proteins, one sensitive to pertussis toxin and the other insensitive to it [4].

LTD₄ is important for the pathophysiology of several inflammatory diseases, particularly asthma and inflammatory bowel disease [1,5]. In chronic intestinal inflammation, one of the major concerns is the increased risk of developing colon cancer [6], and in this context it is of interest that patients treated with non-steroidal anti-inflammatory drugs (NSAIDs) have a much lower incidence of colon carcinoma [7]. The mode of action of NSAIDs is inhibition of cyclo-oxygenase 2 (COX-2) and thereby also inhibition of the production of the main COX-2 product prostaglandin E₂ [8]. COX-2 was suggested as a possible link between

inflammation and cancer owing to it being up-regulated in colon cancer tissue [9]. In further support of this concept, we were able to show that LTD₄ caused up-regulation of COX-2 in non-transformed intestinal epithelial cells [10] and that treatment with the COX-2-specific inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl)methane sulphonamide (NS398) induced apoptosis [10]. Interestingly, the LTD₄ receptor CysLT₁ and COX-2 are both increased in colon cancer tissue [11].

The intricate network of apoptotic signalling pathways is often dysregulated in cancer cells. One example of this is up-regulation of the anti-apoptotic protein Bcl-2 in human colon-cancer cells [12]. The Bcl-2 protein family is important for the regulation of apoptosis, and it includes anti-apoptotic members such as Bcl-2 and Bcl-X_L, as well as pro-apoptotic members like Bax, Bak, Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death) and Bid. Their main site of action is believed to be the mitochondria, where they facilitate or impede the release of cytochrome *c* [13]. The released cytochrome *c* then participates in the formation of the apoptosome together with Apaf-1 and procaspase 9, leading to the activation of caspase 9 and further engagement of downstream executioner caspases such as caspase 3 [14]. It is well known that death-receptor ligation activates caspase 8, which then, depending on the cell type, either directly activates other caspases or cleaves Bid, producing an active form, termed truncated Bid (tBid), which translocates to the mitochondria and contributes to

Abbreviations used: Bad, Bcl-2/Bcl-X_L-antagonist, causing cell death; COX-2, cyclo-oxygenase 2; Int 407 cell, human intestinal epithelial cell; LTD₄, leukotriene D₄; NSAID, non-steroidal anti-inflammatory drug; NS398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methane sulphonamide; Z-DEVD-FMK, Z-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-fluoromethylketone; Z-IETD-FMK, Z-Ile-Glu(Ome)-Thr-Asp(Ome)-fluoromethylketone.

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the release of cytochrome *c* [15,16]. Recent results suggest that death-receptor-independent signalling pathways may also employ the activation of Bid [17,18].

In a previous study [10], we observed increased levels of Bcl-2 in non-transformed intestinal epithelial cells when stimulated with LTD₄. The effect was detectable already at 30 min and was still significant at 5 h, and this increase was partially sensitive to treatment with NS398. This is in good agreement with COX-2, directly or indirectly through the production of prostaglandin E₂, being responsible for the effect on Bcl-2 [12,19]. We have reported previously [10] that NS398 induces apoptosis in intestinal epithelial cells and that this induction can be decreased by stimulation with LTD₄. In the present study, we further addressed the issue of how LTD₄-induced cell-survival signalling can influence programmed cell death.

