Acetylsalicylic Acid Inhibits Cell Proliferation by Involving Transforming Growth Factor-β

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Background—Acetylsalicylic acid (ASA) inhibits cell proliferation. This may be mediated by transforming growth factor-β (TGF-β). TGF-β directly stops cell proliferation, restrains cells in G₀, and inhibits the uptake of platelet-derived growth factor and insulin-like growth factor. These effects are identical to those observed with ASA treatment.

Methods and Results—We cultured rat thoracic aorta vascular smooth muscle cells and measured cytotoxicity, cell proliferation, cell cycle, transcription of TGF-β₁, and concentration of TGF-β₁ in supernatant medium. ASA dose-dependently restrained cells in G₀ phase with no cytotoxic effect and inhibited cell proliferation by 30.86%. Anti-TGF-β₁ reversed this inhibition by 30.21%. However, ASA treatment decreased TGF-β₁ transcription and had no significant effect on TGF-β₁ concentration.

Conclusions—TGF-β seems to play an important role in ASA-mediated inhibition of cell proliferation. Therefore, treatment with ASA prevents coronary disease not only by means of its antiplatelet properties but also by an important inhibition of plaque growth. This relationship between ASA and TGF-β explains many other effects, such as cancer chemoprevention, immunomodulation, and wound healing. The aim of this study was to demonstrate this link. (Circulation. 2003;107:626-629.)

Key Words: aspirin ■ cells ■ aorta ■ transforming growth factor beta

A cetylsalicylic acid (ASA) has been demonstrated to stop vascular smooth muscle cell (VSMC) proliferation. This effect and its well-known antiplatelet action could explain its clinical benefits, already observed, in both heart stroke and angina.

Several studies link this antiproliferative action with alterations in growth factors, the role of which in atheromatosis and plaque rupture is widely accepted. It has been demonstrated that trapidil, an antithromboxane drug, inhibits platelet-derived growth factor (PDGF) cell uptake in mesangial samples. Besides, ASA can reduce circulating levels of PDGF in human platelet-rich plasma. In VSMCs, PDGF is accepted to be a major growth factor, and this fact could explain the antiproliferative action of ASA. Some studies in Caco-2 colorectal cancer cells also show insulin-like growth factor II (IGF-II) inhibition as an important pathway in the antiproliferative effect of ASA in these cells.

On the other hand, the pleiotropic cytokine transforming growth factor (TGF)-β shows the same simultaneous inhibition of several growth factors. We postulate that ASA stimulates the autocrine secretion of TGF-β, which would be the first step of this sequence. TGF-β receptors belong to a superfamily of serine/threonine kinase receptors involved in VSMC development and hypertrophy. It not only stops cell proliferation by means of the p27 and p53 pathway, thus restraining cells in G₀, but also inhibits PPAR-γ, which could mediate tyrosine kinase receptor downregulation (including PDGF and IGF). Moreover, in vivo studies show that ASA can reduce plasmatic levels of proinflammatory cytokines in patients with chronic stable angina and also suggest that TGF-β could mediate this effect.

The aim of this study was to demonstrate that TGF-β plays a major role in the antiproliferative actions of ASA in VSMCs.

Methods

General Materials

Rats were supplied by the Unit of Laboratory Animals of the Universidad Complutense (Madrid, Spain). All animal procedures followed the guidelines for animal care issued by the Universidad Complutense.

DMEM, FCS, antibiotic-antifungal solution (G penicillin, streptomycin, and amphotericin B), and trypsin 0.05% EDTA were obtained from Gibco Life Technologies. Collagenase and BSA were obtained from Sigma.

Cell Cultures

Primary cultures of VSMCs were prepared from enzymatically dissociated rat thoracic aortas according to a method previously described. The cells were cultured in DMEM containing 10% FCS supplemented with 100 IU/mL penicillin G (sodium salt) and 100 μg/mL streptomycin (antibiotic solution, Gibco).

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626
Drugs
We used purified ASA from Sigma, dissolved in dimethyl sulfoxide and diluted in 10% FCS medium (1:100) to obtain final concentrations of 0.5, 1, 2, 5, and 10 mmol/L of ASA.

