Failure of apoptosis and activation on NFκB by celecoxib and aspirin in lung cancer cell lines

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Abstract. Recent studies have demonstrated that antineoplastic activity of Cox-2 inhibitors may depend on targets other than Cox: among those, nuclear factor κB (NFκB) seems the most promising. Although preclinical studies have suggested that aspirin and Cox-2 inhibitors may influence the progression of lung cancer, the molecular mechanisms of these protective effects in this tumor type has not been fully elucidated. We investigated the effects of celecoxib and aspirin in the induction of apoptosis and in the ability to activate NFκB in three non-small cell lung cancer cell lines. Apoptosis was evaluated by FACS, caspase activation assay and expression of apoptosis-related genes by RT-PCR, while NFκB activation was assessed by immunofluorescence. No apoptotic response was observed after treatment with both high and low dose of celecoxib. Nevertheless, celecoxib at both concentrations induced a strong NFκB activation, with increased expression of NFκB-dependent genes, such as *bcl-2*, $bcl\text{-}X_L$ and *survivin*. Similarly, aspirin at both concentrations did not induce any apoptotic response, but activated NFκB in a dose-dependent manner. This study supports the hypothesis that NFκB activation is an important effect of NSAIDs in lung cancer, leading to apoptosis resistance. This effect of both aspirin and celecoxib may be considered undesirable in lung cancer chemoprevention.

Introduction

Several lines of evidence strongly suggest that COX-2 overexpression plays a role in lung tumorigenesis, and COX-2 inhibition seems to be a promising target for cancer prevention and therapeutic strategies in non-small cell carcinoma of the lung (1,2).

Preclinical studies have shown that selective and non-selective non-steroidal anti-inflammatory drugs (NSAIDs) may be protective against lung cancer development, and selective inhibition of COX-2 by celecoxib decreased lung tumor growth *in vitro* and *in vivo* independently of its ability to block the COX-2 enzyme (3). Among NSAIDs, aspirin shows efficacy in the prevention of colon cancer, and clinical trials indicate that aspirin at a low dose is as effective as a higher dosage in reducing risk for colorectal cancer development (4,5). Although in some studies regular aspirin use has been associated to a significant reduction in risk and incidence of lung cancer (6), evidence on the effects of low-dose aspirin on lung cancer risk is limited. A recent clinical trial suggests a protective effect of low-dose aspirin (100 mg) in lung cancer, which was significant for lung cancer deaths (7).

Recently, some phase II clinical trials have been started in order to evaluate the potential chemopreventive efficacy of celecoxib in lung cancer patients (8,9). The observation that celecoxib exhibits the greatest efficacy for growth inhibition in COX-2 negative cell lines suggests that the anti-neoplastic activity of celecoxib is independent of COX-2 expression and depends on a target other than COX-2 (10-12). Among these targets, nuclear factor κB (NFκB) seems the most promising. NFκB is involved in the control of the transcription of many proinflammatory genes including adhesion molecules, cytokines and enzymes, and functions as a regulator of cell death mainly inhibiting apoptosis, although the ability of NFκB to induce or block apoptosis seems to depend on cell type (13). NSAIDs have been described to suppress NFκB and NFκB-dependent genes, such as cyclin D1, leading to a block of proliferation of tumor cells (14-18). The suppression of NFκB has been also described for celecoxib by Shishodia *et al* (19), who suggested that the suppression of NFκB by celecoxib may explain its role in chemoprevention and induction of apoptosis; a downregulation of NFκB by celecoxib has also been demonstrated in K562 cells (20).

Nevertheless, other authors have suggested that the effects of celecoxib on NFκB seem to strictly depend on the dose used. Celecoxib at a high (50 μM), but not at low concentration

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Table I. Primer sequences and amplification conditions.