EXPERIMENTAL

Materials

Antibodies against Bax and Bcl-X_L were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Cytochrome *c* antibody (clone 7H8.2C12) was from Pharmingen (San Diego, CA, U.S.A.) and porin antibody was from Calbiochem (La Jolla, CA, U.S.A.). The antibody against total Bid was from R&D Systems (Minneapolis, MN, U.S.A.), and the antibody that recognizes the activated form, tBid (15 kDa), was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). P-Bad (Ser-112), P-Bad (Ser-136), P-Bad (Ser-155) and Bak antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.), caspase 8 antibody was from Alexis Biochemicals (San Diego, CA, U.S.A.) and the antibody against actin was from ICN Biomedicals (Aurora, OH, U.S.A.). LTD₄ was obtained from Cayman (Ann Arbor, MI, U.S.A.) and ZM198,615 (ICI-198,615) was a gift from Dr R. Metcalf (Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K.). The COX-2-specific inhibitor NS398 [20] was purchased from Biomol (Plymouth Meeting, PA, U.S.A.) and the selective inhibitors against caspases 3 and 8, namely Z-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-fluoromethylketone (Z-DEVD-FMK) and Z-Ile-Glu(Ome)-Thr-Asp(Ome)-fluoromethylketone (Z-IETD-FMK) respectively, were from Enzyme Systems Products (Livermore, CA, U.S.A.). Hoechst 33342 fluorescent chromatin stain and the Mitotracker probe were purchased from Molecular Probes (Eugene, OR, U.S.A.). Peroxidase-linked goat anti-mouse, rabbit anti-sheep, goat anti-rabbit and rabbit anti-goat originated from Dako (Copenhagen, Denmark). Hyperfilm was from Amersham International (Buckinghamshire, U.K.). All other chemicals were of analytical grade and obtained from Chemicon International (Temecula, CA, U.S.A.).

Cell culture

Human intestinal epithelial (Int 407) cells, which exhibit typical epithelial morphology and growth, were cultured as a monolayer to approx. 80% confluence for 5 days in Eagle's basal medium, supplemented with 15% (v/v) newborn calf serum, 55 µg/ml of streptomycin and 55 IU/ml of penicillin. The cells were incubated at 37 °C in a humidified atmosphere of CO₂/air (1:19) and were regularly tested to ensure the absence of mycoplasma contamination.

Separation of mitochondria and cytosol

The cells were cultured for 5 days, washed and left in serum-free culture medium for 30 min. LTD₄ (40 nM) and/or NS398 (100 µM) were added and the cells were incubated for the

indicated periods of time. The different treatments were terminated by the addition of RSB buffer [10 mM NaCl/1.5 mM MgCl₂/10 mM Tris/HCl (pH 7.5)] and the flasks were left on ice for 10 min. The cells were scraped loose, homogenized five times (Dounce; Kimble/Kontes Glass, Deerfield, IL, U.S.A.) and the lysis buffer was added [20 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose, 10 µg/ml protease inhibitor cocktail, 1 mM dithiothreitol and 5% (v/v) Percoll]. The lysates were cleared by two short centrifugations at 750 *g* and then the mitochondria were pelleted by centrifugation at 10000 *g* for 15 min. The supernatant was further cleared of mitochondrial contamination by centrifugation at 200000 *g* for 60 min. The fractionation was checked by assaying citrate synthase and lactate dehydrogenase activity. All samples were assayed and compensated for protein content using the Coomassie Blue protein assay (Pierce, Rockford, IL, U.S.A.) to ensure equal protein content before evaluation for cytochrome *c* content.

Cell lysates

The cells were cultured for 5 days, washed and left in serum-free culture medium for 30 min. LTD₄ (40 nM) and/or NS398 (100 µM) were added and the cells were incubated for the indicated periods of time. In experiments involving the caspase 3 inhibitor, Z-DEVD-FMK, or the caspase 8 inhibitor, Z-IETD-FMK, these were added 30 min before the addition of NS398. The different treatments were terminated by the addition of ice-cold lysis buffer [150 mM NaCl, 20 mM Tris (pH 7.4), 5 mM EDTA, 2 mM Na₃VO₄, 4 µg/ml leupeptin, 60 µg/ml PMSF and 1% Triton X-100]. The flasks were kept on ice for 30 min, and the remaining cell debris was scraped loose into the buffer. The samples were centrifuged at 1000 *g* for 10 min and the resulting supernatants were evaluated and compensated for protein content by using the Coomassie Blue protein assay to ensure equal protein loading.

