

# Cyclooxygenase-2 up-regulation after FLAP transfection in human adenocarcinoma cell line HT29 cl.19A

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**Abstract** Five-lipoxygenase-activating protein (FLAP) is usually described as an essential protein to activate the leukotriene (LTs) synthesis via the 5-lipoxygenase pathway. In the enterocyte model HT29 cl.19 A cell line, 5-lipoxygenase metabolism was found despite the lack of FLAP expression. Therefore HT29 cl.19A represents an original mammalian model to study FLAP-dependent leukotriene synthesis. In FLAP cDNA transfected HT29 cl.19 A cells, FLAP expression led to an increase in cyclooxygenase pathway products (mainly PGE<sub>2</sub>) without an increase in 5-lipoxygenase metabolism. This increase in PGE<sub>2</sub> synthesis was associated with a cyclooxygenase-2 up-regulation in comparison to untransfected HT29 cl.19A cells. These results suggest a possible interaction between the two major pathways of arachidonic acid metabolism.

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**Key words:** Cyclooxygenase-2; 5-Lipoxygenase; Five-lipoxygenase-activating protein; HT29 cl.19A cell line

## 1. Introduction

Arachidonic acid (AA) metabolites named eicosanoids originate from both cyclooxygenase (COX) and lipoxygenase (LO) pathways. They are involved in the mediation of various inflammatory disorders such as gastrointestinal diseases [1–4]. 5-Lipoxygenase (5-LO) catalyzes both the first step in oxygenation of AA to produce 5(*S*)-hydroperoxyeicosatetraenoic acid (5-HPETE) and secondly the production of LTA<sub>4</sub> from 5-HPETE. LTA<sub>4</sub> is the precursor of potent biological effectors such as LTB<sub>4</sub> or sulfidopeptidoleukotrienes (LTC<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub>) [3,4]. A rise in intracellular calcium concentration leads to the activation and translocation of 5-LO to the nuclear cell membrane [5–8]. An 18-kDa integral membrane ‘docking’ protein called FLAP (five-lipoxygenase-activating protein), facilitates the transfer of substrate to 5-LO (AA or 5-HPETE) resulting in increased LT synthesis [4,6,9–13]. Cyclooxygenases (COX) are the key enzymes which catalyze the committed step in the formation of prostaglandins (PGs) and thromboxanes (TXs) [1]. There are two COX isoenzymes: COX-1 expressed constitutively in most tissues and organs to mediate house-keeping functions and COX-2 which is defined as the inducible isoform [14,15] that is normally absent from cells but expressed transiently in response to growth factors, cytokines, mitogens, etc. [16]. Abnormal COX-2 up-regulation is involved in the increase in PGE<sub>2</sub> synthesis observed in colon cancer, and this up-regulation is an important early event in colon carcinogenesis [17].

The permanently differentiated human intestinal epithelial cell line HT29 cl.19A, deriving from the human undifferenti-

ated colonic adenocarcinoma cell line HT29 after treatment with sodium butyrate [18], is routinely used as an in vitro model of colon carcinoma [19]. Using HT29 cl.19A cells, we have demonstrated that the enterocyte could be both target and producer of AA metabolites through the LO and COX pathways [20]. These cells possess interesting characteristics: (i) HT29 cl.19A have abnormal COX-2 expression which was called ‘constitutive’ and greatly enhanced in the total absence of fetal bovine serum (FBS) or exogenous inducers [21]. Moreover, this COX-2 up-regulation was associated with a resistance to apoptosis [22]. (ii) They express 5-LO activity (mRNA and protein) in the absence of FLAP, which was correlated with a synthesis of metabolites such as 5-HETE, LTB<sub>4</sub> and its isomers [20]. Nevertheless, the absence of FLAP led to limited metabolite synthesis, especially for LTB<sub>4</sub> without peptidoleukotriene synthesis [20]. The absence of FLAP expression has also been observed in other enterocyte cell lines such as HT29-18 and HT29-18.C1 cells or in CaCo-2 [23,24]. Therefore, the HT29 cl.19A cell line represents, by the presence of 5-LO and LTA<sub>4</sub> hydrolase expression, an interesting model for studying FLAP-dependent LT synthesis. We therefore transfected this cell line using a FLAP cDNA recombinant mammalian expression vector.

In positive FLAP expressing transfected clones such as the HTF C31 cell line, an increase in cyclooxygenase pathway products such as PGE<sub>2</sub> was observed, without any increase in 5-lipoxygenase pathway metabolites. Moreover, the results suggested that an up-regulation of COX-2 expression could be involved in the increase of PGE<sub>2</sub> synthesis. This surprising data could indicate a possible relationship between the two major AA pathways leading to an increase in prostaglandin production.

## 2. Materials and methods

### 2.1. Chemicals

Cell line HT29 cl.19A was obtained from M. Labois (INSERM U.239, Paris, France). Culture medium was from Eurobio, fetal bovine serum (FBS) was from BioWitaker. Geneticine (G418), trypsin-EDTA and Superscript Preamplification System were from Gibco-BRL. RNazol was from Bioprobe Systems. Taq polymerase was from Perkin-Elmer. Primers used in polymerase chain reaction (PCR) were from Appligene. [1-<sup>14</sup>C]AA, enhanced chemiluminescence detection system ECL and Hyperfilm ECP were purchased from Amersham. Acetylsalicylic acid (Aspirin), indomethacin, calcium ionophore A23187, standards used in the reverse phase high pressure liquid chromatography (RP-HPLC) and for gas chromatography mass spectrometry (GC-MS) were all from Sigma. The PGE<sub>2</sub> and LTB<sub>4</sub> enzyme immunoassay (EIA), human prostaglandin H synthase-2 monoclonal antibody and REV 5901 were from Cayman. Complete (anti-protease and EDTA cocktail), T4 DNA ligase, restriction endonucleases and RNase DNase free were from Boehringer-Mannheim. Nitrocellulose membrane Optiran BA-S83 reinforced was from Schleicher and Schuell. The pcDNA3(+) vector was from

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Invitrogen. Meloxicam, SC-58125 and L745-337 were a gift from Dr. M. Pairet (Boehringer-Ingelheim). MK-886 (L-663,536,3-[1-(4-chlorobenzyl)-3*t*-butyl-thio-*t*-isopropyl-indol-2-yl]-2,2-dimethylpropanoic acid) was from Calbiochem (La Jolla, CA, USA).