Gene	Sequences 5'→3'		Location	Annealing (t˚C)	Size bp
gadph	Upstream	GTGGGGCGCCCCAGGCACCA	103-122		
	Downstream	CTCCTTAATGTCACGCACGATTTC	619-642	60	516
bcl-2	Upstream	GTGGAGGAGCTCTTCAGGGA	431-450		
	Downstream	AGGCACCCAGGGTGATGCAA	715-734	60	304
bax	Upstream	GGCCCACCAGCTCTGAGCAGA	35-55		
	Downstream	GCCACGTGGGCGTCCCAAAGT	491-511	62	479
bcl-x_l	Upstream	TTGGACAATGGACTGGTTGA	96-115		780
bcl-x_s	Downstream	GTAGAGTGGATGGTCAGTG	842-860	58	591
survivin	Upstream	CAGATTTGAATCGCGGGACCC	5-25		
	Downstream	CCAAGTCTGGCTCGTTCTCAG	192-212	60	206

(1 μM) was described to activate NFκB pathways and NFκB-dependent gene transcription (21); the activation of NFκB by high doses of celecoxib has been described also in cervical cancer cells (22).

An activation of NFκB has been described for aspirin only in colon cancer cells (23); this cell type-specific activation of NFκB by aspirin prevents colon cancer (24) inducing apoptosis by a Fas-mediated pathway. However, the precise mechanism of aspirin-induced apoptosis is not fully understood, and almost all studies have evaluated the effects of high-dose aspirin (1-10 mM). In colon cancer cells, aspirin induces IκB degradation with NFκB activation; this pathway was described to be colon cancer specific (23). In gastric cancer, aspirin seems to induce apoptosis both through mitochondrial and death receptor pathways (25,26).

In lung cancer, no studies have been performed in order to clarify if and how low-dose aspirin (1-100 μM), corresponding to the plasma concentration obtained after administration of 80-100 mg aspirin, which in lung cancer seemed effective in reducing cancer risk and mortality (7), may affect apoptosis.

Also celecoxib-induced apoptosis, which does not require the presence of COX-2 enzyme, seems to occur differently in different cell types; it has been shown that apoptosis in colon cancer (27) and breast cancer (28) is mediated by the inactivation of Akt, while in the Jurkat cell line it seems to pass through the mitochondrial pathway (29). In lung cancer cell lines, the precise mechanism of celecoxib-induced apoptosis has not been clearly elucidated. It has been recently suggested that celecoxib (50 μM) induces apoptosis in non-small cell lung cancer cell lines through the extrinsic death receptor pathway, independent of bcl-2 expression, while at a low concentration (3-8 μM), which corresponds *in vivo* to a single dose of 400-800 mg, apoptosis does not occur (30). Sun *et al* (31) confirmed that celecoxib-induced apoptosis in lung cancer cell line occurs only at a higher concentration, thus, also celecoxib-induced apoptosis seems to mainly depend on the dose used.

In the present study, we evaluated the effects of high and low concentrations of celecoxib and the effects of low-dose aspirin on apoptosis and NFκB activation in three non-small cell lung cancer cell lines.

Materials and methods

Drugs. Celecoxib (Amersham-Pharmacia Biotech, Uppsala, Sweden) and aspirin (Sigma) were dissolved in DMSO as 100 mM and 1 M stock solution, respectively, and stored at -20˚C. Stock solutions were diluted to the appropriate concentrations with grown medium immediately before use.

Cell lines. Human NSCLC cell lines SK-MES-1, SK-LU-1 and COLO 699N were purchased from Interlab Cell Line Collection (Genova, Italy). SK-MES-1 cell line, a squamous cell carcinoma, was grown in MEM (Euroclone) supplemented with 10% FBS, 100 U/ml of penicillin, 100 U/ml of streptomycin and 1% non-essential amino acids. SK-LU-1 cell line, a grade III poorly differentiated adenocarcinoma, was grown in MEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 U/ml of streptomycin, 1% non-essential amino acids and 1 mM Na pyruvate.

COLO 699N cell line derived from an adenocarcinoma of the lung, was grown in RPMI-1640 supplemented with 10% FBS, 100 U/ml of penicillin and 100 U/ml of streptomycin. All cell lines were grown in culture flasks (Falcon, Bedford, MA) in a humidified of 5% CO_2 atmosphere at 37˚C.

The media were changed every 3 days and the cells were separated via trypsinazation using trypsin/EDTA when they reached subconfluence. Each cell line was exposed to 5 and 50 μM of celecoxib dissolved in DMSO for 24, 48 and 72 h to different aspirin concentrations (1 μM, 100 μM and 1 mM) for 24, 48 and 72 h.

