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Atherosclerosis regression and TP receptor inhibition: effect of S18886 on plaque size and composition—a magnetic resonance imaging study

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MRI

Aims Endothelial dysfunction, platelet hyperactivity, and inflammation play a crucial role in atherogenesis. A growing body of evidence suggests that inhibition of the thromboxane A2 (TxA2 or TP) receptor may improve endothelial function and reduce the inflammatory component of atherosclerosis in addition to its demonstrated antiplatelet activity. Consequently, we sought to assess the effect of a novel TP receptor antagonist S18886, on atherosclerotic lesion progression and composition by serial non-invasive magnetic resonance imaging (MRI).
Methods and results S18886 was compared with control in an experimental model of established aortic atherosclerosis in New Zealand White rabbits ($n = 10$). The animals underwent MRI of the abdominal aorta at the time of randomization and at the end of treatment. Subsequently, animals were euthanized and specimens were stained for histopathology and immunohistochemistry with anti-α-actin antibodies for vascular smooth muscle cells (VSMC), anti-RAM-11 for macrophages, anti-caspase-3 for apoptotic cells, anti-MMP-1 for metalloproteinases, and anti-endothelin-1 (ET-1) as a marker of endothelial dysfunction. MRI analysis revealed a significant reduction in total vessel area (TVA) and vessel wall area (VWA) in the S18886 group ($P < 0.05$). Immunostaining analysis showed a significant decrease in RAM-11, caspase-3, MMP-1, ET-1 and an increase in α-actin in the treated group ($P < 0.05$ vs. control).
Conclusion Inhibition of the TP receptor by S18886 causes a regression of advanced atherosclerotic plaques. In addition, the reduction in the markers for macrophages, apoptotic cells, metalloproteinases, and endothelin-1 and the increase in VSMC, suggests that S18886 may not only halt the progression of atherosclerosis, but also transform lesions towards a more stable phenotype. The possibility of combining antithrombotic and antiatherosclerotic activity by means of the administration of TP inhibitors deserves further investigation in a clinical setting.

Introduction

Endothelial dysfunction, platelet hyperactivity, and inflammation play a crucial role in atherogenesis. A growing body of evidence suggests that inhibition of the thromboxane A2 (TxA2 or TP) receptor may improve endothelial function and reduce the inflammatory component of atherosclerosis in addition to its demonstrated antiplatelet activity.[1] The TP receptors are not only stimulated by TxA2 but also by virtually all eicosanoids such as the isoprostanes. They are capable of inducing platelet aggregation, vasoconstriction, stimulating the expression of adhesion molecules in endothelial cells, thus leading to leukocyte adherence, inducing apoptosis, and accelerating progression of atherosclerotic lesions.[2,3]

The role of platelet activation in atherogenesis has been extensively investigated.[4–9] Originally thought to be simple, anucleated particles strictly related to the hemostatic system, activated platelets (through the release of the content of their α-granules) are now increasingly appreciated as a major source of pro-inflammatory mediators.[10] Activated platelets are present in the circulating blood of atherosclerotic patients.[11] They are prone to bind leukocytes, preferentially monocytes, to form platelet–leukocyte aggregates.[12] They may affect endothelial inflammation and leukocyte–endothelial interactions, which are crucial events in the pathogenesis of atherosclerosis.[5] Our group has recently reported on the antithrombotic effect of a specific TP receptor inhibitor (S18886).[13] Therefore, an effective and maintained platelet inhibition may have, in addition to the antithrombotic effect, a significant impact on the natural history of atherosclerotic plaques.

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Emerging evidence suggests that selective inhibition of TP receptors has antiatherosclerotic effects by improving endothelial dysfunction, reducing levels of soluble inflammatory markers (e.g. ICAM-1), preventing macrophage accumulation, and inhibiting platelet activation and aggregation.[14,15] Specifically, Cayatte *et al.*[14] reported a reduction in the development of atherosclerosis in *apoE* deficient mice after 11 weeks of treatment with the selective TP receptor inhibitor S18886. Furthermore, a recent study, using atherosclerosis-prone (*apoE*$-/-$) and TP receptor deficient (*TP*$-/-$) mice, showed that these animals had less platelet reactivity, endothelial dysfunction, leukocyte–endothelial cell interaction, and importantly a reduction of 30% in atherosclerotic plaque formation.[1] Consequently, and based on the available evidence, we decided to evaluate the potential antiatherosclerotic activity of S18886, a novel, orally active, specific TP receptor antagonist in an experimental model of established atherosclerosis characterized by advanced, complex atherosclerotic lesions.