## 2.2. Cell culture

The HT29 cl.19A cell line was cultured as described before [20] in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% FBS and 4 mM glutamine at 37°C in a humidified 6% CO<sub>2</sub>, 94% room air atmosphere. Cells were routinely screened for mycoplasma contamination using Chen's method [25]. Transfected cell lines were grown in the presence of 600 µg/ml G418 and used between passages 15 and 35 after transfection.

## 2.3. Plasmid construction

The pcDNA3/FLAP cDNA expression plasmid was constructed by isolating the 843-bp *Eco*RI fragment containing the entire human FLAP cDNA from pBluescript SK(–) plasmid (gift from Dr. J.F. Evans, Merck-Frosst, Center for Therapeutic Research, Canada) and ligating it into the *Eco*RI site of pcDNA3(+) expression vector. This 843-bp fragment contained the open reading frame for human FLAP gene. This construction placed the cDNA downstream from and under the transcriptional control of the cytomegalovirus immediate early response promoter. The sense orientation of this new construction was confirmed by restriction endonuclease digestion (*Bam*HI and *Bst*EII). The pcDNA3(+) expression vector contained the gene-encoding neomycin resistance which enabled selection for the presence of this plasmid in mammalian cells using the antibiotic G418.

## 2.4. Transfection

For electroporation, cells growing in the mid-log phase were trypsinized, washed, pelleted by centrifugation and resuspended in poration medium (standard culture medium/0.01 M glucose/0.01 M DDT) at a density of 10<sup>6</sup> cells/ml. In an electroporation cuvette, 20 µg of plasmid (pcDNA3(+)) (vector control) or pcDNA3(+)/FLAP cDNA were added to 400 µl of cell suspension and cells were then electroporated, using Easyject Plus electroporator (Equibio, Angleur, Belgium) set at 0.2 kV and 1050 µF. Then, cells were immediately suspended in 5 ml of culture medium and 48 h later selection was begun in G418 at 600 µg/ml. G418 resistant cells were subcultured by trypsinization and seeded at 5 × 10<sup>2</sup> per ml into 130-mm dishes. Isolated G418 resistant clones (pcDNA3(+)) or pcDNA3(+)/FLAP cDNA transfected cells were individually transferred, subcultured and amplified separately from 96-well dishes to 75-cm<sup>2</sup> plastic flasks.

## 2.5. Human FLAP recombinant baculovirus preparation

As previously described [26], the recombinant transfer vectors pBac PAK8-FLAP and pBac PAK8-5-LO were constructed by isolating the *Eco*RI fragment containing the entire human FLAP cDNA (843 bp) or the human 5-LO cDNA (2497 bp), respectively, obtained from pBluescript SK(–) or pUC13 plasmids (gift from J. Evans, Merck-Frosst Center for Therapeutic Research, Quebec, Canada). As described by Clontech, we isolated, purified, selected (specific RT-PCR mRNA expression and protein Western blotting) and amplified pure recombinant baculovirus stocks named, respectively, HFLAP-C1 and H5LO-C4. As described [26], monolayers of Sf9 cells (8 × 10<sup>6</sup>) in 175-cm<sup>2</sup> flasks, were inoculated with either H5LO-C4 alone or with H5LO-C4 and HFLAP-C1 at a multiplicity of infection (M.O.I.) of five for each virus. Sf9 cells were harvested 48 h post-infection and the exogenous unlabelled AA (0.5 mM) metabolism was monitored as described below.

## 2.6. RT-PCR analysis

Total cellular RNA was isolated with an RNA extraction kit (RNAzol) [27] and 2 µg were reverse transcribed into cDNA using Superscript Preamplification System. Target cDNA was amplified under the conditions previously described [20] using 10 µl of the reverse transcribed cDNA, 20 pmol of sense (5'–3') and antisense (3'–5') primers (Table 1), 0.2 mM dNTP, MgCl<sub>2</sub> and 1 U Taq polymerase in 1 × PCR buffer. The primers were designed to be RNA-specific [9,20,28–30]. The mixture was overlaid with mineral oil and amplified in a thermal cycler (Crocodyl II, Appligene) under the following conditions: (i) 94°C for 45 s, 62°C for 45 s, and 72°C for 1 min, for 30 cycles for 5-LO, FLAP, COX-1; and (ii) 95°C for 2 min, for 1 cycle, and 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min,

for 35 cycles for COX-2. With standard negative and positive controls supplied in the Superscript Preamplification System [20] additional positive controls were included, using COX-1 and COX-2 cDNAs (gifts from Dr. T. Hla, Department of Molecular Biology, Holland Laboratory, Rockville, USA) and FLAP cDNA (gift from Dr. J.F. Evans, Merck-Frosst Center for Therapeutic Research, Quebec, Canada). PCR products were extracted, visualized and analyzed by electrophoresis [20]. The products resulting from PCR were verified by digestion (Table 1), under previously described conditions [20].

## 2.7. COX-2 Western blot analysis

The cell membrane protein fractions were prepared as described [31] and 25 µg of protein was submitted to SDS-polyacrylamide gel (12%) under reducing conditions (5% β-mercaptoethanol), and electrotransferred onto nitrocellulose membrane Optiran BA-S83 reinforced NC 0.2 µm (Schleicher and Schuell). Western blot analyses [32] were carried out using mouse anti-human prostaglandin H synthase-2 monoclonal antibody (Cayman Chemical) at 1:1000 dilution for 1 h at 37°C. Nitrocellulose membrane was incubated with anti-mouse IgG conjugated to horseradish peroxidase at 1:2000 dilution for 1 h at 37°C. The signal was revealed using enhanced chemiluminescence detection system ECL, and the membrane was exposed to Hyperfilm according to the manufacturer's instructions (Amersham).