Cell viability assay. Trypan blue exclusion method was used to determinate the cell viability of both celecoxib and aspirin treatment. After treatment, cells growing in adherence were collected by pooling cells from the medium (i.e. dead cells) and adherent (live) cells obtained by trypsinization. Cells were then centrifuged (10 min at 500 g) and resuspended in

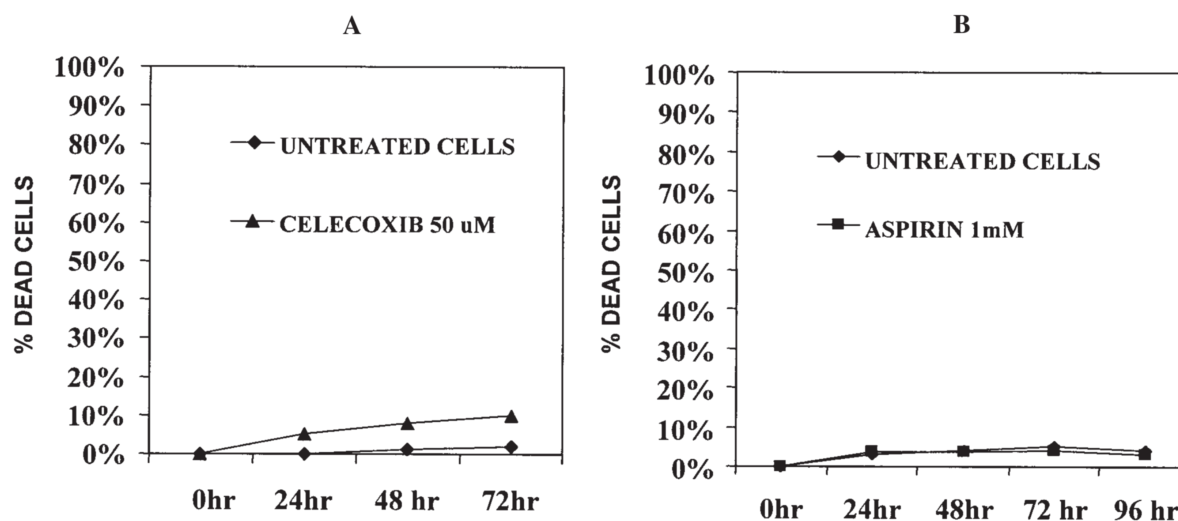

Figure 1. Cell viability assay. (A) Cell viability assay of untreated COLO 699N cells and COLO 699N treated with 50 μM celecoxib after 24, 48 and 72 h. (B) Cell viability assay of untreated COLO 699N cells and COLO 699N treated with 1 mM aspirin after 24, 48 and 72 h.

PBS (50-100 μl). An aliquot of each cell line suspension was diluted 1:1 with 0.4% trypan blue. After 5 min, cells were loaded on a hemocytometer, and both live (unstained) and dead (blue-stained) cells were counted under a light microscope. The percentage of dead cells was then determined. Each treatment condition was tested at least in triplicate, and the mean value (% dead cells) was determined.

RT-PCR. For each μg of total RNA extracted from the frozen cells with Trizol (Invitrogen, Carlsbad, CA) a reverse transcription assay was performed in a finale volume of 20 μl containing 20 mM Tris HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 pmol random examer, 50 units of MuLV reverse transcriptase (Invitrogen) according to the manufacturer's guidelines. Then, 5 μl of cDNA were amplified in PCR buffer containing 25 pmole of each upstream and downstram primer and 1.25 units of Platinum Taq polymerase (Invitrogen) in a final volume of 50 μl. To detect the RNA expression of *bcl-2, bax, bcl-X,* and *survivin*, different PCR amplifications were performed.

Amplifications were performed on a Techne Progene (Cambridge, UK) amplifier. The amplification conditions are described in Table I. All the recommended precautions were taken to avoid the possibility of false-positive results and the preparation of reaction mixture and the analysis of amplified products were carried out in separate rooms.

