Effect of Low-Dose Aspirin on Vascular Inflammation, Plaque Stability, and Atherogenesis in Low-Density Lipoprotein Receptor–Deficient Mice

Tillmann Cyrus, MD; Syuan Sung, BSc; Lei Zhao, MD; Colin D. Funk, PhD; Syun Tang, MD; Domenico Praticò, MD

Background—Atherosclerosis is a complex vascular inflammatory disease. Low-dose aspirin is a mainstay in the prevention of vascular complications of atherosclerosis. We wished to determine the effect of low-dose aspirin on vascular inflammation, plaque composition, and atherogenesis in LDL receptor–deficient mice fed a high fat diet.

Methods and Results—In LDL receptor–deficient mice fed a high fat diet compared with control mice, low-dose aspirin induced a significant decrease in circulating levels and vascular formation of soluble intercellular molecule-1, monocyte chemoattractant protein-1, tumor necrosis factor-α, interleukin-12p40, without affecting lipid levels. This was associated with significant reduction of the nuclear factor κB activity in the aorta. Low-dose aspirin also significantly reduced the extent of atherosclerosis. Finally, aortic vascular lesions of the aspirin-treated animals showed 57% reduction (P<0.05) in the amount of macrophage cells, 77% increase in smooth muscle cells (P<0.05), and 23% increase in collagen (P<0.05).

Conclusions—Our results suggest that in murine atherosclerosis, low-dose aspirin suppresses vascular inflammation and increases the stability of atherosclerotic plaques, both of which, together with its antiplatelet activity, contribute to its antiatherogenic effect. We conclude that low-dose aspirin might be rationally evaluated in the progression and evolution of human atherosclerotic plaque.

Key Words: atherosclerosis • aspirin • inflammation • plaque

Low-dose aspirin is used to prevent complications of atherosclerotic cardiovascular disease such as myocardial infarction and stroke, the No. 1 and No. 3 causes of death, respectively, in the United States.¹ The therapeutic effect of aspirin is generally attributed to its platelet-inhibitory function.²,³ Whether aspirin has in vivo a more profound and complex action, particularly on the vasculature, remains to be fully elucidated. The past decade has been characterized by growing evidence that atherosclerosis is an inflammatory disease of the vasculature.⁴ Thus, circulating levels of inflammatory markers predict the risk of cardiovascular events in the atherosclerotic disease,⁵ and aspirin intake reduces these levels and the risk of vascular events.⁶ Previously, we have shown that indomethacin, an aspirin-like drug, suppressed thromboxane (Tx)A₂ biosynthesis and reduced atherosclerosis.⁷ In that study, indomethacin also significantly reduced prostacyclin (PGI₂), which has anti-inflammatory activity in vitro. It inhibits platelet activation, leukocyte-adhesive interactions, and vascular smooth muscle cell migration and proliferation, mechanisms that may all have antiatherogenic effects.⁸ To the best of our knowledge, the effect of aspirin and other anti-inflammatory drugs on the vascular inflammatory component of atherogenesis and plaque composition is not known.

The present studies were designed to address two questions. First, we wished to determine whether low-dose aspirin had any effect on vascular inflammation during atherogenesis. Second, we wished to determine the effects of this pharmacological intervention on plaque composition in LDL receptor–deficient (LDLR−/−) mice on a high fat diet. Long-term administration of aspirin significantly reduces vascular inflammation and aortic atherosclerotic lesions. Furthermore, it decreases macrophage-foam cell content but increases the amount of collagen and the number of smooth muscle cells present in the atherosclerotic plaques. These findings demonstrate that aspirin manifests a potent antiatherogenic effect not only by inhibiting platelet activation but also by suppressing vascular inflammation and increasing plaque stability.

