Aspirin Reduces Apolipoprotein(a) (Apo(a)) Production in Human Hepatocytes by Suppression of Apo(a) Gene Transcription*

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High serum lipoprotein(a) (Lp(a)) is a risk factor for vascular disorders. Our preliminary observations suggest that, in some patients with coronary heart disease with high serum Lp(a) levels, administration of aspirin reduced Lp(a) levels. Therefore, we aimed to analyze the effects of aspirin on the production of apo(a), the expression of apolipoprotein(a) (apo(a)) mRNA and the transcriptional activity of apo(a) gene promoter. Aspirin (5 mM) reduced the apo(a) levels in culture medium of human hepatocytes and suppressed apo(a) mRNA expression to 73% and 85% of the controls, respectively. Aspirin also reduced the transcriptional activity of apo(a) gene transfected into HepG2 hepatoma cells in a dose-dependent manner, with a maximal effect at 5 mM (44.3 ± 1.5% of the control). Sodium salicylate (5 mM) also reduced apo(a) gene transcription, whereas indomethacin (10 μM) had no effect. Deletion analysis of apo(a) gene promoter showed that promoter region extending from −30 to +138 is critical for the effect of aspirin. Furthermore, enhanced production, mRNA expression, and gene transcription of apo(a) by interleukin-6 were also inhibited by aspirin. These results demonstrate that aspirin reduces apo(a) production from hepatocytes via reduction of the transcriptional activity of apo(a) gene with suppression of apo(a) mRNA expression. The suppression of apo(a) production by aspirin may at least in part play a role in the anti-atherogenic effect of aspirin in vascular disorders.

Lipoprotein(a) (Lp(a)) is a low density lipoprotein-like lipoprotein in which apolipoprotein B-100 is disulfide-linked to an additional high molecular weight glycoprotein, apolipoprotein(a) (apo(a)) (1). Lp(a) has been shown to be deposited in atherosclerotic plaques (2–4). Because apo(a) is highly homologous to plasminogen (5), it competes with plasminogen for binding to its receptor, resulting in the inhibition of plasmin formation (6, 7) and transforming growth factor-β activation (8, 9). These properties of Lp(a) lead to retardation of clot lysis and acceleration of cell growth. Serum Lp(a) level is strongly influenced by genetic backgrounds and is not influenced by age, foods, or environmental conditions (10). However, in several disease states such as inflammatory disorders, serum Lp(a) levels are elevated (11). Epidemiological studies demonstrated that patients with cardiovascular or cerebrovascular diseases show higher serum Lp(a) levels (12, 13), and that elevated serum Lp(a) is an independent risk factor for coronary heart disease in both men and women aged 55 years and younger (14, 15). It has been reported that nicotinic acid can lower serum Lp(a) levels by as much as 38% (16), although several adverse effects by this agent hampers general clinical use for patients with high serum Lp(a) levels.

Aspirin has been used widely in patients with atherosclerotic diseases, and its efficacy in preventing coronary heart disease has been established. Although the effect of aspirin is thought to be mainly due to an inhibition of platelet aggregation, other possibilities have not been ruled out. Because these patients are often complicated with hyperlipidemia, many investigators examined the influence of aspirin on serum cholesterol and triglyceride levels with negative results (17).

Recent investigations on the action of aspirin have revealed novel mechanisms that aspirin affects transcriptional factors and an antioxidant protein. In humans, aspirin inhibits the activities of transcriptional factors such as nuclear factor-κB (NF-κB) (18–20) and activator protein 1 (AP-1) (21, 22) and activates the heat shock transcriptional factor (23). These effects may explain the anti-atherosclerosis, anti-carcinogenesis, and anti-inflammatory mechanisms of aspirin. In addition, aspirin increases ferritin synthesis in bovine pulmonary artery endothelial cells (24), whereby aspirin may enhance endothelial resistance to oxidative damage.