Flow cytometry analysis of apoptotic cells. Flow cytometry was used to detect quantitatively the apoptotic rate and distribution of the cell cycle. After incubation with celecoxib and aspirin at the appropriate concentrations, cells were harvested, washed twice with ice-cold phosphate buffer (PBS) and then $1.5x10^6$ were fixed in 2 ml ethanol 70%, and incubated for 1 h at 4˚C. Cellular pellet was dissolved in 0.5 ml of a solution containing propidium iodide 5 μg/ml and RNasi A 1 mg/ml in PBS. The stained cells were incubated at room temperature for 30 min in the dark. The DNA content of the cells was analyzed by FACS Calibur flow cytometry using the CellQuest analysis program. The DNA content in the sub G1 population was considered to represent apoptotic cells.

Enzyme-linked immunosorbent assay for detection of caspase-3, -8 and -9. The cleavage activity of Ile-Glu-Thr-Asp conjugated to *p*-nitroanilide (IETD-pNA), Leu-Glu-His-Asp conjugated to *p*-nitroanilide (LEDH-pNA), and Asp-Glu-Val-Asp conjugated to *p*-nitroanilide (DEVD-pNA) was measured by using FLICE/caspase-8, caspase-9/Mch6 and caspase-3/CPP32 colorimetric assay kit (Bio vision). About $3x10^6$ cells were pelletted, washed twice in PBS and resuspended in 50 μl chilled lysis buffer. The formations of *p*-nitroanilide were measured by ELISA microtiter reader (Corning Incorporated) at 405 nm after samples were incubated at 37˚C for 2 h with appropriate substrates. Percent increase in caspase activities was determined by comparing results with controls.

Immunofluorescence. Cells were grown on Labteck chamber slides (Nunc, Naperville, IL, USA) and treated with celecoxib (5, 50 μM) and aspirin (1, 100 μM and 1 mM) for 24 and 48 h. After treatment, cells were washed with PBS and fixed with absolute methanol for 5 min at -20˚C. Cells were then incubated for 1 h with rabbit polyclonal antibody to NFκB p65 (Santa Cruz), rinsed 3 times with PBS and then incubated with FITC-conjugated anti-rabbit IgG (Sigma) for 1 h. Cells were then rinsed 3 times with PBS and mounted with Prolong anti-fast reagent and the fluorescence was analysed by an Olympus BX52 fluorescence microscope. The images were acquired and elaborated with the IAS 2000 software.

Results

Effects of celecoxib on lung cancer cell lines. The cell viability assay performed on each cell line after the treatment with 50 μM celecoxib for 24, 48 and 72 h revealed a low percentage of dead cells compared to the controls (Fig. 1A).

Flow cytometry analysis shows that celecoxib (50 μM) did not induce apoptosis in all the cell lines analyzed. When treated with 50 μM celecoxib, the apoptotic cell percentage