Methods

Animals
LDLR−/− mice (back-crossed 10 times to C57BL/6 mice) were obtained from Jackson Laboratories (Bar Harbor, Maine) at 6 weeks of age. All procedures and care of animals were approved by the

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IACUC of the University of Pennsylvania. After 2 weeks of acclimatization, they were fed a high fat diet (normal chow supplemented with 0.15% cholesterol and 20% butter fat) for the entire study. At this time, animals were divided into 2 groups (n = 14 each) and randomized to receive placebo or aspirin (30 mg/L) in their drinking water, which was replaced with fresh water every other day. Considering that each animal drinks in average 3 to 4 mL of water per day, this would be equal to 90 to 120 μg aspirin per day for a mouse of 30-g of weight. On a body scale-adjusted scale, this amount would be equal to 180 to 240 mg/d if the animals weighed 60 kg. Urine and plasma were collected before aspirin was started (baseline, 8 weeks of age) and at the end of the study (26 weeks of age), as previously described.7,9

COX-1 Activity Ex Vivo

COX-1 activity ex vivo was assessed by measurement of serum TbB 2 and platelet aggregation induced by arachidonic acid (100 μM), as previously described.7,9

Biochemical Analyses

Serum TbB 2, urinary 2,3-dinor TbB 2, and 2,3-dinor 6-keto PGF 1α were measured by gas chromatography/mass spectrometry assays, as previously described.7,9 Plasma cholesterol and triglyceride levels were determined enzymatically by using Sigma reagents (Sigma Chemical Co). Soluble intercellular adhesion molecule-1 (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-12 p40 (IL-12p40) levels were measured by ELISA kits, following the manufacturer’s instructions (Pierce Endogen).

Aortic Formation of Cytokines

After the mice were killed, the aortic tree was perfused with PBS containing EDTA (2 mM/L) and butylated hydroxytoluene BHT (20 μmol/L), pH 7.4, by inserting a cannula into the left ventricle and allowing free efflux from an incision in the inferior vena cava. After removal of the surrounding adventitial fat tissue, the thoracic aorta was separated from the arch and the abdominal regions. One centimeter of the thoracic aorta (n = 5) was cut and incubated in serum-free medium at 37°C for 24 hours, with a gentle shaking. At the end of the incubation time, supernatants were collected and assayed for cytokine levels.

Electrophoretic Mobility Shift Assay

Nuclear extracts from mouse aortas (abdominal regions) were dissociated with the use of a mortar and pestle and extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co). Protein concentrations were determined by the Bradford method (Bio-Rad Laboratory). Double-stranded nuclear factor-κB (NF-κB) consensus oligonucleotide (5′-AGTGAAGGGGGA-CTTCCAGGC-3′), (Promega Corp, Madison, Wis) was used as probe after 5′-end labeling and purification. Binding reactions were performed as follows: nuclear extracts (10 μg protein) were incubated with radiolabeled DNA probes (70 fmol, 2.5 × 106 cpm) at room temperature for 30 minutes in 20 μL of binding buffer (4% glycerol, 1 mM/L MgCl 2, 0.5 mM EDTA; 0.5 DTT; 50 mM/L NaCl; 10 mM/L Tris-HCl, pH 7.5; 0.05 mg/mL poly-d-IdC). Competitor unlabeled oligonucleotide was added to the reaction at 50-fold molar excess. For supershift assay analyses, samples were incubated with anti-p50, anti-p65, anti-C-Rel, or anti-p52 rabbit antibodies (Santa Cruz Biothecnol, Santa Cruz, Calif). The products of the binding reaction were electrophoresed through 5% nondenaturing polyacrylamide gels in 1 x TB buffer. Gels were dried and analyzed by autoradiography. The assays were always performed in a blinded fashion.

Preparation of Mouse Aortas and Quantification of Atherosclerosis

After the final blood collection, mice were killed, and the aortic tree was perfused for 10 minutes with ice-cold PBS, as previously described.7,9 The aorta was opened longitudinally from the aortic root to the iliac bifurcation, fixed in formal-sucrose (4% paraformaldehyde, 5% sucrose, 20 μmol/L BHT and 2 mmol/L EDTA, pH 7.4), then stained with Sudan IV (n = 9). The extent of atherosclerosis was determined by using the en face method.7,9 Atherosclerosis was also quantified in the aortic root cross sections from fresh-frozen OCT-embedded hearts, as previously described.9 Briefly, alternate 10-μm frozen sections on the aortic root covering 300 μm of the proximal aorta, starting at the sinus, were fixed in acetone, rehydrated, and stained for atherosclerotic lesions with oil red O. Images were captured digitally and analyzed always in a blinded fashion, as previously described.7,9