Cell Proliferation
We used the tetrazolium salt XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis[4-methoxy-6-nitro]benzene sulfonic acid hydrate) test cell proliferation technique (Roche). XTT measures cell proliferation by absorbance (450 nm wavelength). Doses of 0.5, 1, 2, 5, and 10 mmol/L ASA were added to a 96-well multplate, with 100 µL/well and 1000 cells/mL (Neubauer’s counter). All of the results were registered in quadruplicate at days 1, 3, and 6 after seeding. We also performed this experiment with ASA-free medium using DMEM, 0.2% BSA (Sigma).

We used recombinant rat PDGF-BB (Sigma), which was reconstituted with sterile PBS and added directly to samples at a final concentration of 10 ng/mL. We also used a specific anti–TGF-β neutralizing antibody (R&D Systems, Madrid, Spain), which was reconstituted with sterile PBS and directly added to samples (50 µg/mL final concentration).

LDH Measurements
To assess cytotoxicity, we measured LDH levels using a scanning multiwell spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader at 450 nm. The reference wavelength was 690 nm. Cells were seeded in a 12-well multplate and allowed to attach for 24 hours. After this period, cells were placed in 10% FCS plus vehicle or 10% FCS plus different concentrations of ASA (0.5, 1, 2, 5, and 10 mmol/L). After 24 hours, the supernatant was used to measure LDH by an ELISA reader. The absorbance is expressed as milliopacitodensities per minute (mOD/min).

Cell Cycle Experiments
We measured cell percentages at each phase of cell cycle following the VSMC-adapted protocol.16 To estimate the proportion of cells in each phase of the cell cycle, cellular DNA contents were measured by flow cytometry (FACS). Cells were plated, allowed to attach overnight, and placed in DMEM plus 0.4% FCS for 48 hours as described above. ASA (0.5, 1, and 2 mmol/L) was added in a serum-free medium composed of DMEM with 0.2% BSA and 10 ng/mL PDGF-BB. After 18 hours, the cells were harvested by trypsinization, washed with PBS, pelleted, and resuspended in PBS containing 0.6% Nonidet P-40 and 100 µg/mL propidium iodide, to which RNase was added to a final concentration of 100 µg/mL. Flow cytometric analysis was carried out with a FACSscan (Becton-Dickinson) flow cytometer equipped with a 15-mW Argon laser emitting at 488 nm. Propidium iodide fluorescence was determined through a 575/24-28 filter; 10,000 cells were acquired per sample, and a double discriminator module was used to ensure detection of single cells. We also performed these experiments adding 50 µg/mL of anti–TGF-β to the medium.

Northern Blot Assays
The expression of TGF-β mRNA was studied in serum-deprived VSMCs that were stimulated with 10 ng/mL of PDGF-BB in the presence or absence of 2 mmol/L of ASA. Total RNA was extracted from the cells using Trizol reagent (Gibco). Total RNA (20 µg) was separated in a 6% formaldehyde–1.2% agarose gel, blotted onto Hybond N+ membranes in 10×standard saline citrate (SSC). Membranes were washed in 2×SSC fixed by ultraviolet irradiation and baked at 80°C for 2 hours. Hybridization was performed overnight at 65°C in 5×SSC, 0.2% sodium dodecyl sulfate, 50 µmol/L sodium phosphate, 10×Denhardt’s solution, and 20 µg/mL salmon sperm. Blots were hybridized to a random-primed specific DNA probe for rat TGF-β and then exposed to Kodak X-Omat film.

ELISA Measurements
To determine TGF-β levels in the conditioned medium of control and treated samples, a solid-phase TGF-β–specific sandwich ELISA

Results
XTT Results
As shown in Figure 1, ASA inhibits proliferation of VSMCs. Nevertheless, a dose-dependent effect is only observed with concentrations of 0.5, 1, and 2 mmol/L. The maximum effect takes place on day 6, when the 2-mmol/L dose restrains proliferation by 30.86% compared with the PDGF control.

Specific anti–TGF-β1 (at a constant concentration of 50 µg/mL) reverses this inhibition, the final effect being independent of the ASA concentration. On the sixth day, a 50-µg/mL dose of antibody increased proliferation by 30.21% with respect to 2 mmol/L ASA (Figure 2).

LDH Measurements
Concentrations of 0.5, 1, 2, 5, and 10 mmol/L ASA did not increase LDH levels significantly (data not shown). Therefore, the antiproliferative effect of ASA cannot be attributed to cytotoxicity.

Cell Cycle Assays
It has been shown that TGF-β stops VSMC proliferation, restraining cells in G1 phase.17 Our results with 0.5, 1, and 2 mmol/L ASA show an increase in the number of cells in G1 and a decrease in the S phase. Both effects are dose-dependent (Figure 3).