## 2.8. Subcellular FLAP and 5-LO localization

Freshly trypsinized cells (HT29 cl.19A and HTF C31) were resuspended in lysis buffer (15 mM Tris-HCl, pH 7.5, 2 mM MgCl<sub>2</sub>, 1 × Complete solution, a protease and EDTA cocktail). Cells were lysed in a dry ice ethanol bath by sonication using a Bioblock Scientific Vibra Cell 72434 (4 × 15 s at a power setting of 60%). Sonicates were centrifuged (12000 × g, 10 min, 4°C) and the 12000 × g pellet was referred to as the nuclear fraction. The resulting supernatants were centrifuged (100000 × g, 60 min, 4°C). The 100000 × g supernatant (referred to as cytosol), the corresponding pellet (referred to as cellular membranes) and the 12000 × g pellet were evaluated for protein concentration by the Lowry method [33] using BSA as a standard. Each fraction protein (25 µg) was submitted to SDS-PAGE and immunoblot analysis as described for COX-2. Western blots were carried out using 1:1000 dilution for 1 h at 37°C of human FLAP polyclonal rabbit antiserum designated H4TB6 (gift by Dr. P. Vickers, Merck-Frosst Center for Therapeutic Research, Quebec, Canada) or using 1:1000 dilution for 1 h at 37°C of human 5-LO polyclonal rabbit antiserum designated L-859 S8 (gift by Dr. C. Creminon, CEA, Service de Pharmacologie et d'Immunologie, DRIPP, Centre d'Etude Saclay, France). Nitrocellulose membrane was incubated with anti-rabbit IgG conjugated to horseradish peroxidase at 1:2000 dilution for 1 h at 37°C. The signal was revealed using ECL as described before.

## 2.9. Immunocytochemistry

Freshly trypsinized cells were seeded onto poly-L-lysine coated glass coverslips in 6-well plates. For interleukin-1β (IL-1β) dependent protein expression assay, cells were analyzed 18 h after addition of 10 ng/ml of IL-1β. For subsequent immunofluorescent labelling of COX-2 protein, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. The non-specific Fc-binding sites were blocked by incubation of all coverslips with 3% (w/v) BSA in PBS, pH 7.4, for 30 min before incubation for 4 h with mouse anti-human prostaglandin H synthase-2 monoclonal antibody (1:100 in PBS/3% (w/v) BSA), or with mouse preimmune serum (1:100 in PBS/3% (w/v) BSA) as control. After washing three times with PBS, cells were labelled for 1 h with FITC-conjugated goat anti-mouse IgG (1:100 in PBS/3% (w/v) BSA/0.02% (w/v) Evans blue), washed again and mounted with Immu-mount (Shandon).

## 2.10. HT 29 cl.19A arachidonic acid metabolism

After three weeks of culture, 55 × 10<sup>6</sup> cells were trypsinized, washed twice with 0.05 M sodium phosphate buffer, pH 8.2, resuspended in 500 µl of the same buffer containing 2 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> and preincubated for 5 min at 37°C. Calcium ionophore A23187 (0.5 µg) and exogenous [<sup>14</sup>C]AA (1.5 µCi, 54 µM) were added in an ethanolic solution that never exceeded 0.4% and did not affect AA metabolism. The same experiments were performed (i) on HT29 cl.19A cells; (ii) on HTF C31 cells which is a representative clone among those expressing FLAP protein; (iii) on HTF C31 cell mono-

Table 1

Oligonucleotides used for amplification of the mRNA species studied, restriction endonucleases used for PCR product verification and expected PCR product size before and after restriction

mRNA species	5' Primers	3' Primers	PCR products (bp)	Restriction endonucleases and fragment sizes (bp)
COX-1	TGC CCA GCT CCT GGC CCG CCG CTT	GTG CAT CAA CAC AGG CGC CTC TTC	303	<i>Tru91</i> (79 and 224)
COX-2	TTC AAA TGA GAT TGT GGG AAA ATT GCT	AGA TCA TCT CTG CCT GAG TAT CTT	305	<i>HhaI</i> (92 and 213)
FLAP	ATG GAT CAA GAA ACT GTA CGC	ATG AGA AGT AGA GGG GGA GAT G	479	<i>BstEII</i> (181 and 298)

All primers are displayed 5' to 3'.

layers preincubated for 8 h at 37°C with 10 ng/ml of IL-1 $\beta$ , a classical COX-2 gene expression inducer; (iv) on HTF C31 cells preincubated for 30 min at 37°C with 10<sup>-5</sup> M aspirin or 5  $\times$  10<sup>-5</sup> M indomethacin, non-selective COX-1 and COX-2 inhibitors, with 10<sup>-5</sup>–10<sup>-8</sup> M meloxicam, a selective COX-2 inhibitor, with 10<sup>-7</sup> M SC-58125 or 10<sup>-7</sup> M L745-337 which are selective COX-2 inhibitors; (v) on HTF C31 cells preincubated for 5 min with 10<sup>-6</sup> M MK-886 and 10<sup>-6</sup> M REV-5901, LT synthesis inhibitors; (vi) on HTF C1 and HTF C11, two other positive FLAP expressing clones; (vii) on HT29 cl.19A cells transfected by pcDNA3(+) (vector control). After 15 min at 37°C, the reaction was stopped by the addition of 500  $\mu$ l of methanol containing 50 ng/ml of PGB<sub>2</sub> as internal standard. Exogenous [1-<sup>14</sup>C]AA metabolism was analyzed by RP-HPLC using a tertiary methanol-acetonitrile-water gradient, as previously described [20,34] and using 5-, 12-, 15-HETEs, PGE<sub>2</sub>,  $\Delta$ 6-*trans* LTB<sub>4</sub>,  $\Delta$ 6-*trans*-12 epi LTB<sub>4</sub>, LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub> and 12-hydroxyheptadecatrienoic acid (12-HHT) as synthetic standards. Prior RP-HPLC analysis of [1-<sup>14</sup>C]AA metabolism, the primary COX pathway derivatives (PGE<sub>2</sub>, PGD<sub>2</sub>, PGB<sub>2</sub>, TXB<sub>2</sub>, 6-keto-PGF<sub>1 $\alpha$</sub> , PGF<sub>2 $\alpha$</sub> ) were identified by GC-MS analysis [35] after incubation with 1 mM unlabelled AA.