We have been evaluating the effect of aspirin on serum Lp(a) levels in several patients with coronary heart diseases or old cerebral infarction with high levels of serum Lp(a), and preliminary observations suggest that serum Lp(a) levels decrease after treatment with 81 mg/day of aspirin by 15–20% (2). Because serum Lp(a) levels are determined mainly by the synthesis of apo(a) protein in the liver (25, 26), and because the synthesis of apo(a) is mostly regulated by the expression of apo(a) gene (27), we examined whether aspirin modulates the apo(a) production, apo(a) mRNA expression, and transcriptional activity of apo(a) gene promoter using normal human hepatocytes and HepG2 hepatoma cells.

EXPERIMENTAL PROCEDURES

Materials—Aspirin (Wako Chemical Co., Osaka, Japan) and indomethacin (Wako Chemical Co.) were dissolved in absolute ethanol and added to cultures at a final ethanol concentration of 0.1%. Sodium salicylate and interleukin (IL)-6 (Wako Chemical Co.) were dissolved in Dulbecco’s phosphate-buffered saline (Life Technologies, Inc.).

Analysis of Apo(a) Production from Cultured Hepatocyte—Human hepatocytes from several individuals were obtained (Cell Systems Corp., Kirkland, WA) and cultured in collagen-coated 10-cm dishes and

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The abbreviations used are: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); IL, interleukin; COX, cyclooxygenase; NF-κB, nuclear factor-κB; AP-1, activator protein 1.


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maintained in 5% CO₂ in CS-C serum-free medium (Cell Systems Corp.) according to manufacturer’s instructions. Hepatocytes capable of producing the highest amounts of Lp(a) were selected by using a TintElize Lp(a) kit (Biopool AB, Umeå, Sweden), propagated, and stocked for the following study. At subconfluence, culture medium was removed and fresh 10% containing aspirin and/or IL-6 was added, and the cells were incubated for an additional 48 h. Culture medium was then collected, 10 μM benzamide was added, and the medium was centrifuged at 1,300 × g for 5 min to remove cellular debris. The medium was concentrated by centrifugation using Centricon Plus-20 Centrifugal Filter Devices (Millipore Corp., Bedford, MA). Apo(a) levels in the medium were measured by TintElize Lp(a) kit. Because this enzyme-linked immunosorbent assay kit uses polyclonal anti-human apo(a) antibodies for catching and detecting the apo(a) protein, both free and bound forms of apo(a) can be detected.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Apo(a) mRNA—Total RNA was isolated from normal human hepatocytes treated with or without aspirin and/or IL-6 for 24 h using a commercially available kit (Qiagen Inc., Chatsworth, CA). RNA samples were quantitated by absorbance at 260 nm, and 1 μg of RNA in a 20 μl volume was reverse transcribed with oligo(dT)₁₇ primer using an Advantage RT-for-PCR kit (CLONTECH). One micro-liter of the reverse transcription reaction mixture was then subjected to PCR for Taq polymerase (Takara Shuzo Co., Shiga, Japan) using both the forward primer 5'-ACCTGAGCAAAGCCATGTG-3' corresponding to nucleotide numbers 99–117 (numbering according to McLean et al. (Ref. 5)), and the reverse primer 5'-AGTACTCCCACTCTGACCG-3', corresponding to 324–343, according to a protocol described by Rouy et al. (28).

Linearity of the PCR reaction was tested by amplification of 200 ng of total RNA per reaction from 15 to 50 cycles. The linear range was found to be between 15 and 40 cycles. In no case did the amount of RNA used for PCR reaction exceed 200 ng/reaction. The sample were amplified for 25–30 cycles using the following denaturation, annealing, and extension conditions: 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 90 s. The amplified products were confirmed to be an apo(a) cDNA by sequencing analysis. Glyceraldehyde-3-phosphate dehydrogenase primers were used as a control.