Gel electrophoresis and immunoblotting

Cell lysates and cytosolic or mitochondrial fractions were solubilized by boiling at 100 °C for 5 min in a sample buffer [62 mM Tris (pH 6.8), 1% SDS, 10% (v/v) glycerol, 15 mg/ml dithiothreitol and 0.05% Bromophenol Blue]. The solubilized proteins were subjected to electrophoresis on 12 or 15% homogeneous polyacrylamide gels in the presence of SDS. The separated proteins were electrophoretically transferred to a PVDF membrane. The membrane was blocked in 5% (w/v) non-fat dry milk or 3% (w/v) BSA either overnight at 4 °C or for 1 h at room temperature (20 °C). Subsequently, it was incubated for 90 min or overnight at 4 °C with specific antibodies against actin (1:2000 dilution), Bcl-X_L, Bak, Bid, tBid, caspase 8 (all 1:500 dilution), Bax, phospho-Bad (Ser-112), phospho-Bad (Ser-136), phospho-Bad (Ser-155), cytochrome *c* or porin (all 1:250 dilution). Thereafter, the membrane was washed extensively, incubated for 1 h at room temperature with peroxidase-linked antibodies (1:5000), washed, incubated with ECL[®] (enhanced chemiluminescence) and exposed to hyperfilm-ECL[®] to visualize immunoreactive protein. Densitometric analysis was performed with a Fluor-S quantitative imaging system (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Immunostaining

The cells were cultured on coverslips in 35 mm Petri dishes. After stimulation, preheated medium containing 100 nM Mitotracker Red was added and the cells were incubated at 37 °C for 30 min

after which the medium was removed and the dishes washed several times with PBS. The cells were fixed with 4% (w/v) paraformaldehyde for 15 min at 37 °C and subsequently permeabilized with 0.2% Triton X-100 for 3 min at 4 °C. Blocking was performed in 2% non-fat dry milk, 2% BSA and 0.1% Tween 20 for 1 h at room temperature. The primary antibody was diluted 1:50 in a solution containing 1% BSA and was incubated for 90 min at room temperature. The secondary antibody used was a 1:100 dilution of 488 Alexa-conjugated anti-mouse in PBS with 1% BSA, and the incubation lasted for 1 h at room temperature. Between all the steps mentioned above, extensive washing with PBS was performed. Finally, the coverslips were mounted on glass slides and the cells were examined in a Nikon fluorescence microscope at $\times 60$ magnification. Images were obtained and processed using a Hamamatsu Photonics (Hamamatsu City, Japan) digital camera and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, U.S.A.).

Hoechst staining and Trypan Blue exclusion

The cells were cultured on glass coverslips for Hoechst staining and directly in 35 mm $\times$ 10 mm Petri dishes for Trypan Blue exclusion, and were exposed for 5 days to 100 μ M NS398 with or without 20 μ M caspase 3 inhibitor, Z-DEVD-FMK, or 20 μ M caspase 8 inhibitor, Z-IETD-FMK. During the course of the experiment, fresh media and inhibitors were added. After completion of the treatment, the cells on coverslips were washed several times with PBS, fixed with 4% (w/v) paraformaldehyde for 15 min at 37 °C and then incubated for 15 min in 10 μ g/ml of Hoechst 33342 in PBS. The coverslips were washed and mounted on glass slides. The cells were examined, and images were recorded and processed using the same equipment as described above for immunostaining.

To determine viability, the cells growing directly in Petri dishes were first photographed using a Nikon phase-contrast microscope (TMS) and a Nikon F-300 camera at $\times 10$ magnification. Thereafter, the cells were harvested and stained with 0.2% Trypan Blue in PBS, and the percentage of viable cells was calculated.

RESULTS

Cytochrome *c* release in Int 407 cells

In the light of NS398 inducing apoptosis via caspase 3 activation, we investigated whether this was preceded by cytochrome *c* release. Cells were fractionated and the cytosolic and mitochondrial fractions were evaluated for cytochrome *c* content. As seen in Figure 1(A), NS398 induced a release of cytochrome *c* to the cytosol that reached a peak at approx. 3–4 h, and simultaneously with the increase in the cytosol the cytochrome *c* levels decreased in the mitochondrial fraction. The membranes were re-probed with actin and porin respectively to determine equal protein loading. The timing of the release is in good agreement with our previous finding of significant caspase 3 activation after 4 h of NS398 treatment [10]. The release of cytochrome *c* on COX-2 inhibition was also confirmed by microscopic studies, where we stained the cells with a mitochondria-specific dye (Mitotracker Red) and cytochrome *c* antibody simultaneously (Figure 2). In the control cells, cytochrome *c* exhibited a scattered distribution that coincided with the staining of the mitochondria (Figure 2). In the NS398-challenged cells, on the other hand, the cytochrome *c* staining was diffuse, due to its leakage into the cytosol (Figure 2). Furthermore, addition of LTD₄ could, to a significant extent,