#### 2.11. Enzyme immunoassay (EIA)

HT29 cl.19A, HTF C31 and vector control cells were cultured as described and the daily changed medium was centrifuged at 1000  $\times$  g for 5 min at 4°C, and stored until analysis at -80°C. The amount of PGE<sub>2</sub> released by cell monolayers was measured by a commercially available EIA kit according to the manufacturer's instructions (Cayman) using serial dilutions of previously conditioned medium. The amount of LTB<sub>4</sub> produced by cell monolayers was measured by a commercially available EIA kit according to the manufacturer's instructions (Cayman) using serial dilutions of previously conditioned medium obtained from unstimulated cells (endogenous AA, no calcium ionophore, no calcium) or from stimulated cells incubated for 20 min with calcium ionophore A23187 (1  $\mu$ M) in the presence of CaCl<sub>2</sub> (2 mM), MgCl<sub>2</sub> (0.5 mM), using endogenous AA.

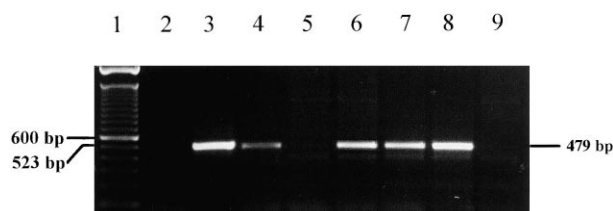


Fig. 1. RT-PCR analysis of FLAP mRNA. Total RNA extracted from cell monolayers was reverse transcribed, amplified by PCR using specific primers and PCR products were analyzed by electrophoresis on 0.8% agarose gel. Lane 1: Molecular weight ladders: 100 bp DNA ladder; lane 2: negative control: cDNA from chloramphenicol acetyl transferase mRNA with FLAP primers (no band); lane 3: positive control cDNA from chloramphenicol acetyl transferase with corresponding primers (band at 523 bp); lane 4: cDNA FLAP positive control (band at 479 bp); lane 5: mRNA from HT29 cl.19A cells; lanes 6–8: mRNA from HTF C1, C11 and C31 cells; lane 9: mRNA from HT29 cl.19A cells transfected by pcDNA3 (vector control).

### 3. Results

#### 3.1. RT-PCR analysis

HT29 cl.19A and HTF C31 cells exhibited a positive RT-PCR signal for COX-1 and COX-2 (data not shown). A FLAP positive signal corresponding to a specific FLAP mRNA expression could be detected only in positive FLAP expressing clone of transfected cells (HTF C1, C11 or C31) and, as described [20], no positive signal was observed for untransfected HT29 cl.19A cells or for vector control cells (Fig. 1). In each case, when we observed a specific band at the same level as expected and as the amplified control cDNA, we verified the nature of the amplification products by digestion with specific restriction enzymes (Table 1). In each case, the same fragments for control cDNAs or for amplification products were obtained.

#### 3.2. FLAP expression in baculovirus-Sf9 cell system

The chromatogram profiles demonstrated that uninfected Sf9 cells did not possess any arachidonic acid metabolism. Sf9 cells coinfecting with HFLAP-C1 and H5LO-C4 generated high levels of both 5-HPETE and LTB<sub>4</sub> isomers (data not shown) compared to cells simply infected with H5LO-C4. In addition to allowing efficient use of exogenous arachidonic acid by 5-lipoxygenase, FLAP also affected, as previously described [10], the ratio of metabolites synthesized by activation of LTA<sub>4</sub> synthase activity. The ratio of 5-HPETE/LTB<sub>4</sub> isomers was approximately 3:1 for cells expressing 5-lipoxygenase alone and 1:1 for cells expressing 5-lipoxygenase and FLAP (data not shown).

#### 3.3. Transfection of HT29 cl.19A cells

Using RT-PCR analysis, Western blot and RP-HPLC profile, 10 positive FLAP expressing clones (named HTF C1, 4, 9, 10, 11, 18, 23, 26, 31, 32) were identified from 50 initial clones and expanded (Figs. 1–4). Pure isolated clones of pcDNA3(+)

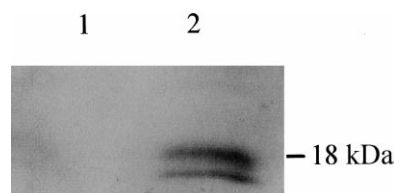


Fig. 2. Western blot analysis of FLAP protein. Western blot analysis of COX-2 was carried out using human FLAP polyclonal antibody (H4TB6) and anti-mouse IgG conjugated to horseradish peroxidase. The signal was revealed using enhanced chemiluminescence detection system ECL (see Section 2). Lane 1: 25  $\mu$ g protein from HT29 cl.19A cells; lane 2: 25  $\mu$ g protein from HTF C31 cells. Position of molecular weight ladders is indicated by arrows.