Ribonuclease Protection Assay—Apo(a) probe corresponding to the region spanning 99 to 343 of apo(a) cDNA was prepared as described above and was ligated into the pcRT<sub>2</sub>g2.1 vector (Invitrogen, San Diego, CA). The resulting plasmid containing apo(a) probe inserted in the inverted orientation (3' to 5' in front of the Sp6 promoter) was digested with EcoRV and used as a template for the synthesis of the antisense RNA probe. Radioactive transcripts were prepared using [γ⁻³²P]UTP and T7 RNA polymerase RPA II<sup>TM</sup> kit (Ambion, Austin, TX). Ten micrograms of RNA and 50,000 cpm/sample of the apo(a) probe or 20,000 cpm/sample of the β-actin probe were co-precipitated, resuspended in hybridization buffer, and incubated overnight at 42 °C. Unbound RNA was then digested with RNase, and the remaining radioactive RNA was precipitated, resuspended in loading buffer, and separated on a 6% polyacrylamide gel. Gels were dried and exposed to X-Omat<sup>TM</sup> AR film (Eastman Kodak Co.) at –80 °C. The intensities of the protected bands were quantitated by densitometric scanning.

Construction of Luciferase Reporter Plasmid and DNA Transfection—Chimeric luciferase plasmids were constructed with modification of a previously reported method (29). In brief, 1,438-, 1,088-, 698-, 441-, and 168-base pair fragments of apo(a) gene promoter extending from nucleotides –1,300 to +138 (pGL2/apo/–1300), –950 to +138 (pGL2/apo/–950), –560 to +138 (pGL2/apo/–560), –303 to +138 (pGL2/apo/–303), and –30 to +138 (pGL2/apo/–30) (numbering according to Wade et al. (Ref. 30)) were ampliﬁed with both the forward primer 5'-GGAAATTCTATTGCGGAAAGATTTG-3' (italics indicate an added XhoI site present in the promoter sequence), 5'-CCCTAGTTTTTATTTTATTTAATA-3' (italics indicate an added DraI site in the promoter sequence), 5'-GATTTGATATTATAAACATTTA-3' (italics indicate an added EcoRV site in the promoter sequence), 5'-TTGGAAAAACCTTGGAGGAGCTATTGATTG-3' (italics indicate an added HindIII site in the promoter sequence), or 5'-GGGATATGCGATCTATATCTCAAGATTTA-3' (italics indicate an added EcoRV site) and the reverse primer 5'-CCCTCTGGAGGACTCTCTCCGAGCCTGCGAGCTGCGCCAG-3' (italics indicate an added XhoI site). The ampliﬁed PCR fragments were cloned into the EcoRI/XhoI, EcoRV/XhoI, EcoRV/XhoI, HindIII/XhoI, or EcoRV/ XhoI site of pBS/KS<sup>+</sup> (Stratagene, La Jolla, CA). The resultant clones were digested with SmaI (3' of the EcoRI, DraI, EcoRV, and HindIII poly linker sites of pBS/KS<sup>+</sup>) and XhoI. The SmaI/XhoI fragments were cloned into the SmaI/XhoI site of the luciferase reporter vector pGL2/Basic (Promega, Madison, WI). HepG2 cells were cultured and cotransfected with 2 μg of a chimeric luciferase plasmid and 1 μg of pSV-β-galactosidase (Promega) using LipofectaMINE reagent (Life Technologies, Inc.), as described previously (29).

Results of Aspirin and IL-6 on Lp(a) Levels in Culture Medium of Normal Human Hepatocytes—Apo(a) levels were measured in 10 ng/ml were added to subconfluent hepatocytes in collagen-coated 10-cm dishes and cultured for 48 h. Culture medium was then collected and concentrated. Lp(a) levels in concentrated medium were measured using a TintElize Lp(a) kit. Data are means ± S.E. for four experiments. * significantly different from the control Lp(a) levels in culture medium (p < 0.01). **, significantly different from the control and the IL-6 treated Lp(a) levels in culture medium (p < 0.01).

