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.

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Key Words: aspirin ■ cells ■ aorta ■ transforming growth factor beta
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-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test to measure cell proliferation (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 containing 100 μL/well and 1000 cells/mL. 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 microtiter plate spectrophotometer assay. 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 PDGF-BB. After 24 hours, the supernatant was used to measure LDH by an ELISA reader. The absorbance is expressed as absorbance measured (A450 nm) and expressed as absorbance measured (A405 nm - A630 nm).

Statistical Analysis
The results are expressed as mean±SEM and accompanied by the number of observations. A statistical analysis of the data was carried out by a Student’s t test or by a two-way ANOVA, when necessary. Differences with a P<0.05 were considered statistically significant.

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. 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.\textsuperscript{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.\textsuperscript{20} Because TGF-β also increases MAPK/ERK activity,\textsuperscript{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.\textsuperscript{21} This hypothesis is in agreement with the recent findings demonstrating increased COX-2 transcription in TGF-β–treated VSMCs.\textsuperscript{22} TGF-β activates the ERK pathway in this manner, which induces the expression of new TGF-β via the AP-1 complex,\textsuperscript{18} reinducing ERK activation in highly proliferating cell lines. Therefore, PKC inhibitors, such as delapril, angiotensin II type 1 receptor antagonists,\textsuperscript{18} retinoids,\textsuperscript{20} and probably ASA, inhibit TGF-β expression despite their antiproliferative action. In other words, cells activate the TGF-β pathway when TGF-β is required and its expression is decreased, and therefore the TGF-β pathway and TGF-β transcription are inversely regulated.

In atherosclerosis, TGF-β is an important growth factor involved in plaque stability,\textsuperscript{23} playing a major role in trans-

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**Figure 2.** Comparison of cell proliferation of VSMCs with ASA and specific anti–TGF-β1 versus ASA alone. At the highest dose of ASA (2 mmol/L), specific anti–TGF-β1 (50 μg/mL) reversed ASA-mediated inhibition of cell growth by 30.21%.

**Figure 3.** Time-dependence of cell cycle progression in synchronized rat VSMCs measured by flow cytometry. Cells were plated, allowed to attach, and placed in 0.4% FCS-DMEM for 48 hours to synchronize them as described in the text. In 5 bottles, medium was replaced by DMEM with BSA 0.2%, with or without PDGF-BB (10 ng/mL), and BSA+PDGF plus different concentrations of ASA. In the other 5 bottles, we repeated the same procedure, adding anti–TGF-β1 (50 μg/mL). Hatched bars indicate percent of cells in G2/M; solid bars, percent in S; and dotted bars, percent in G0/G1. Results are from 3 independent experiments, each conducted in duplicate. C indicates control; Ab, anti–TGF-β1.
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 but 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 categories of patients. 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

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