Figure 1 Cytochrome *c* release in fractionated Int 407 cells

Int 407 cells were treated with or without 100 μ M NS398 and/or 40 nM LTD₄, lysed, fractionated, separated by SDS/PAGE and immunoblotted. (A) The cytosolic and mitochondrial fractions of cells treated with NS398 for the indicated time periods were probed with an antibody against cytochrome *c* and, thereafter, stripped and re-probed with antibodies against actin and porin respectively. (B) The cytosolic fraction of cells treated with or without NS398 and/or LTD₄ for 3.5 h was probed with an antibody against cytochrome *c*, and re-probed for actin.

inhibit this release of cytochrome *c* as shown by both immunoblotting (Figure 1B) and microscopy (Figure 2).

Characterization of the involvement of other Bcl-2 family members in the apoptotic signalling of Int 407 cells

We have shown previously that Bcl-2 accumulates in the membrane fraction of these cells on stimulation with LTD₄ [10]. This accumulation is significant after 2 h and is still high after 5 h of LTD₄ stimulation. To elucidate which other players are involved in the cell-survival signalling of LTD₄, we evaluated cell lysates treated with LTD₄ as well as NS398 for the presence of the anti-apoptotic protein Bcl-X_L. In control cells, as well as those stimulated for up to 3 h, no Bcl-X_L could be detected even when the blots were overexposed (Figure 3A). It was found that the pro-apoptotic protein Bax was also not expressed in Int 407 cells (results not shown), whereas Bak, on the other hand, was expressed, but the levels of the protein were unaffected by LTD₄ as well as NS398 (Figure 3A). We then turned our attention to the pro-apoptotic protein Bad. Bad plays an important role in several signalling pathways via phosphorylation on distinct serine residues [21]. Using antibodies specific to the forms phosphorylated on Ser-112, Ser-136 and Ser-155 residues respectively, we evaluated cell lysates stimulated for different time points with

Figure 2 Cytochrome *c* release in Int 407 cells

Int 407 cells were grown on coverslips, treated with or without 100 μ M NS398 and/or 40 nM LTD₄ for 3.5 h and stained with the mitochondria-specific dye (Mitotracker Red). Thereafter, the cells were fixed, permeabilized and immunostained for cytochrome *c* using an Alexa 488-conjugated secondary antibody. Fluorescence micrographs of the cells are shown. The upper panels show untreated control cells. In the middle panels, the cells have been exposed to 100 μ M NS398 for 3.5 h. In the lower panels, cells were pretreated for 30 min with 100 μ M NS398 and then 40 nM LTD₄ was added and incubation was continued for 3 h.

Figure 3 Characterization of the involvement of other Bcl-2-family members in the apoptotic signalling of Int 407 cells

(A) Representative Western blot of cell lysates of Int 407 cells treated with 40 nM LTD₄ or 100 μ M NS398 for the indicated time periods, separated by SDS/PAGE, immunoblotted and probed with antibody against Bcl-X_L or Bak. A mouse myeloma cell lysate was used as positive control ('C') for Bcl-X_L and a human A431 cell lysate was used as positive control for Bak. (B) Cell lysates of Int 407 cells treated with 40 nM LTD₄ for the indicated time periods, separated by SDS/PAGE and immunoblotted. Western blot probed with antibodies against Bad phosphorylated at Ser-112 or Ser-155.

LTD₄ (Figure 3B). Although the phosphorylated forms of Bad were present, we could detect no change in their amounts on LTD₄ stimulation, and the levels remained unchanged after 24 h of stimulation (results not shown).