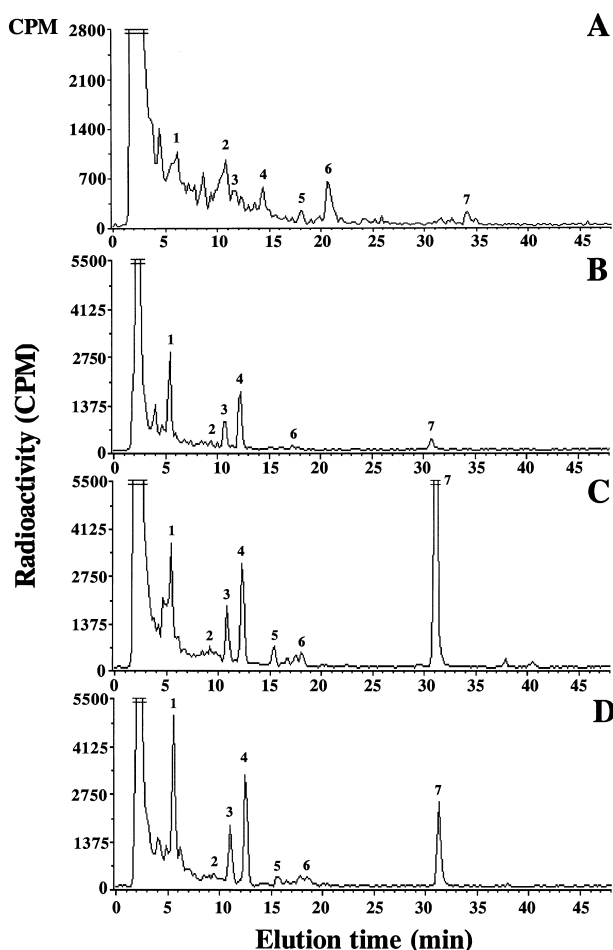


Fig. 3. Reverse-phase HPLC chromatogram of exogenous  $[1-^{14}\text{C}]$ arachidonic acid metabolism. Freshly trypsinized cells ( $55 \times 10^6$ ) HT29 cl.19A cells (A), HTF C1 cells (B), HTF C11 cells (C) and HTF C31 cells (D) were incubated for 15 min at  $37^\circ\text{C}$  in the presence of  $[1-^{14}\text{C}]$ AA (1.5  $\mu\text{Ci}$ , 3 mM). The metabolites synthesized were analyzed by RP-HPLC (see Section 2). Metabolites identified were:  $\text{PGE}_2$  (peak 1),  $\text{LTB}_4$  and 6-*trans*- $\text{LTB}_4$  isomers (peak 2), 12-HHT (peak 3), compound X (peak 4), 15-HETE (peak 5), 5-HETE (peak 6) and AA (peak 7). The data presented are representative of four experiments.

transfected cells (vector control) were obtained. Therefore, the results of our studies using the representative HTF C31 clone are principally presented here.

### 3.4. COX-2 protein expression

When HTF C31 cells were analyzed by Western blot, specific COX-2 protein expression was detected in the form of triplet at 74-kDa, 72-kDa and 66-kDa bands. At the same time, we observed two specific COX-2 protein bands at 74 kDa and 66 kDa in HT29 cl.19A cells (Fig. 5). Semi-quantitative evaluation of protein corresponding band by measurement of absorbance with Densylab Ver. 2.05 software (Bio-probe system) showed that HTF C31 clone synthesized about 2.5–4.5 times more protein than untransfected cells, and that the 74-kDa band was the major enzyme form produced by HTF C31 clone.

Results obtained from immunocytochemistry studies conducted on confluent monolayers showed that no immunofluorescence could be detected in control cells incubated

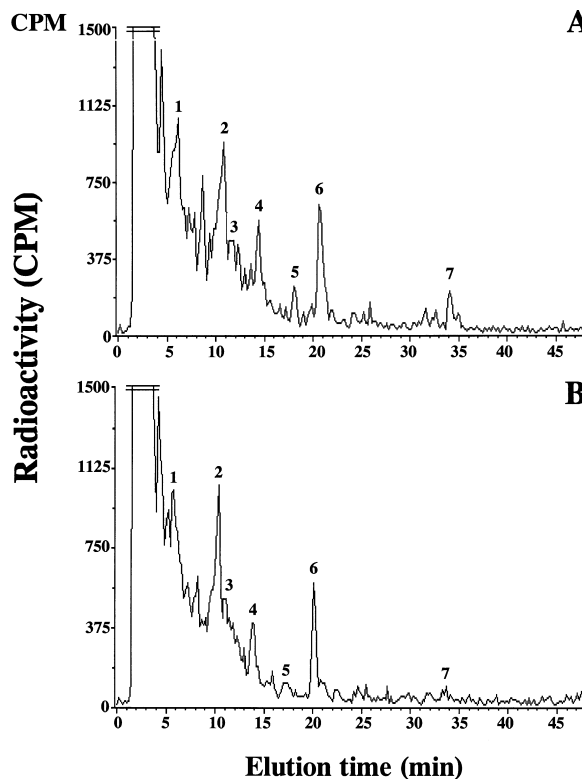


Fig. 4. Reverse-phase HPLC chromatogram of exogenous  $[1-^{14}\text{C}]$ arachidonic acid metabolism. Freshly trypsinized cells ( $55 \times 10^6$ ) HT29 cl.19A cells (A) and HT29 cl.19A pcDNA3 transfected cells (vector control) (B) were incubated for 15 min at  $37^\circ\text{C}$  in the presence of  $[1-^{14}\text{C}]$ AA (1.5  $\mu\text{Ci}$ ). The metabolites synthesized were analyzed by RP-HPLC (see Section 2). Metabolites identified were:  $\text{PGE}_2$  (peak 1),  $\text{LTB}_4$  and 6-*trans*- $\text{LTB}_4$  isomers (peak 2), 12-HHT (peak 3), compound X (peak 4), 15-HETE (peak 5), 5-HETE (peak 6) and AA (peak 7). The data presented are representative of four experiments.

with buffer or mouse preimmune serum (Fig. 6A). Specific immunofluorescence could be detected in a few untransfected HT29 cl.19A and vector control cells (Fig. 6B and C) and was increased in HTF C31 cells (Fig. 6D). After IL- $1\beta$  incubation, the same fluorescence could be detected in a larger portion of HTF C31 cells (data not shown).