Histology and Immunohistochemistry

Briefly, serial 6-μm frozen sections of the aortic root covering 300 μm of the proximal aorta, starting at the sinus, were fixed in 1% paraformaldehyde, rehydrated, and stained for collagen type I and type III, as previously described.10,11 Briefly, sections were incubated for 90 minutes in 0.1% sirius red F3BA (Polysciences Inc) in saturated picric acid. After rinsing in 0.1% HCl and in distilled water, sections were dehydrated with 70% ethanol and analyzed by polarization microscopy.11 Immunostaining for macrophage content was performed as previously described.10,11 Briefly, the avidin-biotin-alkaline phosphatase method (Vector Laboratories and Boehringer Mannheim, GmbH), using rat mAb to mouse macrophages (MOMA-2; Accurate Chem Sci Corp) diluted in PBS 1:30 was used. For smooth muscle cells, monoclonal mouse anti-human smooth muscle α-actin (clone 1A4, dilution 1:400, Sigma Chemical Co) was used as the primary antibody followed by a secondary peroxidase-conjugated rabbit anti-mouse antibody (P-0260, dilution 1:300, Dako). Antibody reactivity was detected with the use of a Nova red substrate kit (SK-4800, Vector Laboratories Inc). Cross sections were counterstained with hematoxylin. As control, no primary antibody was added to the same sections. Macrophage, smooth muscle cell, and collagen-positive regions were quantified in sections by determination of the area that stained positive for the respective markers.9,12 Image acquisition and analysis were always performed in a blinded fashion.

Statistical Analysis

Results are expressed as mean±SEM. Data were analyzed by ANOVA and subsequently by Student’s unpaired 2-tailed t test, as indicated. Probability values <0.05 were considered significant.

Results

Starting at 8 weeks of age, LDLR−/− mice were fed a high fat diet for the entire study. At that time, mice were randomized to receive placebo or aspirin (30 mg/L daily) in their drinking water. Assuming that each mouse drinks 3 to 4 mL of water per day, the estimated daily intake of the drug was calculated as ~5 mg/kg per day. There were no apparent differences between the two groups in terms of mobility, behavior, and food intake during the entire study. As expected, after the mice were given a high fat diet for 10 weeks, we observed an increase in weight and plasma lipid levels that was not different between the two groups (not shown). At the end of the study, that is, at 26 weeks of age, both groups also had similar increases in plasma cholesterol, triglyceride levels, and body weight (Table). Compared with baseline, excretion was increased both in 2,3-dinor TbB 2, the major murine TxA 2 metabolite,9 and 2,3-dinor-6-keto PGF 1α, the PGJ 2 metabolite,9 in atherosclerotic mice (Table). Mice receiving aspirin had reduced 2,3-dinor TbB 2 urinary excretion after 10 weeks (15 ±1.5 versus 52 ±4 mg/kg creatinine, P <0.01), which was suppressed along with serum TbB 2 and ex vivo platelet aggregation by the end of the study (Table). In contrast, after the mice received aspirin for 10 weeks, we observed a small
Body Weight, Plasma Cholesterol, Triglycerides, Serum TxB\(_2\), Arachidonic Acid–Induced Platelet Aggregation, Urinary 2,3-dinor TxB\(_2\), and 2,3-dinor 6keto PGF\(_{1\alpha}\) Levels in LDLR\(^{-/-}\) Mice at 8 Weeks of Age (Base) and After 18 Weeks on a High-Fat Diet (Final), With or Without Low-Dose Aspirin (n=14 Animals for Each Group)