COX-2 inhibition causes activation of caspase 8 and cleavage of Bid

There is increasing evidence that the cleavage of Bid might play an important role even in non-death-receptor-mediated apoptosis [17,18]. Int 407 cells were treated with NS398 and the cell lysates were blotted with an antibody against Bid. As shown in Figure 4(A), the amount of total Bid (22 kDa) was found to decrease on NS398 treatment. When using an antibody that recognizes tBid [22], we could detect increasing amounts of this activated 15 kDa form of the protein (Figure 4A). After only 2 h, the level of tBid was significantly increased and the activation seemed to reach a peak at 4 h. The protease most commonly associated with cleavage of Bid is caspase 8, but since other players such as calpains have also been suggested [23], we studied the effect of NS398 treatment on caspase 8 activation. This was done using an antibody which, in our cells, detected the entire pro-caspase (approx. 55 kDa), as well as an activation-associated cleavage product that corresponds to 41 kDa. We conclude that caspase 8 was activated by treatment with NS398 (Figure 4B), in a time scale consistent with the appearance of tBid as seen previously (Figure 4A). To ensure equal protein loading, all the blots were

re-probed against actin, and the ratios between the densitometric values of tBid or caspase 8 and actin were calculated.

LTD₄ reduces Bid cleavage and caspase 8 activation

We have seen previously that LTD₄ has the ability to reduce apoptosis induced by NS398 [10], and to elucidate further this signalling pathway, we studied the effect of LTD₄ addition on the generation of tBid and cleavage of caspase 8. Intriguingly, treatment with LTD₄ could not only reduce the amount of Bid cleavage induced by NS398, but it also dramatically lowered the basal level of tBid (Figure 5A). The time scale of this effect was evaluated, and as can be seen in Figure 6(A), a significant reduction was detectable after 2 h of LTD₄ stimulation. The cleavage and activation of caspase 8 induced by NS398 was similarly decreased by co-treatment with LTD₄. The inhibitory effect of LTD₄ alone on Bid cleavage was reflected by a reduction in caspase 8 activation in intestinal epithelial cells stimulated with LTD₄ (Figure 5B), but in this case, the effect was easily detectable after 1 h stimulation (Figure 6C). The ability of LTD₄ to lower the level of activated caspase 8 and tBid below baseline was in both cases shown to be mediated by receptor-specific events, as it could be blocked with the LTD₄ receptor antagonist ZM198,615 (Figures 6B and 6D). All the blots were re-probed for actin, and the ratios between the densitometric values of activated tBid or caspase 8 and actin were calculated.

Caspase 3 activation is a downstream event to caspase 8 activation, tBid generation and release of mitochondrial cytochrome *c*

Many of the caspases can activate each other and also reinforce activation cascades through a type of positive-feedback loop. In the present study, we have seen that NS398 treatment induces activation of caspase 8 in Int 407 cells, but since we have detected previously caspase 3 activation after the inhibition of COX-2, we examined the role of these caspases in the apoptotic signalling pathway. Employing specific inhibitors of caspases 3 and 8, we treated the cells for 5 days with NS398, with or without the inhibitors. As seen from the nuclear staining in Figure 7(A), as well as the whole cell morphology in Figure 7(B), both these inhibitors have the capacity to block radically the apoptosis induced by NS398. We also performed Trypan Blue exclusion experiments to validate these results, and the inhibitory effect on apoptosis was confirmed to be highly significant for caspase 3 as well as caspase 8 (Figure 7C). In Figure 7(D), we studied the effect of caspase inhibition on the individual proteins in the cascade, and as shown, inhibition of caspase 3 had no effect on the NS398-induced activation of caspase 8 or Bid, and the release of cytochrome *c* to the cytosol was similarly unaffected. On the other hand, tBid generation as well as cytochrome *c* release were blocked by inhibition of caspase 8. The fact that no effect is seen on caspase 8 activation is not surprising, since the inhibitor does not interfere with its activation but rather its action, as seen with the effect on tBid and cytochrome *c*. In summary, these results suggest a string of events from NS398, via caspase 8 activation and tBid to cytochrome *c* release, all of which can be decreased by LTD₄.