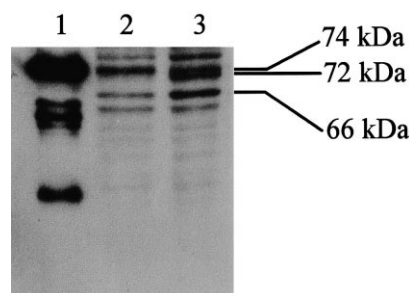


Fig. 5. Western blot analysis of COX-2 protein. Western blot analysis of COX-2 was carried out using human prostaglandin H synthase-2 monoclonal antibody and anti-mouse IgG conjugated to horseradish peroxidase. The signal was revealed using enhanced chemiluminescence detection system ECL (see Section 2). Lane 1: Purified sheep COX-2 (3  $\mu\text{g}$ ); lane 2: 25  $\mu\text{g}$  protein from HT29 cl.19A cells; lane 3: 25  $\mu\text{g}$  protein from HTF C31 cells. Position of molecular weight ladders is indicated by arrows.

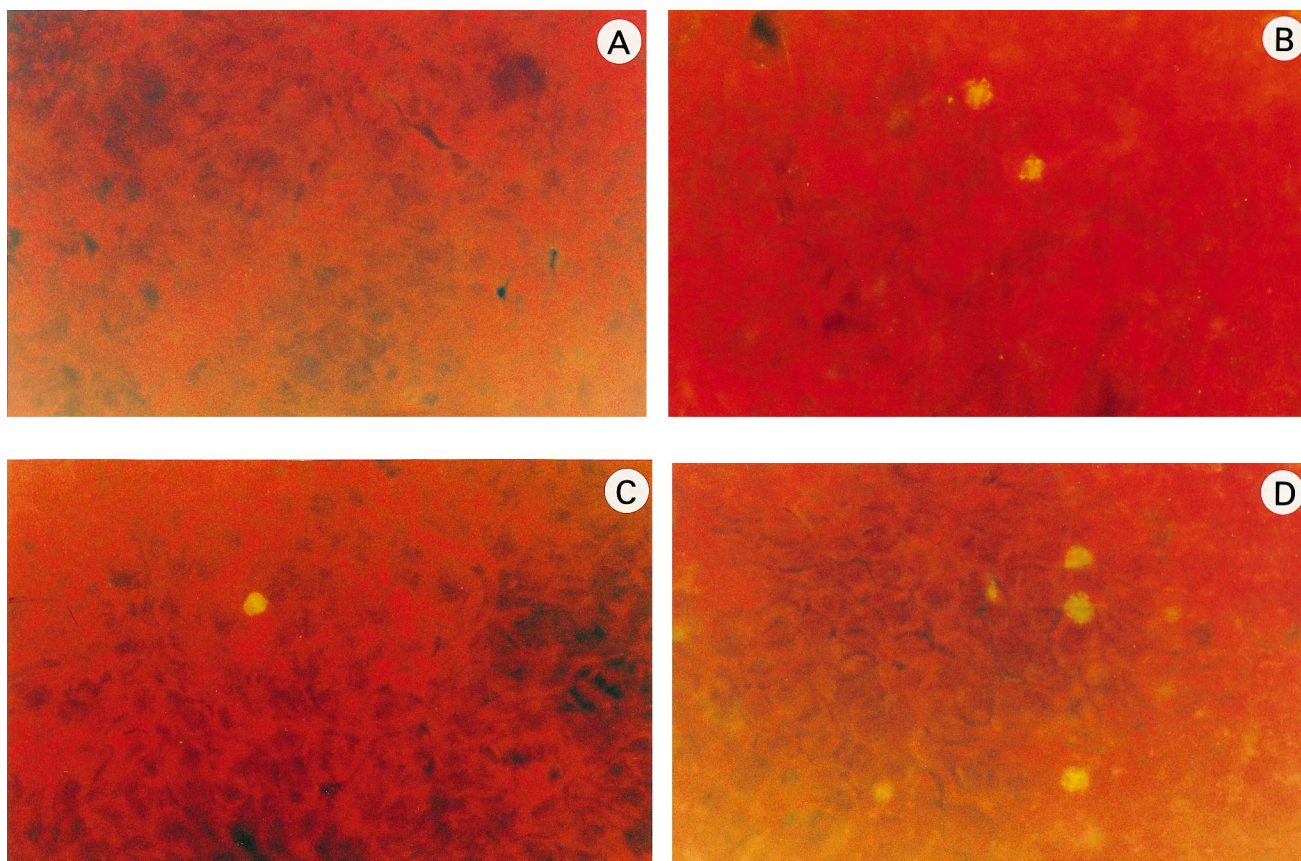


Fig. 6. Immunocytochemical detection of COX-2 protein. Subsequent immunofluorescent labelling of COX-2 protein was done on fixed and permeabilized cell monolayers. The non-specific Fc-binding sites were blocked with 3% (w/v) BSA, before incubation with mouse human prostaglandin H synthase-2 monoclonal antibody, or with mouse pre-immune serum as control (A). Cells were labelled with FITC-conjugated goat anti-mouse IgG and stained with Evans blue. Immunofluorescence pictures were taken with a Nikon microscope OPTIPHOT-2 (magnification 500 $\times$ ). B: HT29 cl.19A cells; C: HT29 cl.19A pcDNA3 transfected cells (vector control); D: HTF C31 cells.

### 3.5. Subcellular FLAP and 5-LO localization

Western blot analysis showed that FLAP was absent from all cellular fractions derived from HT29 cl.19A cells (data not shown). In HTF C31 cells, a positive FLAP signal was principally observed in the nuclear fraction and for a slight part in cytoplasmic fraction (data not shown). A 5-LO positive signal was observed in the cytoplasmic fraction in both HT29 cl.19A and HTF C31 cells (data not shown).