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Aspirin</th>
</tr>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>20(\pm)1.5</td>
<td>22(\pm)2.0</td>
</tr>
<tr>
<td>Final</td>
<td>37.5(\pm)3</td>
<td>38.5(\pm)2.1</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>190(\pm)20</td>
<td>205(\pm)16</td>
</tr>
<tr>
<td>Final</td>
<td>1750(\pm)40</td>
<td>1850(\pm)50</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>85(\pm)12</td>
<td>95(\pm)10</td>
</tr>
<tr>
<td>Final</td>
<td>800(\pm)45</td>
<td>850(\pm)55</td>
</tr>
<tr>
<td>TxB(_2), ng/mL</td>
<td>180(\pm)15</td>
<td>198(\pm)17</td>
</tr>
<tr>
<td>Final</td>
<td>195(\pm)10</td>
<td>10(\pm)2.4*</td>
</tr>
<tr>
<td>Platelet aggregation, LT%</td>
<td>80(\pm)15</td>
<td>8(\pm)2*</td>
</tr>
<tr>
<td>2,3-dinor TxB(_2), ng/mg creatine</td>
<td>6.8(\pm)1</td>
<td>6.2(\pm)0.8</td>
</tr>
<tr>
<td>Final</td>
<td>95(\pm)4</td>
<td>11(\pm)1.8*</td>
</tr>
<tr>
<td>2,3-dinor 6keto PGF(_{1\alpha}), ng/mg creatine</td>
<td>0.32(\pm)0.03</td>
<td>0.30(\pm)0.02</td>
</tr>
<tr>
<td>Final</td>
<td>2.8(\pm)0.5</td>
<td>2.1(\pm)0.3</td>
</tr>
</tbody>
</table>

LT indicates light transmission.

Results are expressed as mean\(\pm\)SEM.

\(^*P<0.001\) vs placebo.

reduction in 2,3-dinor 6 keto PGF\(_{1\alpha}\) urinary excretion (1.6\(\pm\)0.1 versus 1.8\(\pm\)0.1, \(P>0.05\)). By the end of the study, we found a reduction of \(\approx 25\%\), which did not reach statistical significance, compared with placebo (Table).

Vascular Inflammatory Responses

To explore the vascular inflammatory responses associated with atherogenesis in LDLR\(^{-/-}\), we performed two sets of experiments. First, we determined circulating levels of several inflammatory cytokines that have been involved in atherosclerosis.\(^{12}\) At the end of the study, LDLR\(^{-/-}\) mice (n=10 for each group) on a high fat diet had a significant increase in s-ICAM-1 (5.1\(\pm\)1 versus 13.5\(\pm\)1.2 ng/mL, \(P<0.01\)), MCP-1 (90\(\pm\)10 versus 185\(\pm\)15 ng/mL, \(P<0.01\)), TNF-\(\alpha\) (50\(\pm\)10 versus 300\(\pm\)20 pg/mL, \(P<0.001\)), and IL-12 p40 (35\(\pm\)5 versus 150\(\pm\)10 pg/mL, \(P<0.01\)) circulating levels. Low-dose aspirin significantly reduced circulating levels of s-ICAM-1 (10\(\pm\)1, \(P<0.05\)), MCP-1 (120\(\pm\)18 ng/mL, \(P<0.01\)), TNF-\(\alpha\) (120\(\pm\)15 pg/mL, \(P<0.01\)), and IL-12 p40 (70\(\pm\)10 pg/mL, \(P<0.01\)). At the end of the study, segments of thoracic aortas (n=5 animals for each group) were isolated and incubated in serum-free medium for 24 hours. Supernatants were collected and assayed for s-ICAM-1, MCP-1, TNF-\(\alpha\), and IL-12 p40 levels. Compared with animals receiving placebo, supernatants from aortas of animals receiving aspirin had significantly reduced levels of all of the cytokines considered (Figure 1).

To explore the molecular consequence of this therapy regimen in vivo, we sought evidence for modulation of NF-\(\kappa\)B because this transcription factor has been linked to modulation of vascular inflammatory response genes.\(^{13}\) Mice were killed at the end of the study; the aortas were harvested, and nuclear extracts were isolated and assayed for NF-\(\kappa\)B binding activity. Gel mobility shift assay results indicated the functional presence of this factor in aortic nuclear extracts from 26-week-old LDLR\(^{-/-}\) mice on a high fat diet by using an NF-\(\kappa\)B consensus sequence probe (Figure 2). This was significantly reduced (34\%) in nuclear extract samples from aspirin-treated animals (Figure 2). The identity of the NF-\(\kappa\)B band was verified by competition studies in which 50-fold excess unlabeled NF-\(\kappa\)B probe completely blocked NF-\(\kappa\)B binding activity (Figure 2). Supershift studies with antibodies against p65 and p50 and incubation with anti-p52 and anti-C-Rel further confirmed the specificity of NF-\(\kappa\)B binding activity (Figure 2).