Treatment of Transfected Cells—At 16 h after transfection, the medium was replaced by 2 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 20 μl of i-glutamine and various agents, and the cells were incubated for an additional 48 h. All cultures without aspirin, sodium salicylate, indomethacin, or IL-6 contained the respective vehicles.

Assay of Luciferase and β-Galactosidase Activities—Cell lysates were prepared as previously reported (29). Luciferase activity was measured with a luminometer (model 1253; Bio-Oribit Oy, Turku, Finland) by mixing 100 μl of luciferase substrate solution (Toyobo, Tokyo, Japan) with 20 μl of cell lysates. For measurement of β-galactosidase activity, 20 μl of cell lysates diluted 100-fold with 0.1 M potassium phosphate buffer was mixed with 200 μl of Galactone (Tropix, Bedford, MA), which was previously diluted 100-fold with 0.1 M potassium phosphate, 1 mM magnesium chloride pH 7.8, for 1 h at room temperature. Then, β-galactosidase activity was measured following addition of 300 μl of Em- red (Tropix). Luciferase activity was normalized with β-galactosidase activity.

Statistical Analysis—Values are expressed as means ± S.E. Statistical analyses of the data were performed by a paired t test for serum Lp(a) levels (Fig. 1B), and by one-way analysis of variance followed by Bonferroni’s test for in vitro experiments (Figs. 1–5). p values less than 0.05 were considered statistically significant.

RESULTS

Effects of Aspirin on the Production of Apo(a) Protein—To examine whether aspirin affects the production of apo(a) from the liver, the apo(a) levels in culture medium from normal human hepatocytes were measured in the presence or absence of aspirin. The results showed that 5 mM aspirin reduced the apo(a) levels in culture medium to 73% of the control (Fig. 1).

Effects of Aspirin on the Expression of Apo(a) mRNA and the
Transcriptional Antivity of Apo(a) Gene—Because the levels of apo(a) gene expression are known to play a major role in determining the hepatic production and serum levels of Lp(a) (27), we next examined the effects of aspirin on apo(a) mRNA expression and transcriptional activity of apo(a) gene promoter, using RT-PCR analysis, ribonuclease protection assay, and luciferase reporter assay. As shown in Fig. 2A (lanes 1 and 2), 5 mM aspirin suppressed the expression of apo(a) mRNA, calculated from density ratio of apo(a)/glyceraldehyde-3-phosphate dehydrogenase cDNA, to 60% of the control value. To quantitate more accurately the changes of apo(a) mRNA levels, ribonuclease protection assay was performed and demonstrated that 5 mM aspirin also suppressed the expression of apo(a) mRNA to 85% of the control values (Fig. 2B, lanes 1 and 2). Then, luciferase reporter plasmid containing the 5’ flanking region of apo(a) gene promoter extending from nucleotides −1300 to +138 (relative to the transcription start site) was constructed (pGL2/apo−1300) and was transfected into HepG2 human hepatoma cells to assess the luciferase activity in the presence or absence of aspirin. The luciferase activity of pGL2/apo−1300 in the absence of aspirin was taken as a control. As shown in Fig. 3A, aspirin treatment reduced the luciferase activity. A significant reduction of luciferase activity was observed at the lowest dose of aspirin (0.05 mM), and a maximal effect was obtained at 5 mM (44.3 ± 1.5% of the control value). The suppressive doses of aspirin are relevant to the serum concentrations in patients taking aspirin (1–3 mM) (19).

Effects of Sodium Salicylate and Indomethacin on the Transcriptional Activity of Apo(a) Gene—When sodium salicylate, a metabolite of aspirin, was added at similar concentrations, the luciferase activity was also reduced in a dose-dependent manner (Fig. 3B). However, it appears that a slightly higher concentration of sodium salicylate (0.5 mM) was required to cause a significant reduction in luciferase activity. Although aspirin is known to inhibit cyclooxygenase (COX)-1, sodium salicylate does not inhibit the enzyme. In order to confirm that the effect of these anti-inflammatory agents was not mediated via an inhibition of prostaglandin synthesis, the effect of a potent inhibitor of COX-1, indomethacin, was examined (Fig. 3C). At concentrations comparable to the plasma levels in patients receiving effective doses of indomethacin (1–20 μM) (19), no inhibitory effect of indomethacin was observed on the transcriptional activity of apo(a) gene. These results are consistent with the assumption that aspirin and sodium salicylate can reduce the transcriptional activity of apo(a) gene independent of prostaglandin synthesis.