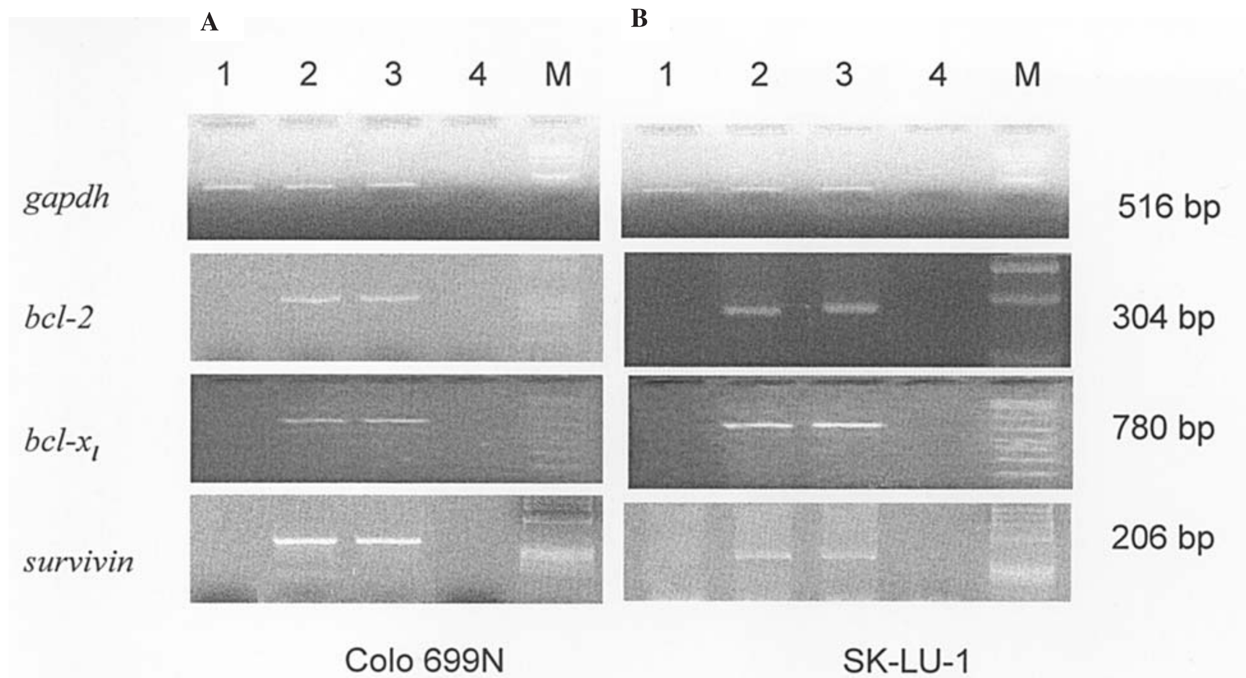

Figure 2. (A) *Gadph, bcl-2, bcl-X* and *survivin* RT-PCR products separated on a 2% agarose gel followed by ethidium bromide staining. Lane 1, COLO 699N untreated; lane 2, COLO 699N 50 μM celecoxib 48 h; lane 3, positive control (RNA from M14 cell line); lane 4, negative control (sample without RNA). M, molecular weight marker. (B) *Gadph, bcl-2, bcl-X* and *survivin* RT-PCR products separated on a 2% agarose gel followed by ethidium bromide staining. Lane 1, SK-LU-1 untreated; lane 2, SK-LU-1 μM aspirin 48 h; lane 3, positive control (RNA from M14 cell line); lane 4, negative control (sample without RNA). M, molecular weight marker.

reached 7% in COLO 699N, 6% in SK-LU-1 and 3% in SK-MES-1 after 48 h. At lower concentration (5 μM) apoptosis was not induced. The ELISA assay performed on COLO 699N, SK-LU-1 and SK-MES-1 with 50 μM celecoxib for 48 h revealed no activation of caspase-3, -8 and -9. Gene expression analysis performed by RT-PCR demonstrated in all three cell lines an overexpression of *bcl-2, bcl-*X_L and *survivin* after 24 h of treatment, while bax decreased at 24 h and its expression disappeared at 48 and 72 h. This effect was more evident in COLO 699N cell line, which was negative for *bcl-2* and *bcl-X* expression before treatment (Fig. 2A).

The activation of NFκB was based on the immuno-fluorescent detection of its translocation into cell nuclei from its initial localization in the cytoplasm, where it exists as inactive form. NFκB was detected in the cytoplasm of ~100% of the cells in all three cell lines used. COLO 699N cell line treated with 50 μM celecoxib for 24 h showed translocation of NFκB into the nucleus in ~30% of cells, showing an intense staining into the nucleus, while after treatment with 5 μM celecoxib NFκB was not activated.

In both SK-MES-1 and SK-LU-1 cell lines treatment with 5 μM celecoxib at 24 h induced a nuclear translocation of NFκB in 30%, whereas the treatment with 50 μM celecoxib for 24 h showed NFκB activation in 70% of both cell lines (Fig. 3).

Effects of aspirin on lung cancer cell lines. The cell viability assay performed on each cell line after the treatment with 1 mM aspirin for 24, 48 and 72 h revealed a low percentage of dead cells compared to the controls (Fig. 1B). In all three cell lines used we failed to find any significant percentage of apoptotic cells by flow cytometry assay after treatment with aspirin at 1 μM, 100 μM and 1 mM for 24, 48 and 72 h.