In highly proliferating cell lines, TGF-β causes apoptosis by the p53 pathway. In our experimental conditions, apoptosis was not observed, as indicated by LDH levels.

Anti–TGF-β1 was unable to reverse this inhibition at 18 hours, perhaps because in cell proliferation experiments, the inhibition was observed on the sixth day but not at 24 hours.
Northern Blot
We found an important ASA-mediated decrease of TGF-β1 mRNA (Figure 4), similar to that observed after incubation with other antiproliferative drugs.18,19

ELISA Measurements
We first tried to detect the active form of TGF-β1 in control and ASA-treated samples. However, as expected by its short half-life (2 minutes), this proved impossible.

Levels of the latent form were 417±3.4 pg/mL for the control group (VSMCs with 10 ng/mL PDGF) and 439±5.3 pg/mL for the ASA-treated group (VSMCs with 10 ng/mL PDGF plus 2 mmol/L ASA). Although levels were slightly increased by ASA, the difference was not significant at a level of P<0.05.

Discussion
The involvement of ASA in the autocrine loop of TGF-β seems to have been demonstrated, although the molecular pathway has yet to be elucidated. We postulate that ASA inhibition of both forms of COX causes a retrograde inhibition of phospholipase A2 (PLA2). Protein kinase C (PKC) is a bifunctional mediator, activating both PLA2 and mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) (MAPK/ERK). If PKC is involved in PLA2 stimulation, it does not activate the MAPK/ERK pathway, which is essential for regulation of both cell proliferation and gene expression through early response genes, such as ELK-1, c-fos, and c-myc.20 Because TGF-β also increases MAPK/ERK activity,13 it might be released to balance this ASA effect, because a direct increase in MAPK/ERK activity through serine/threonine kinase receptors is a specific pathway of the TGF-β superfamily members.21 This hypothesis is in agreement with the recent findings demonstrating increased COX-2 transcription in TGF-β–treated VSMCs.22 TGF-β activates the ERK pathway in this manner, which induces the expression of new TGF-β via the AP-1 complex,18 reinducing ERK activation in highly proliferating cell lines. Therefore, PKC inhibitors, such as delapril, angiotensin II type 1 receptor antagonists,18 retinoids,20 and probably ASA, inhibit TGF-β expression despite their antiproliferative action. In other words, cells activate the TGF-β1 pathway when TGF-β1 is required and its expression is decreased, and therefore the TGF-β pathway and TGF-β1 transcription are inversely regulated.

In atherosclerosis, TGF-β is an important growth factor involved in plaque stability,23 playing a major role in trans-
forming a soft plaque (unstable angina, high risk of stroke) into a fibrotic plaque (stable angina, low risk of stroke). According to our findings, the preventive action of ASA in coronary disease may be attributable not only to its well-known antiplatelet effect and also to its induction of TGF-β pathway and inhibition of VSMC growth.

TGF-β seems to be an essential cytokine in VSMC homeostasis. When cells require TGF-β, it is activated by cleavage of its C-terminal sequence and is subsequently released. However, they remain attached to each other by noncovalent bonds, and it is this latent form that is measured by ELISA assays.

On the other hand, association between ASA and TGF-β could extend or restrain its clinical usage. TGF-β seems to be beneficial in colon, stomach, esophageal, pancreatic, skin, and bladder cancer chemoprevention, oral tolerance, cyclosporin treatment, and wound healing in macular hole. However, it is regarded as undesirable in some extended cancers, in infection by intracellular parasites and viruses such as HIV, in keloid scars, and in kidney fibrosis.

We conclude that TGF-β is a necessary factor for the antiproliferative effects of ASA in VSMCs, suggesting it as the major mechanism that ends at cellular restraint. Basic and clinical repercussions should therefore be investigated.

References


Figure 4. Northern blot analysis of rat VSMCs. RNA was extracted from starved VSMCs (line 1), VSMCs stimulated with 10 ng/mL PDGF-BB (line 2), and VSMCs stimulated with 10 ng/mL PDGF in the presence of 2 mmol/L ASA (line 3). Total RNA (20 μg) from each sample was analyzed by Northern blot, using a TGF-β1 probe (top) and subsequently rehybridized with a 7S cDNA probe to control RNA loading (bottom).
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