### 3.6. Arachidonic acid metabolites generated by stimulated cells

The HT29 cl.19A cells metabolite profile demonstrated as previously described [20] that the major 5-LO pathway products were 5-HETE (peak 6, Fig. 3A, Fig. 4A), LTB<sub>4</sub> and its  $\Delta 6$ -trans-LTB<sub>4</sub> isomers which were eluted in an unresolved broad peak (peak 2, Fig. 3A, Fig. 4A). 15-HETE was also detected (peak 5, Fig. 3A, Fig. 4A). We also identified a COX pathway derivative by the fact that its synthesis largely decreased in the presence of COX specific inhibitors. However, we could not identify this compound as being one of the primary COX pathway derivatives. This was then named compound X (peak 4, Fig. 3A, Fig. 4A). The same results were obtained for vector control pcDNA3(+) transfected cells (Fig. 4B) and for pcDNA3/FLAP negative clones (no expression of FLAP mRNA). In the presence of  $10^{-5}$  M aspirin, we also observed an increase in 15-HETE production.

With HTF C31 cells, no significant changes were observed

concerning the LO metabolites (Fig. 3D). We did not observe any increase in 5-LO metabolites. In fact, the most important event observed for transfected cells was a quantitative and qualitative difference concerning the COX pathway. We particularly noticed an increase in PGE<sub>2</sub> synthesis which appeared as the major peak (peak 1, Fig. 3D), compound X (peak 4, Fig. 3D), and 12-HHT (peak 3, Fig. 3D). Similar profiles were observed with other positive FLAP expressing clones such as HTF C1 or HTF C11 (Fig. 3B and C).

When HTF C31 cells were preincubated with meloxicam, the inhibition of global COX metabolite synthesis was  $100 \pm 5\%$  ( $n=4$ ) for  $10^{-5}$  M,  $58 \pm 5\%$  for  $10^{-6}$  M ( $n=4$ ),  $45 \pm 4\%$  for  $10^{-7}$  M ( $n=4$ ) and  $32 \pm 4\%$  for  $10^{-8}$  M ( $n=4$ ). In the presence of  $10^{-7}$  M of SC-58125 or L745-337, inhibition of COX metabolite synthesis was  $52 \pm 4\%$  ( $n=4$ ) and  $70 \pm 6\%$  ( $n=4$ ), respectively (data not shown). When HTF C31 cell monolayers were preincubated with 10 ng/ml of IL-1 $\beta$ , the metabolic profile showed a large increase in COX metabolite synthesis,  $+105 \pm 10\%$  ( $n=4$ ), without qualitative chromatogram profile changes (data not shown).

When HT29 cl.19A or HTF C31 cells were preincubated with  $10^{-6}$  M MK-886 ( $n=4$ ) or  $10^{-6}$  M REV-5901 ( $n=4$ ), we did not observe any changes in COX metabolite synthesis (data not shown). Under these conditions, we observed a slight decrease in LTB<sub>4</sub> and isomer synthesis in HTF C31 cells.



Table 2  
EIA analysis of PGE<sub>2</sub> and LTB<sub>4</sub> production

Eicosanoid	HT29 cl.19A cells	HTF C31 cells	Vector control cells
PGE <sub>2</sub> (pg/10 <sup>6</sup> cells)			
Unstimulated cells	9.0 ± 0.5	17.6 ± 0.7	8.5 ± 0.6
IL-1β	17.0 ± 0.7	24.5 ± 0.8	17.4 ± 0.5
LTB <sub>4</sub> (pg/10 <sup>6</sup> cells)			
Unstimulated cells	3.55 ± 0.17	2.55 ± 0.19	3.70 ± 0.15
A23187	3.94 ± 0.21	3.60 ± 0.16	4.00 ± 0.20

PGE<sub>2</sub> synthesis was evaluated in cell monolayers in the total absence of stimulation by ionophore A23187 and exogenous AA, in the presence or in the absence of 10 ng/ml IL-1β stimulation. The amount of LTB<sub>4</sub> produced by cell monolayers was measured in unstimulated cells (endogenous AA, no calcium ionophore, no calcium) or from stimulated cells incubated for 20 min with calcium ionophore A23187 (1 μM) (CaCl<sub>2</sub>, MgCl<sub>2</sub>, endogenous AA). Values are expressed as the mean ± S.E. (*n* = 3 experiments, *P* < 0.05).

### 3.7. Quantification of PGE<sub>2</sub> by EIA

Table 2 demonstrated a global increase in HTF C31 cell PGE<sub>2</sub> synthesis for each condition tested in comparison with HT29 cl.19A cells and vector control cells. We especially demonstrated that FLAP transfection led to a level of PGE<sub>2</sub> synthesis very similar to that obtained for IL-1β HT29 cl.19A stimulated cells.

EIA analysis (Table 2) also demonstrated that unstimulated HT29 cl.19A cells produced a greater amount of LTB<sub>4</sub> than HTF C31. After calcium ionophore stimulation, LTB<sub>4</sub> production from endogenous AA in HT29 cl.19A cells was not significantly different from that in HTF C31 cells. However, the increase in LTB<sub>4</sub> production was greater in HTF C31 cells (+1.05 pg/10<sup>6</sup> cells) than in HT29 cl.19A cells (0.39 pg/10<sup>6</sup> cells). A result similar to that of HT29 cl.19A cells was obtained with vector control cells in which the increase in LTB<sub>4</sub> production after stimulation was 0.30 pg/10<sup>6</sup> cells (Table 2).

## 4. Discussion

Several eicosanoids (PGE<sub>2</sub>, LTB<sub>4</sub>, 5-HETE) are increased in patients suffering from inflammatory bowel diseases and LTC<sub>4</sub>, 12-, 15-HETE and TXB<sub>2</sub> may be increased in inflamed tissues compared with normal mucosa [1,2,36,37]. It is now well established that ulcerative colitis in particular, as well as Crohn disease predispose to colon cancer [2].