DISCUSSION

COX-2 has been strongly suggested in the emergence and progression of colon cancer [24], and treatment with NSAIDs, which inhibit COX-2, lowers the incidence thereof. NSAIDs also

Figure 4 NS398-induced cleavage and activation of Bid and caspase 8

Int 407 cells were treated with or without 100 μ M NS398 for the indicated time periods, lysed, separated by SDS/PAGE and immunoblotted. (A) The upper panel depicts a representative

induce apoptosis in many colon-cancer cell lines [25], and we were able to show previously [10] that the COX-2-specific inhibitor NS398 induced apoptosis in intestinal epithelial cells. Even more intriguing was the fact that the inflammatory mediator LTD₄ could reduce the apoptosis induced by NS398. In view of this, we were interested in elucidating the signalling pathway of apoptosis induced by COX-2 inhibition, and how LTD₄ could influence it.

The pathways leading to apoptosis vary depending on the cell type and the situation. In some cases, the process is completely independent of mitochondrial involvement and occurs through a direct sequence of caspase-to-caspase activation. On the other hand, death-receptor-mediated pathways, as well as other apoptotic pathways, often proceed through cytochrome *c* release, via apoptosome assembly, to the activation of downstream executioner caspases [14]. Li et al. [26,27] showed that NS398 induces apoptosis via a cytochrome *c*-dependent pathway in colon-cancer as well as oesophageal-cancer cells. Therefore we were not surprised to find that the apoptotic response of Int 407 cells to NS398 was mediated by release of cytochrome *c* from mitochondria. Furthermore, this release was strongly decreased by the addition of LTD₄, indicating that LTD₄ exerts its anti-apoptotic effect upstream of or at the mitochondrial level. Although the exact mechanisms underlying the tight regulation of mitochondrial membrane integrity and release of apoptotic proteins such as cytochrome *c* are not clear, this regulation seems to be one of the main functions of the Bcl-2 family of proteins. We showed previously that LTD₄ induces membrane accumulation of Bcl-2 [10], providing a possible explanation for the anti-apoptotic effect of LTD₄ at the mitochondrial level. We also investigated the possibility that LTD₄ might influence Bcl-2 activity through phosphorylation events, but the phosphorylation status was unaffected. Next we proceeded to study some of the other family members.

The anti-apoptotic Bcl-X_L and its pro-apoptotic counterpart Bax have been attributed varying levels of importance in the emergence of colon cancer. In some colon-cancer cell lines, they have been found to be unaffected [12,28], whereas in other cases, absence of Bax abolished the apoptotic response to NSAIDs and Bcl-X_L expression was inhibited by the same treatment [29]. In the non-transformed intestinal epithelial cells we have studied, the former seems to be true as neither of the two is expressed. Another potentially important Bcl-2 family member in the context of colon carcinogenesis is the pro-apoptotic protein Bak. Bak has been shown to be down-regulated in transformed intestinal epithelial cells [30], and it exhibits decreased expression levels in tumours [31]. We could show that although Int 407 cells express Bak, the level was unaffected by treatment with NS398 or LTD₄.

There are three important serine-phosphorylation sites on the pro-apoptotic protein Bad, and phosphorylation of any of them leads to the cancellation of its pro-apoptotic function [21,32], due to the thereby induced interaction with 14-3-3 proteins, and sequestration in the cytosol [33]. Bad is phosphorylated on

Western blot of the full-length (22 kDa) Bid. The middle panel shows a representative Western blot of the activated form of Bid, tBid (15 kDa), and the panel below this shows the same blot re-probed against actin with the ratios between the densitometric values calculated. The molecular masses of the marker bands are shown on the right-hand side. The lower panel shows densitometric analysis of tBid levels and the data represent means $\pm$ S.E.M. for at least three separate experiments. **P* < 0.05, ***P* < 0.01. (B) The upper panel shows a representative Western blot of caspase 8, depicting the pro-caspase as well as an activation-associated cleavage product of 41 kDa. The molecular mass of the marker bands is shown on the right-hand side. The lower panel shows densitometric analysis of activated caspase 8 levels and the data represent means $\pm$ S.E.M. for at least three separate experiments. **P* < 0.05.