Analysis of Aortic Atherosclerosis

The aortic atherosclerotic lesion area was quantified by two independent methods, the en face and the cross section analysis of the proximal aorta. The average (percentage mean) size of the aortic atherosclerotic lesion area in the placebo group was 14\(\pm\)2.3\% of the total aortic surface; this was significantly reduced in the aspirin-treated mice to 5.1\(\pm\)1.1\% (\(P=0.001\)) (Figure 3). Similar to the en face analysis, treatment with aspirin resulted in a significant reduction in the cross section analysis of lesion size compared with the control group (565 670\pm\1 884 versus 400 385\pm\2 557 \(\mu\)/section, \(P=0.03\)).

Histological Studies

Next, to investigate whether low-dose aspirin had any impact on plaque composition, detailed histological studies analyzing macrophage, smooth muscle cells, and collagen content were performed. This treatment reduced the percent of positive area for macrophages by 57\% (\(P<0.05\)) (Figures 4 and 5). Both in control and aspirin-treated mice, collagen covered the major part of total lesion areas. However,
Analysis of picrosirius red-stained areas showed a 23% (P<0.05) increase in collagen content in the aortic lesions of aspirin-treated mice (Figures 4 and 5). Finally, aspirin treatment led to a 77% increase (P<0.05) in the number of smooth muscle cells in the atherosclerotic lesions (Figures 4 and 5). No immunostaining was detected in sections in which the primary antibody was omitted (not shown).

Discussion
The present studies were designed to address two hypotheses. First, we wished to determine the effect of low-dose aspirin on vascular inflammation during atherogenesis, and second, its effect on plaque composition. Low-dose aspirin is widely used in the secondary prevention of the complications of cardiovascular disease, and previous works have shown that this dosage preferentially inhibits platelet-derived TxA2 over endothelial-derived PGI2. This differential effect has theoretical advantages in that unaffected PGI2 production could promote the antithrombotic and anti-inflammatory properties of the endothelium. The first goal of this study, in fact, is the natural extension of our previous observations, in which we reported that an aspirin-like drug, indomethacin, retards atherosclerosis. As expected, we found that low-dose aspirin completely inhibits platelet activation. The animals also had intense systemic and vascular signs of inflammation, as shown by increased levels of sICAM, MCP-1, TNF-α, IL-12 p40, and aortic levels of NF-κB activity. We found that low-dose aspirin did not affect total PGI2 biosynthesis but reduced vascular inflammation. All of these effects were associated with a significant retardation of atherosclerotic lesion development, which was more evident in the entire aortic tree (64%) than in cross sections of the aortic roots (30%). This difference is consistent with other published studies that have used these two independent methods to evaluate the extent of atherosclerosis. We are aware that based on our data, it is not possible to tell whether the antiatherogenic effect of aspirin is secondary to the antiplatelet activity alone or also to the reduction of the vascular inflammation. However, conflicting results were shown in previous studies in apolipoprotein E-deficient mice given a higher dosage of aspirin, which would have suppressed platelet activation.

Figure 2. NF-κB activity is reduced in aortas from aspirin-treated LDLR−/− mice. DNA binding activity of nuclear extracts (10 μg) from aortas of LDLR−/− on placebo or aspirin was tested by a labeled NF-κB consensus probe in electrophoretic mobility shift assay. Binding was competed by incubation with a 50-fold excess of unlabeled NF-κB. Nuclear extracts were supershifted with antibodies against p50, p65, C-Rel, and p-52. Bands for anti-p50 and anti-p65 are indicated with arrow and double arrowhead, respectively.

Figure 3. Percentage of total aortic atherosclerotic lesion areas in LDLR−/− mice given a high fat diet receiving placebo or low-dose aspirin at end of study (26 weeks of age) (n=9 for each group; P=0.001).