In previous studies concerning the role of enterocytes in AA metabolism [20], we showed that the HT29 cl.19A cell line, used as an enterocyte model, could be both a target and producer of AA metabolites through the LO and COX pathways [20], and that this cell line exhibited an unusual constitutive COX-2 expression which was linked to resistance to apoptosis [21,22]. Moreover, this cell line exhibited an active 5-LO pathway, despite the absence of FLAP expression, which led essentially to the production of 5-HETE, Δ<sup>6</sup>-LTB<sub>4</sub> isomers and LTB<sub>4</sub> at a low level [20]. This phenomenon has been reported for a small number of cell lines which were all derived from intestinal carcinomas [23,24].

In this work, in agreement with a previous report [6], we found 5-LO to be exclusively located in the cytosolic fraction. A23187 stimulation enhanced 5-LO enzymatic activity with increased LTB<sub>4</sub> synthesis from endogenous AA (EIA analysis) or from exogenous [<sup>14</sup>C]AA (RP-HPLC analysis). This result corresponded to normal 5-LO calcium dependent activity; nevertheless, the absence of FLAP led to a limited metabolite synthesis, especially for LTB<sub>4</sub> without peptidoleukotriene synthesis.

In order to study FLAP-dependent leukotriene synthesis,

we obtained, by transfection with a human FLAP cDNA, new clones characterized by their specific FLAP mRNA and protein expression. The capacity of this cDNA to be transcribed and translated into an active FLAP protein was controlled by using the Sf9-baculovirus system during cotransfection studies with 5-LO: we demonstrated activation of 5-LO by an increase in 5-HETE and LTB<sub>4</sub> isomer synthesis.

In the first step of our work, we isolated, selected and amplified FLAP positive clones as HTF C1, C11 or C31 in which we expected an increase in 5-LO activity. On the contrary, in HTF C1, C11 and particularly in C31 cells, we noticed a surprising COX pathway activation. Several studies have demonstrated that COX-2, but not COX-1, mRNA and protein are elevated in human colon cancer [17,38–42], and authors considered that abnormal COX-2 up-regulation involved in the increase in PGE<sub>2</sub> synthesis observed in colon cancer was an important early event in the disease process [41,42]. So, with regard (i) to the AA metabolism studies in positive FLAP expressing clones, and (ii) to the particular COX-2 expression in HT29 cl.19A cell line [21,22], we explored COX-2 expression under various conditions using the HTF C31 clone (most representative FLAP expressing clone), in comparison with HT29 cl.19A results.

HTF C31 cells showed a COX-2 mRNA expression associated with specific COX-2 protein synthesis demonstrated by the presence on Western blot of the classically described forms of COX-2: the 66-kDa form which is the inactive non-glycosylated enzyme and the 72- and 74-kDa forms which represented the active enzymes, respectively glycosylated 3 and 4 times [43,44]. On the contrary, in HT29 cl.19A cells we observed a lower protein synthesis and the absence of the 72-kDa form. This was in agreement with immunohistochemistry studies in which specific COX-2 fluorescence could be detected in a larger part in HTF C31 than in HT29 cl.19A cells. These results were also associated with the presence of a more active AA metabolism, via COX pathway, in HTF C31 clones, in comparison with HT29 cl.19A cells. We particularly showed a large increase in PG synthesis with ionophore A23187 stimulated cells. PGE<sub>2</sub> EIA measurements of unstimulated (neither ionophore A23187 nor exogenous AA) cells clearly indicated a higher level of PGE<sub>2</sub> synthesis by the HTF C31 clone corresponding to an increase in COX-2 protein synthesis. The inducible nature of COX-2 expression in HTF C31 clone was supported by the following arguments. Preincubation with IL-1β, a COX-2 gene inducer [45], led to a specific increase in protein synthesis, as shown by immunofluorescence studies, associated with an increase in COX-2 metabolites (especially PGE<sub>2</sub>), without any qualitative modification of the chromato-

gram. This could indicate, in association with an inhibitory effect of meloxicam, SC-58125 or L-745-337, all considered selective COX-2 inhibitors, that the major part of COX activity in HTF C31 clone was due to COX-2 activity. Our results also indicated that FLAP cDNA transfection increased COX-2 gene transcription but the exact mechanism by which COX-2 gene expression was increased after FLAP transfection has not yet been defined.

In this work, we showed that HTF C31 cells exhibited FLAP expression (mRNA, protein), which is located in the nuclear fraction, in agreement with a previous report [6]. Moreover, even if unstimulated HTF C31 cells produced less LTB<sub>4</sub> than unstimulated HT29 cl.19A cells, EIA analysis demonstrated that the presence of FLAP in transfected cells enhanced LTB<sub>4</sub> synthesis after A23187 and calcium stimulation.

Our results showed that simple transfection of cells with pcDNA3 alone did not modify metabolism in comparison with untransfected cells. Our results also demonstrated that FLAP transfection enhanced the COX pathway as observed in different positive FLAP expressing clones such as HTF C31, C1 or C11. Thus, many hypotheses could be suggested to explain COX-2 activation after FLAP transfection. The absence of inhibitory effects of both MK-886, which blocks the AA binding site of FLAP, and REV-5901 which blocks FLAP-dependent LT synthesis, could rule out COX-2 up-regulation by a direct FLAP-dependent mechanism. Another hypothesis could be that FLAP-dependent LT synthesis is implicated in increased COX-2 gene expression. This can be eliminated by EIA LTB<sub>4</sub> analysis run on unstimulated monolayers in which HT29 cl.19A cells produced more LTB<sub>4</sub> than HTF C31. Thus, no increase in FLAP-dependent LT synthesis is implicated in COX-2 gene expression. The lesser capacity of HTF C31 cells to produce LTB<sub>4</sub> could be due to an increase in AA metabolism by the COX-2 pathway.

Taken together, RT-PCR, Western blot, immunochemistry, RP-HPLC and EIA AA analysis showed that HTF C31 cells exhibited, under our experimental conditions, a specific increase in COX-2 expression and activity in comparison with HT29 cl.19A cells. This could constitute a possible interaction between the major pathways of AA metabolism, acting to increase eicosanoid synthesis by an unknown mechanism.

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