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Inhibition of the growth of 12V-*ras*-transformed rat fibroblasts by acetylsalicylic acid correlates with inhibition of NF-κB

Sofia Ljungdahl, Maria C Shoshan and Stig Linder
Radiumhemmet's Research Laboratory, Department of Oncology–Pathology, Radiumhemmet, Karolinska Institute and Hospital, 171 76 Stockholm, Sweden. Tel: (+46) 8-729 54 08; Fax: (+46) 8-33 90 31.

Epidemiological studies have demonstrated a correlation between regular aspirin (acetylsalicylic acid; ASA) use and a decreased risk for the development of cancer. We here show that ASA inhibits the growth of 12V-*ras*-transformed rat fibroblasts *in vitro* at pharmacological concentrations. This effect appeared to be unrelated to inhibition of cyclooxygenase, since other cyclooxygenase inhibitors did not inhibit cell growth. A number of nuclear transcription factors have been implicated as mediators of transformation. ASA has recently been reported to inhibit the activation of one such factor, NF-κB. We found that NF-κB binding activity was decreased in ASA-treated 12V-*ras*-transformed cells. Inhibition of NF-κB activation was not due to a general inhibitory effect, since AP-1 binding activity was not affected. We conclude that ASA inhibits the growth of 12V-*ras*-transformed fibroblasts, possibly via inhibition of NF-κB.

Key words: Acetylsalicylic acid, cyclooxygenase, NF-κB, *ras* transformation.

Introduction

Epidemiological studies have demonstrated a correlation between regular aspirin (acetylsalicylic acid; ASA) use and a decreased risk for the development of fatal colorectal cancer. In a recent study, rats that received 0.1% ASA in their diet developed significantly fewer tumors after nitrosamine treatment compared to rats that did not receive ASA.[1] ASA is a well-documented inhibitor of cyclooxygenase (CO), an enzyme which catalyzes the conversion of arachidonic acid (AA) to prostaglandin H2. Mitogens increase prostaglandin synthesis in fibroblasts.[2] Furthermore, it has been reported that phospholipase A_2 (cPLA2) and CO expression is constitutively increased in some transformed cells,[3] and that some prostaglandins are affected by transformation.[2]

NF-κB is a ubiquitous transcription factor which binds to specific decameric DNA sequences and regulates transcription of multiple genes. The activity of NF-κB is regulated by an inhibitory protein, IκB, which sequesters NF-κB in the cytoplasm. A large variety of agents, including growth factors, the tumor promoter phorbol 12-myristate 13-acetate and the cytokine tumor necrosis factor-α, initiate signal transduction pathways that converge upon the NF-κB/IκB complex, resulting in the dissociation of IκB and the translocation of NF-κB to the nucleus. Expression of activated forms of Ras or of the serine/threonine kinase Raf-1 results in the activation of NF-κB.[4] Overexpression of IκB has been shown to result in reversion of transformation in the NIH-3T3 cell system. ASA has been shown to inhibit the activation of NF-κB[5] by preventing the degradation of IκB. This results in NF-κB being retained in the cytosol.

We show here that ASA inhibits the growth of rat embryo fibroblasts (REF) transformed by 12V-*ras*. In contrast, a series of other CO and lipoxygenase (LO) inhibitors did not cause growth inhibition, suggesting that the effect of ASA was not due to inhibition of AA metabolism. However, ASA was observed to inhibit NF-κB activity in *ras*-transformed REF cells, raising the possibility that growth inhibition was due to inhibition of NF-κB-dependent gene expression.

Materials and methods

Cell cultures

The transformed rat fibroblast cell lines (BRN-1 and A14) have been described previously.[6,7] Cells were maintained in Dulbecco's modified Eagle's medium

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Correspondence to S Linder

supplemented with 5% fetal calf serum (Gibco, Life Technologies, Paisley, UK).

Proliferation assays were done by plating cells at a concentration of $20-50 \times 10^3$ cells/50 mm Petri dish. Cells were detached from one plate and counted the following day, and inhibitors were added from appropriate stock solutions to the remaining dishes. Cells were detached, and counted 2 and 5 days later. Cells were counted in a hemocytometer and the means of 10 counted 0.1 mm^2 squares are shown.