Effects of Aspirin on the Production, mRNA Expression, and Transcriptional Activity of Apo(a) in the Presence of IL-6—We have shown that IL-6 enhances the transcriptional activity of apo(a) gene promoter (29), which may be the mechanism for the elevation of serum Lp(a) levels under inflammatory conditions. In fact, we confirmed in the present study that IL-6 (10 ng/ml) increased the production of apo(a) from hepatocytes (Fig. 1) and markedly enhanced the expression of apo(a) mRNA (Fig. 2, A
and B, lane 3). Therefore, we examined whether aspirin can suppress the increase in apo(a) production, apo(a) mRNA expression, and transcriptional activity of apo(a) gene by IL-6. The results demonstrated that the increased production of apo(a) from hepatocytes in the presence of IL-6 (10 ng/ml) was significantly suppressed by aspirin (5 mM) (Fig. 1). As shown in Fig. 2 (A and B), RT-PCR analysis and ribonuclease protection assay also revealed that aspirin (5 mM) markedly suppressed the induction of apo(a) mRNA by IL-6 (10 ng/ml). In addition, IL-6 (10 ng/ml) increased the luciferase activity to 193.6 ± 7.5% of the control, and simultaneous addition of aspirin (5 mM) along with IL-6 (10 ng/ml) completely inhibited the IL-6-induced increase and further reduced the luciferase activity to 65.2 ± 1.0% of the control (Fig. 4).

**Deletion Analysis of Apo(a) Gene Promoter**—In order to further elucidate which region(s) of apo(a) gene promoter is responsible for the actions of aspirin, four different lengths of deletion luciferase plasmids containing the promoter regions extending from −950 to +138 (pGL2/apo−/−950), −560 to +138 (pGL2/apo−/−560), −303 to +138 (pGL2/apo−/−303), and −30 to +138 (pGL2/apo−/−30) were constructed, and changes in luciferase activity in the presence or absence of aspirin were assessed. As shown in Fig. 5, with all four shorter constructs examined, aspirin reduced the luciferase activity to a similar degree to that with pGL2/apo−/1300, although basal transcriptional activity varied with different lengths of constructs. These results suggest that the effect of aspirin on apo(a) gene transcription is mediated via a promoter sequence from −30 to +138, and that aspirin may exhibit its effect by modulating a transcriptional factor(s) that can bind to this region.

**DISCUSSION**

The present studies demonstrated that therapeutically relevant concentrations of aspirin can effectively reduce the production of apo(a) from cultured normal human hepatocytes by suppressing the apo(a) mRNA expression and gene transcription. In addition, aspirin also reduced the enhanced apo(a) gene transcription and mRNA expression along with the elevated apo(a) production from hepatocytes induced by IL-6. Accumulated evidence indicates the close relationship between inflammation and atherosclerosis. In a prospective study by Ridker et al. (31), elevated serum levels of C-reactive protein are shown to predict the risk of future myocardial infarction and stroke, and aspirin can reduce the risk of a first myocardial infarction with a significant decrease in C-reactive protein levels. Biasucci and colleagues (32) demonstrated that serum levels of IL-6 in patients with unstable angina are commonly elevated, are correlated with C-reactive protein levels, and are associated with the prognosis of the disease. In the light of these observations, there is a possibility that the inflammation-associated risk of vascular events is mediated at least in part by an IL-6-induced increase in serum Lp(a) levels. Therefore, one of the plausible mechanisms for aspirin to prevent these events may be to reduce serum Lp(a) levels, and large scale clinical studies for analyzing the effect of aspirin on serum Lp(a) levels are warranted.