In SK-LU cell line aspirin at 100 μM and 1 mM induced expression of *bcl-2, bcl-*X_L and *survivin* at 48 h (Fig. 2B); in the other cell lines we observed a slight increase in mRNA expression of *bcl-2, bcl-X* and *survivin* after treatment with 1 mM aspirin.

NFκB was detected by immunofluorescence in the cytoplasm in all the cell lines used, as previously described. After treatment with 1 μM, 100 μM and 1 mM aspirin for 24 h we did not observe evident nuclear staining in COLO 699N and SK-MES-1 cell lines, whereas in SK-LU-1 we observed a partial activation in 30% of the cells at 100 μM aspirin; on the contrary, the treatment with 1 mM aspirin induced nuclear translocation of NFκB in 5, 10 and 80% of COLO 699N, SK-MES-1 and SK-LU-1, respectively (Fig. 3).

Discussion

Evidence from animal studies suggests that aspirin and NSAIDs, including COX-2-specific inhibitors, may influence the progression of lung cancer. Recently, some phase II clinical trials have been started in order to evaluate the potential chemopreventive efficacy of aspirin and COX-2-specific inhibitor celecoxib in lung cancer patients, where the regular aspirin use was by some authors described associated with reduced risk.

While a pathway of celecoxib-induced apoptosis has been described in lung cancer, aspirin-induced apoptosis seems to

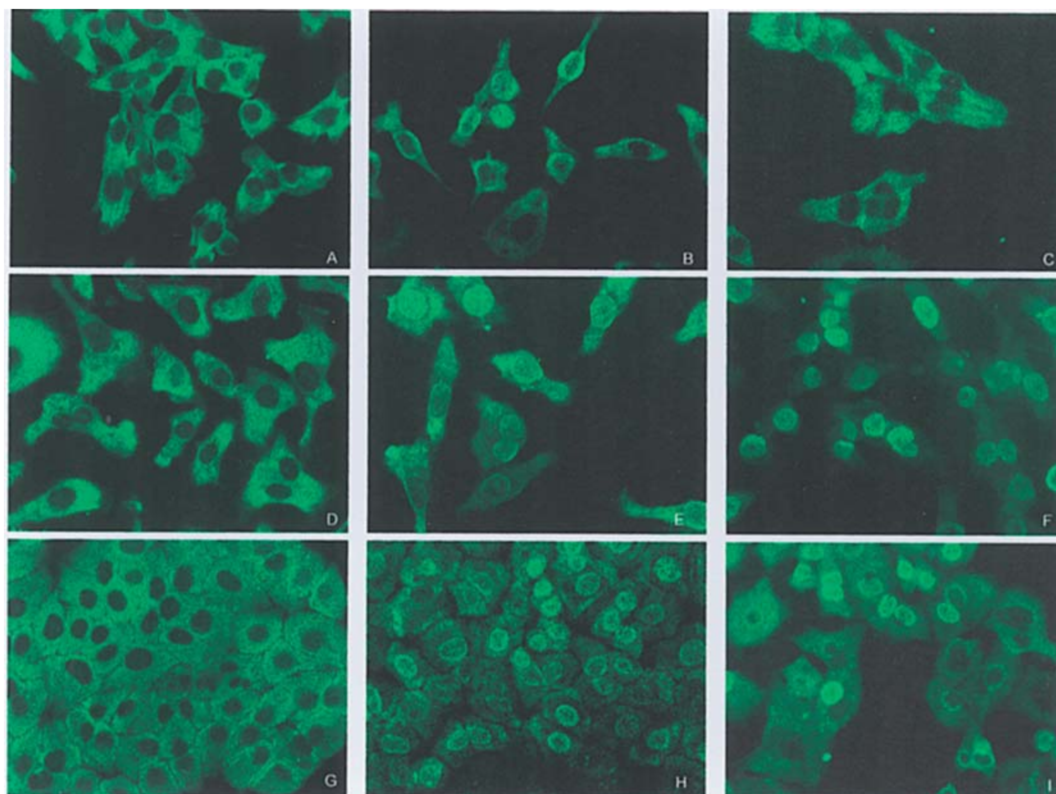

Figure 3. Immunofluorescence of p65 NFκB of untreated COLO 699N (A), SK-LU-1 (D) and SK-MES-1 (G); 50 μM celecoxib-treated COLO 699N (B), SK-LU-1 (E) and SK-MES-1 (H); 1 mM aspirin-treated COLO 699N (C), SK-LU-1 (F) and SK-MES-1 (I).

be restricted to colon cancer cells, suggesting that its effects may have particular relevance only in colon cancer chemoprevention (23).