Gel mobility shift analyses

Nuclear cell extracts were prepared as described.[8] Protease inhibitors aprotinin, pepstatin, leupeptin and phenylmethylsulfonyl fluoride were included at all steps of the procedure. First, 1 ng of double-stranded ^{32}P-labeled NF-κB (CGTCCCGGAGTTTCCTA) or collagenase TRE (CGTGTCTGACTCATGCTTG) oligonucleotide probes was added to cell extracts and incubated for 15 min at room temperature. Extracts were loaded onto 6% polyacrylamide/0.5 × TBE gels and electrophoresis was performed at 10 V/cm for 2 h.

PLA_2 assay

The activity of PLA_2 was assayed as the release of ^{14}C-labeled AA (Amersham Sweden, Stockholm, Sweden) from prelabeled cells. Briefly, cells were incubated with AA (0.1 μCi/ml in growth medium) for 16–18 h, washed three times and incubated for 30 min at 37°C in fresh medium. The supernatants of triplicate samples were collected, centrifuged and assayed for free AA by scintillation counting. Total incorporated AA was assayed by trypsinizing labeled and washed cells before quantification by scintillation counting.

Results

Inhibition of the growth of 12V-*ras*-transformed fibroblasts by ASA

REF have been widely used in transformation studies. REF are more phenotypically stable than mouse fibroblasts and transformed lines often remain diploid. We examined the effect of ASA on the proliferation of transformed rat fibroblasts cell lines. The data for one such cell line (BRN-1) is shown in Figures 1 and 2. Significant growth inhibition was observed at a concentration of 0.1 mM ASA. At 1 mM, the population doubling time of BRN-1 cells was approximately 42 h, compared to 18 h for untreated cells. Similar data was obtained using another cell line (A14; not shown).

Effects of other CO and LO inhibitors

The effect of a number of CO inhibitors on the proliferation of BRN-1 cells was examined (Figure 2). No growth inhibition was observed with piroxicam, ibuprofen or naproxen.

5,8,11-Eicosatriynoic acid (ETI), an inhibitor of 5-LO and 12-LO,[9] did not inhibit the proliferation of BRN-1 cells (Figure 2). Neither did meclofenamic acid, an inhibitor of CO, 5-LO and 15-LO, have any growth inhibitory effects. Finally, combinations of

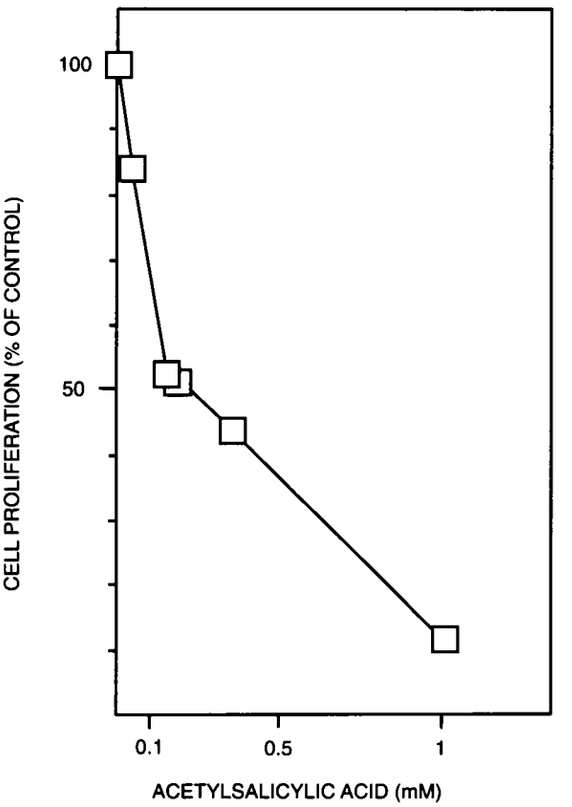

Figure 1. Effect of ASA on the growth of BRN-1 cells. Cells were grown in the presence of various concentrations of ASA for 5 days, detached and counted. The number of cells in the untreated control represents 100%.