Figure 4. Representative aortic sinus lesion area of atherosclerotic LDLR−/− mice. Photomicrographs: Aortic root cross sections in LDLR−/− mice given a high fat diet receiving placebo (right panel) or low-dose aspirin (left panel). Sections were stained for lipid lesions (oil red O) (Oro), immunostained for macrophage content, smooth muscle cells (SMC), or collagen (C).
In general, the therapeutic effect of aspirin is attributed to its immediate platelet inhibitory function. Percutaneous revascularization with balloon angioplasty results in endothelial damage, which can favor acute thrombotic occlusion. In this situation, aspirin is demonstrated to be beneficial. Aspirin may have additional biological properties on the vasculature; however, its efficacy in late complications such as restenosis is less clear. Atherosclerosis is a complex and multifactorial disease, and inflammation is involved in its pathogenesis and evolution. Increased levels of several cytokines have been reported in human and murine atherosclerosis, and evidence for their role in the disease has been provided. Activated NF-κB is present in atherosclerotic lesions both in human and in apolipoprotein E-deficient mice. In our study, we found that several inflammatory cytokine levels and aortic NF-κB activity were significantly increased during atherogenesis in LDLR−/− mice and that aspirin significantly reduced both of them. It is possible that this effect could be secondary to the antiplatelet and antiatherosclerotic effects of the drug and that the decrease of aortic NF-κB activity be secondary to the reduction of the disease per se (macrophage content of the plaque). Furthermore, it is known that aspirin, after blocking COX activity, enables this enzyme to produce potent anti-inflammatory mediators such as lipoxins, which activate their own receptor. Interestingly, the activation of these receptors is associated with inhibition of cytokines at the transcriptional level. This could be another mechanism by which aspirin indirectly could influence vascular inflammation.

Although high-dose aspirin inhibits the activation of NF-κB in vitro, we found that low-dose aspirin reduced this activation in vivo. Several aspects differentiate our study from the majority of the investigations on this issue. Most of them have been performed in vitro by using one cell type, high doses of aspirin, and most importantly, one–time point observation, which would be equal to an acute effect of the drug. Conversely, we used a complex cell system (mouse) and a much lower amount of aspirin but a long-term oral administration, which would be equal to a chronic effect of the drug. All these considerations could explain the different results. Nonetheless, we speculate that the observed reduction of vascular NF-κB activity could not be directly dependent on the pharmacological action of aspirin but a secondary event.

In recent years, it has become evident that the composition of the atherosclerotic plaque together with its susceptibility to rupture are two important aspects of the disease. Here, we report for the first time that low-dose aspirin has a significant effect on plaque composition. It increases the number of smooth muscle cells and the content of collagen, but reduces the foam cells within the aortic atherosclerotic lesions. These findings strongly suggest that aspirin treatment results in the development of a more stable plaque phenotype. In the present study, we cannot discriminate between a direct effect of aspirin on plaque composition and a secondary effect on plaque because of its antiatherosclerotic activity. However, we previously showed that indomethacin did reduce macrophages but did not influence smooth muscle cell content of atherosclerotic lesions. Furthermore, it is known that the reduction of atherosclerosis (lesion size) does not necessarily correspond to a more stable phenotype.

In summary, our studies demonstrate that aspirin, besides its antiplatelet action, may have additional in vivo properties on the vasculature that can also contribute to its antiatherogenic effect. These include suppression of vascular inflammation and development of smaller atherosclerotic lesions that contain more collagen and smooth muscle cells and less macrophages and cholesterol, consistent with a more stable plaque phenotype. A potential limitation of our study is that despite the similarity of our aspirin regimen with the human condition of administration of low-dose aspirin, there are aspects that need to be taken into account before our results are transferred to human atherosclerosis. First, the pharmacokinetics of aspirin in mice could be different from that in humans; second, whereas humans get a once-daily administration, the animals in our study had a multiple daily dosing. Considering the existence of relatively little literature on the effect of this drug on atherosclerosis, our observations suggest that a reevaluation of aspirin in atherosclerotic plaque progression and composition in humans is timely.

Acknowledgments

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References
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