Figure 5 Effect of LTD₄ on NS398-induced cleavage and activation of Bid and caspase 8

Int 407 cells were treated with or without 100 μ M NS398 and/or 40 nM LTD₄ for 2 h as indicated, lysed, separated by SDS/PAGE and immunoblotted. (A) The upper panel shows a

Ser-112, Ser-136 and Ser-155 already in unstimulated cells. The pathways commonly implicated in these phosphorylation events are extracellular signal-regulated protein kinase 1/2 [34] and cAMP respectively [21]. Much to our surprise, although LTD₄ stimulation induces strong activation of extracellular signal-regulated protein kinase 1/2 in Int 407 cells [34], the levels of phosphorylated Bad protein remain unchanged upon LTD₄ stimulation. This indicates that the inhibitory effect of LTD₄ on programmed cell death is not mediated by an increase in the ratio of anti- to pro-apoptotic Bcl-2 family members at the mitochondrion, with the possible exception of Bcl-2 itself. This theory also fits well with the level of Bak remaining unchanged at the mitochondrial level on LTD₄ stimulation.

The activation of caspase 8 and, later, the cleavage of Bid, have previously been linked mainly to death-receptor-induced apoptosis. However, more evidence is now emerging that non-death-receptor-activated pathways of apoptosis also employ this system [17,18]. In the present study, we further contribute to this notion by showing that in non-transformed intestinal epithelial cells, the COX-2 inhibitor NS398 causes an activation of caspase 8. This activation reaches a significant level within 2 h of treatment, and immediately thereafter the pro-apoptotic protein Bid is cleaved and the resulting 15 kDa large tBid, which translocates to mitochondria and facilitates release of cytochrome *c*, is produced. The activation of caspase 8 in non-death-receptor-related situations has been suggested to be secondary to the activation of other executioner caspases, such as caspase 3, and thus simply a part of a positive feed-forward regulation of the apoptotic machinery [35]. However, evidence has also been shown of situations in which this event is truly the primary and thereby regulatory step of the process [36]. We believe that this is also the case with NS398-induced apoptosis of Int 407 cells, due to the fact that a significant increase in caspase 8 activation and Bid cleavage is seen before the first detectable increase in cytochrome *c* in the cytosol after 3 h. We have seen previously that caspase 3 is significantly activated 4 h after the addition of NS398, although it does not culminate until after 6–12 h [10]. Although it is possible that this activated caspase 3 might function to enhance further the activation of caspase 8, it is not the initiating step, since inhibition of caspase 3 through the addition of Z-DEVD-FMK did not inhibit caspase 8 and had no effect on NS398-induced activation of Bid and cytochrome *c* release. In contrast with the caspase 3 inhibition showing no effect, when caspase 8 was inhibited, tBid generation and cytochrome *c* release were completely blocked. It should be pointed out that both these inhibitors were highly effective in stopping apoptosis, and so inhibition of caspase 8 alone is sufficient to block the apoptosis-inducing function of NS398. The risk of unspecific or incomplete effects of inhibitors should always be considered, as has been done in this case. The ability of both caspase inhibitors to inhibit completely the apoptosis induced by NS398, even after prolonged exposure, together with the inhibitory effect of the caspase 8 inhibitor on Bid cleavage and cytochrome *c* release, is in favour of a satisfyingly specific action, even though complete specificity cannot be achieved.

representative Western blot of the activated form of Bid, tBid (15 kDa), and the panel below this shows the same blot re-probed against actin with the ratios between the densitometric values calculated. The lower panel shows densitometric analysis of tBid levels and the data represent means $\pm$ S.E.M. for at least three separate experiments. * P < 0.05, ** P < 0.01. (B) The upper panel shows a representative Western blot of caspase 8, depicting the activation-associated cleavage product of 41 kDa. The lower panel shows densitometric analysis of activated caspase 8 levels and the data represent means $\pm$ S.E.M. for at least three separate experiments. * P < 0.05.