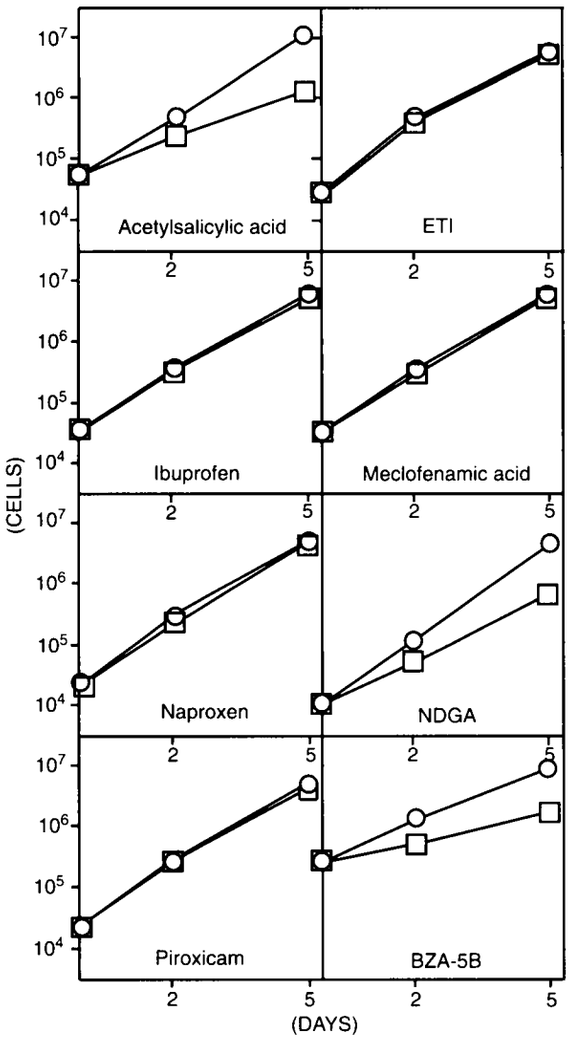

Figure 2. Effect of CO and LO inhibitors and the Ras inhibitor BZA-5B on BRN-1 cell growth. Cells were grown in the presence of 10^{-3} M ASA (CO), 10^{-4} M ibuprofen (CO), 10^{-6} M naproxen (CO), 10^{-5} M piroxicam (CO), 10^{-5} M ETI (5-LO, 12-LO); 10^{-5} M meclofenamic acid (CO, 5-LO, 15-LO), 2×10^{-5} M NDGA (5-LO, 12-LO, cytochrome P450 monooxygenase) and 5×10^{-5} M BZA-5B (Ras). Shown is the growth of cells between 2 and 5 days after addition of inhibitor. (○) Cell growth in absence of inhibitor. (□) Cell growth in presence of inhibitor.

piroxicam and ETI or naproxen and ETI did not affect proliferation of BRN-1 cells (data not shown). These data suggest that the effect of ASA on BRN-1 cell growth cannot be attributed to inhibition of CO.

Nordihydroguaiaretic acid (NDGA), which inhibits the 5-LO, 12-LO and cytochrome P450 monooxygenase systems, reduced cell growth significantly at a concentration of 20 mM (Figure 2).

PLA_2 activity is not elevated in transformed REF

The activity of PLA_2 was determined in transformed BRN-1 and A14 cells. As shown in Figure 3, PLA_2 activity in BRN-1 cells was similar to that of untransformed FR3T3 cells. A14 cells showed lower PLA_2 activity.

ASA decreases NF-κB binding activity in BRN-1 cells

Nuclear extracts were prepared from BRN-1 cells grown in the presence and absence of ASA. Electrophoretic mobility shift assays were performed using ^{32}P-labeled oligonucleotide probes. As shown in Figure 4, NF-κB binding activity was reduced in ASA-treated cells. In contrast, AP-1 (Jun/Fos) binding activity was not affected by ASA treatment. We used an inhibitor of Ras farnesyltransferase, BZA-5B, as a control for the AP-1 binding assay. In BZA-5B-treated cells, AP-1 activity was decreased (Figure 4).

Discussion

In the present study, we did not observe an increased PLA_2 enzyme activity in 12V-*ras*-transformed REF cells compared to untransformed FR3T3 cells. This result is in contrast to other studies showing elevated PLA_2 enzyme activity in transformed cell lines (see Lin *et al.*[10] for references). The finding of

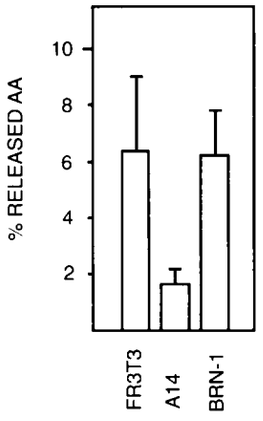

Figure 3. PLA_2 activity. The activity of PLA_2 in intact cells seen as release of [^{14}C]AA during 30 min from prelabeled cells, as described in Materials and methods. Shown is released label in quadruplicate samples as percent of total incorporated label. Similar results were obtained in two more experiments.