In the present study, we investigated the proapoptotic effects of a high and low dose of celecoxib and those of low-dose aspirin in three NSCLC cell lines. Low-dose aspirin failed to induce apoptosis in all the cell lines analysed; the data are in accord to that described by Din *et al* (24), who suggested that the substantial difference in the anti-tumor effects of aspirin strictly depends on the cell type. Nevertheless, we observed a dose-dependent activation of NFκB in aspirin-treated cells more evident in the SK-LU-1 cell line, suggesting that induction of NFκB by aspirin is not restricted to colon cancer cells, as described by Din *et al*, but is presumably dependent on the cell type.

Furthermore, activation of NFκB by aspirin in lung cancer cells is not correlated to apoptosis induction, since we did not observe any apoptotic response in SK-LU-1, where NFκB was found activated in 80% of cells. On the contrary, the induction of *bcl-2, bcl-*X_L and *survivin* mRNA in the SK-LU-1 cell line, where NFκB is strongly activated after treatment, suggests that, at least in this cell line, NFκB activation may result in a block of apoptosis through the upregulation of anti-apoptotic members of *bcl-2* and IAP family. On the other hand, in the SK-MES-1 and COLO 699N cell lines, where we observed a less evident dose-dependent upregulation of anti-apoptotic genes (evident only after treatment with 1 mM aspirin), NFκB was also partially translocated into the nucleus. Thus, NFκB activation by aspirin does not necessarily promote apoptosis, as described in colon cancer, but, in a cell-type-dependent manner, it may also be responsible for apoptosis resistance, through the transcription of genes involved in the suppression of cell death by mitochondrial pathway. This block of apoptosis observed in lung cancer aspirin-treated cell lines may be deleterious in cancer chemoprevention. The difference we observed among the cell lines in the dose of aspirin necessary for NFκB induction and apoptosis resistance may reflect the intrinsic characteristics of the cell lines, suggesting that each tumor, with its own cellular characteristic, may differently respond to the same chemopreventive approach, and that the same dose may not necessarily be effective in all patients with the same cancer type. This observation reflects the different response of patients to the same treatment.

We observed after celecoxib treatment a clear apoptosis resistance at both low and high dose in all the cell lines analysed. This failure in apoptosis induction was revealed by FACS, caspase activity and cytotoxicity assay. Concerning NFκB, it was generally found activated in all cell lines at both doses, except in the COLO 699N cell line where a low dose of celecoxib did not induce any activation. Our findings are partially in contrast with those by Niederberger *et al* (21) who found that a low dose of celecoxib decreased NFκB activation, while a high dose had the opposite effect. In lung cancer cell lines we found that NFκB activation may be more dependent on the cell type than on the dose used. In fact, in SK-MES-1 and SK-LU-1 celecoxib induced NFκB activation at both doses used, while in COLO 699N this activation was weaker and evident only after treatment with 50 μM

celecoxib. Several mechanisms have been described for celecoxib-induced apoptosis: the activation of caspase-8 and -9 using NFκB as a possible target in cervical cancer, caspase-9-dependent mitochondrial pathway in lymphoma, while in lung cancer the activation of extrinsic death receptor pathway has been described by Liu *et al* (30). Since we failed to find an apoptotic response in celecoxib-treated cells, we may speculate that the activation of NFκB induced by celecoxib at high and low dose may induce in these lung cancer cell lines a general tendency to apoptosis resistance through the upregulation of anti-apoptotic genes such as *bcl-2*, *bcl-X_L* and *survivin*, similarly to that observed after treatment with aspirin. This study supports the hypothesis that NFκB activation by aspirin and celecoxib in lung cancer may represent a survival pathway leading to apoptosis resistance. Furthermore, the role of NFκB in regulation of apoptosis appears strictly drug-dependent or cell context-dependent.

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