The rate of production but not the catabolism of Lp(a) plays a major role in determining serum Lp(a) levels (25, 26). The rate of Lp(a) production could be affected by transcriptional efficiency (29), stability of apo(a) mRNA, post-translational processing of apo(a) protein (33), and efficiency of apo(a)-apo-
lipoprotein B-100 complex formation (34, 35). Among these steps, many previous studies support the importance of the expression levels of apo(a) mRNA to determine the serum LP(a) levels. There is a correlation between serum LP(a) levels and hepatic apo(a) mRNA levels in cyromulogus monkeys (27), baboons (36), and humans (37). Ramharack et al. (38) reported that gemfibrozil suppresses the expression of apo(a) mRNA, which leads to a significant reduction in plasma LP(a) levels. In addition, we have previously demonstrated that IL-6 and all-trans-retinoic acid enhance transcriptional activity of apo(a) gene in vitro, and treatment with all-trans-retinoic acid elevates serum LP(a) levels in vivo (29). The present studies demonstrate that aspirin can reduce the production of apo(a) from hepatocytes as well as apo(a) mRNA expression with a suppression of the transcriptional activity of apo(a) gene both in the presence and absence of IL-6. From these results, it is plausible to assume that serum LP(a) levels are modulated by the expression levels of apo(a) mRNA, and that the levels of apo(a) mRNA are regulated mainly by transcriptional activity of apo(a) gene. The present results also suggest that new agents which are capable of reducing serum LP(a) level can be created by screening their effects on apo(a) gene promoter activity.

The molecular mechanism of action of aspirin in platelets, vessel walls, stomach, and kidneys is well characterized. Aspirin is shown to acetylate the hydroxyl group of a serine residue at position 529 in the polypeptide chain of COX-1, resulting in decreased conversion of arachidonate to prostaglandin G2 and ultimately to prostaglandin H2 and thromboxane A2 (39). How- ever, because inhibition of COX-1 by indomethacin had no effect on the transcriptional activity of apo(a) gene (Fig. 3C), the mechanism of action of aspirin on apo(a) gene transcription appears to be different. In addition, sodium salicylate, which does not inhibit COX-1, suppressed the transcription of apo(a) gene. It has been reported that sodium salicylate affects prosta glandin-independent signaling process via an inhibition of NF-κB (18, 40, 41). Aspirin is also reported to inhibit the activation of NF-κB by preventing the degradation of IκB (18) or by blocking the induction of NF-κB (19) and the activity of AP-1 via an elevation of intracellular H+ concentration (21) or via blocking of activation of mitogen-activated protein kinase family members (22). Thus, there is a possibility that these agents affect the transcriptional activity of apo(a) gene by a similar mechanism(s).

Apo(a) gene contains putative binding sites for several transcriptional factors including NF-κB at nucleotide −1074, AP-1 at +82, NF-IL6 at −1132, −999, −740, −606, −139, −82, and +97, and C/EBP at −770, −112, −54, and +102 of apo(a) gene promoter (Fig. 5) (30, 42). As mentioned above, because aspirin and sodium salicylate can inhibit the activation of NF-κB and AP-1, the suppression of apo(a) gene transcription may be mediated by these transcriptional factors. The resulted obtained by deletion analysis suggests that the negative regulatory element(s) elicited by aspirin exists between nucleotide −30 and +139 of apo(a) gene promoter, and that a transcriptional factor(s) binding to this region may be affected by aspirin. Wade et al. (30) reported that a possible negative regulatory element was present around a putative AP-1 binding site at +82 of apo(a) gene promoter, but that the binding of nuclear proteins to this site was not displaced by excess amounts of AP-1 oligo-nucleotide competitor. These results provide evidence that an unknown transcriptional factor(s) other than AP-1 is likely to be involved in binding to this site. Although the effect of aspirin on transcriptional activity of apo(a) gene promoter could be mediated by this unknown transcriptional factor, further stud-
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