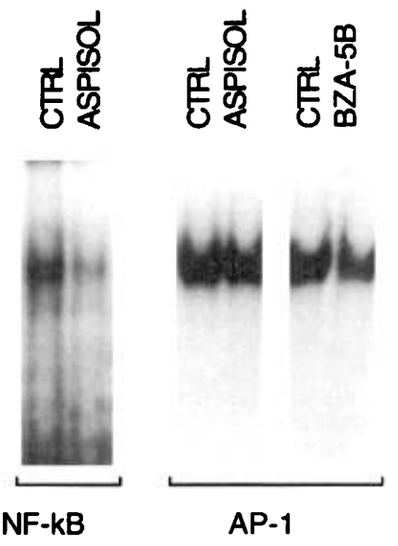

Figure 4. NF-κB activity is decreased in ASA-treated cells. (A) Binding assay performed with a NF-κB probe. (B) Binding assay performed with a collagenase-TRE probe. Cells were grown in the presence of 1 mM ASA for 3 days or BZA-5B for 3 days and nuclear extracts were prepared.

similar (or lower) PLA_2 activity in *ras*-transformed REF as in the untransformed FR3T3 cell line suggests that increases in PLA_2 are not necessary for transformation. AA is primarily metabolized through three pathways, i.e. cyclooxygenase, lipoxygenase and cytochrome P450 monooxygenase.[11] Growth factors such as epidermal growth factor and platelet-derived growth factor have been demonstrated to stimulate the formation of prostaglandins in some cells, and it has been reported that some prostaglandins are affected by transformation.[2]

We examined the effects of a number of CO inhibitors on the growth of 12V-*ras*-transformed rat fibroblasts. ASA inhibited the growth of these cells. Strong inhibition of cell growth was observed at 1 mM, a concentration which is in the same range as that required for anti-inflammatory effects (1–2 mM). In contrast, no effect was observed using other CO inhibitors (piroxicam, naproxen and ibuprofen), suggesting that the effect of ASA was not due to inhibition of CO.

LO-derived AA metabolites are involved in the growth response in some cells.[14] Growth factors stimulate synthesis of 9(*S*)-HODE, 13(*S*)-HODE and 12(*S*)-HETE, and inhibition of the production of these results in cessation of DNA synthesis in some cells. In the present study, we did not observe any effect of the 5- and 12-LO inhibitor ETI on the growth of BRN-1 cells or two other cell lines tested. Furthermore, meclofenamic acid, an inhibitor of 5-LO, 15-LO and CO did not affect cell growth. In contrast, NDGA, an inhibitor of the 5-LO, 12-LO and cytochrome P450 monooxygenase systems, reduced BRN-1 cell growth. This finding is in accord with a previous report showing that inhibition of COs and LOs separately does not affect serum stimulated cell growth, whereas NDGA significantly reduced cell growth.[15] Together, these data suggest that in 12V-*ras*-transformed rat fibroblasts, metabolites generated by cytochrome P450 monooxygenase are important for cell growth, whereas 5-, 12- and 15-LO are dispensible for growth.

Recent studies have shown that ASA has other activities, such as inhibition of NF-κB function.[5] ASA treatment has been shown to result in prevented degradation of the NF-κB inhibitor IκB, resulting in NF-κB being retained in the cytosol. We show here that ASA treatment results in inhibition of NF-κB binding activity in 12V-*ras*-transformed fibroblasts. In such cells, NF-κB activity is constitutively activated, presumably via activation of Raf kinase.[4] It has recently been argued that ASA will inhibit the activity of a number of intracellular kinases and that there is no specific effect on NF-κB.[12] We examined the effect of ASA on AP-1 binding activity, but found no effect. In contrast, a Ras farnesylation inhibitor did decrease AP-1 activity. We conclude, therefore, that although we cannot exclude general unspecific effects of ASA on various cellular kinase systems, we can exclude effects on systems which are involved in induction of AP-1 binding activity.

NF-κB has been implicated in cell transformation. First, one member of the NF-κB family, Rel, is a retrovirally transduced gene. Secondly, activated forms of Ras or Raf-1 result in the activation of NF-κB.[4] Thirdly, genes implicated in the transformed phenotype such as MMP-9[13] have κB binding sites in their promoters. Finally, overexpression of IκB has been shown to result in reversion of transformation in the NIH 3T3 cell system.

We conclude that inhibition of the growth of 12V-*ras*-transformed cells by ASA appears to be independent of CO inhibition. Our data raise the possibility that the reported anti-carcinogenic effects of ASA may be, at least partly, due to inhibition of a transcription factor.

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