Figure 6 Reduction in basal cleavage and activation of tBid and caspase 8 by LTD₄ receptor-mediated signalling

In (A) and (C), the cells were treated with or without 40 nM LTD₄ for the indicated time periods, whereas in (B, D), the cells were treated with or without LTD₄ and the CysLT₁ receptor antagonist ZM198,615 for 2 h. After termination of treatment, the cells were lysed, separated by SDS/PAGE and immunoblotted. (A) and (B) The upper panel shows a representative Western blot of the activated form of Bid, tBid (15 kDa), and the panel below this shows the same blot re-probed against actin with the ratios between the densitometric values calculated. The lower panel shows densitometric analysis of tBid levels and the data represent means $\pm$ S.E.M. for at least three separate experiments. $^{**}P < 0.01$. (C) and (D) The upper panel shows a representative Western blot of caspase 8, depicting the activation-associated cleavage product of 41 kDa. The lower panel shows densitometric analysis of activated caspase 8 levels and the data represent means $\pm$ S.E.M. for at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

From this, we conclude that the most probable sequence of events on COX-2 inhibition of intestinal epithelial cells is the activation of caspase 8, leading to cleavage of Bid and release of cytochrome *c* from mitochondrial, followed by activation of

caspase 3. It cannot of course be excluded that some part of the activation of caspase 3 seen on COX-2 inhibition is a direct consequence of caspase 8 activation, as typically seen in the so-called type 1 cells [37].

Figure 7 NS398-induced apoptosis is mediated through caspase 8, tBid and cytochrome *c*

(A) Representative fluorescence micrographs of Int 407 cells stained with Hoechst 33342 after 5 days of (from the left-hand side) no treatment, 100 μ M NS398, 100 μ M NS398 and 20 μ M caspase 3 inhibitor, Z-DEVD-FMK, and finally, 100 μ M NS398 and 20 μ M caspase 8 inhibitor, Z-IETD-FMK. (B) Representative micrographs of morphology of Int 407 cells after treatment as described in (A). (C) Cells treated as in (A) and (B) were stained with Trypan Blue and the percentage of viable cells was calculated. The values represent means $\pm$ S.E.M. for three separate experiments. * $P < 0.05$, ** $P < 0.01$. (D) Int 407 cells were treated for 3.5 h with 100 μ M NS398, 20 μ M Z-DEVD-FMK and 20 μ M Z-IETD-FMK as indicated, lysed, for cytochrome *c* detection fractionated into cytosol and mitochondria, separated by SDS/PAGE and immunoblotted. The representative Western blots of (from the top) activated caspase 8 (41 kDa), tBid (activated form of Bid, 15 kDa) and leakage of cytochrome *c* to the cytosolic fraction are shown. The blots were re-probed for actin to ensure equal protein loading. All the above results are representative of at least three separate experiments.

In our attempt to clarify the mechanisms underlying the ability of LTD₄ to counteract apoptotic effects induced by NS398, we evaluated which of these effects could be decreased by LTD₄ stimulation. The results showed that although events at the mitochondria cannot be excluded, such as the increased levels of Bcl-2 induced by LTD₄ stimulation, there is also a strong anti-apoptotic effect upstream of the mitochondria. First of all there

is an LTD₄-induced reduction in NS398-generated tBid. This is interesting since it has been suggested that a possible mode of regulation of Bid cleavage by caspase 8 is through phosphorylation of Bid by casein [38] or other kinases. Although this factor cannot be ruled out, the fact that the amount of caspase 8 activated by inhibition of COX-2 was considerably decreased by the addition of LTD₄, favours an effect at this level or further

upstream. Even more intriguing is the fact that we have now detected a level at which LTD₄ not only reduces the apoptotic effects induced by NS398, but also reduces the levels of activated caspase 8 and tBid below what is seen in untreated cells.

In summary, we have been able to elucidate further the pathway of apoptotic signalling employed by the COX-2-specific inhibitor NS398. Caspase 8 is activated, in turn causing cleavage of Bid and generation of tBid, which translocates to the mitochondria, leading to the release of cytochrome *c* into the cytoplasm. Furthermore, we have shown that the inflammatory mediator LTD₄ not only can be confirmed to reduce apoptosis induced by NS398, but also has a negative impact on the induction of apoptosis inherent to the epithelial cells. This, together with our previous finding of an increased rate of proliferation in intestinal epithelial cells on LTD₄ treatment [34], provides the inflammatory mediator with dual modes causing a large impact on the neoplastic potential of the cells.

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