Methods

Experimental model of atherosclerosis

Advanced aortic atherosclerotic lesions were induced in male New Zealand white rabbits ($n = 10$, age = 3 months, weight = 3.5 ± 0.2 kg, Covance, Princeton, NJ, USA) by a combination of 9 months of high-cholesterol (HC) diet and double aortic balloon denudation injury (at months 1 and 3) as previously described.[16–18] All procedures were performed under general anaesthesia by intramuscular ketamine injection (20 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (10 mg/kg, Bayer Corporation, Shawnee Mission, KA, USA). The study protocol was approved by the Internal Review Board of Mount Sinai School of Medicine.

Study design

After 9 months of atherosclerosis induction, the animals were randomized to either S18886 (5 mg/kg/day) plus HC diet, or HC diet alone. The treatment period was 6 months. The experimental model of atherosclerosis utilized in this study has already demonstrated consistency on the development of advanced atherosclerotic lesions.[19]

Magnetic resonance imaging

Nine months after initiation of the atherogenic diet, all rabbits underwent *in vivo* magnetic resonance imaging (MRI) study ('atherosclerosis control'). The animals were anaesthetized and placed supine in a 1.5 T clinical MRI system (Sigma, General Electric) using a conventional phased-array volume coil.[19] Gradient echo coronal images were used to localize the abdominal aorta. Thereafter, sequential axial images (3 mm thickness) of the aorta from the right renal artery to the iliac bifurcation were obtained using a fast spin-echo sequence (total imaging time 45 min) with an in-plane resolution of 300×300 μm [proton density weighted (PDW): TR/TE, 2.300/17 ms; T2W: TR/TE, 2.300/60 ms, T1W: TR/TE, 800/5.6 ms, field of view = 9×9 cm, matrix 256×256, echo train length = 8, signal averages = 4].[16] The procedure was repeated at the end of the treatment period prior to sacrifice ('end of treatment').

Histopathology and immunohistochemistry

At the end of the study, animals were euthanized within 48 h of the MRI by intravenous injection of Sleepaway 5 mL i.v. (Fort Dodge Animal Health) after receiving heparin (100 U/kg) to prevent post-mortem blood clotting. The aorta was removed and fixed in paraformaldhehyde (4% in phosphate-buffered saline), serial sections of the aorta were cut at 3 mm intervals matching corresponding MR images. The selected aortic specimens were paraffin-embedded, and 5 μm thick sections were cut and stained with combined Masson's trichome elastin stain. Macrophage detection was performed by immunohistochemistry with the RAM-11 antibody. Anti-α-actin antibodies was used for vascular smooth muscle cells (VSMC), anti-caspase-3 for apoptotic cells, anti-MMP-1 for metalloproteinases, and anti-endothelin-1 (ET-1) as a marker of endothelial dysfunction. Dilutions were determined using controls at the time of staining.

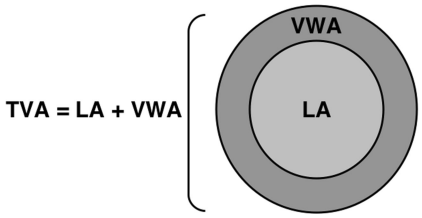

Figure 1 Scheme illustrating the parameters assessed by MRI and histopathology. VWA, vessel wall area; LA, lumen area; TVA, total vessel area.

Image analysis

The MR images were transferred to a Macintosh computer for further analysis. Cross-sectional areas of the lumen and outer boundary of each section were determined by manual tracing with Image Pro-Plus (Media Cybernetics). From these measurements, mean wall thickness (MWT), vessel wall area (VWA), total vessel area (TVA), and lumen area (LA) were calculated (*Figure 1*). Two experienced investigators blindly and randomly analysed each of the MRI and histopathology sections ($n = 327$). In order to match the slices in the both modalities (MRI and Histopathology), the right renal artery was taken as an anatomic reference. The slices for each rabbit were numbered cranio-caudally, the first slice being the one containing the right renal artery and the last section the one containing the bifurcation into the iliac arteries. Histopathology and immunostaining analysis (RAM-11, MMP-1, caspase-3, endothelin-1, and α-actin) were quantified by computer-assisted planimetry with Image Pro-Plus (Media Cybernetics).

Statistical analysis

After we tested for normal distribution and equality of variances with Levene's *F* test, the effectiveness of the treatment was analysed with unpaired *t*-tests by comparing S18886 and placebo groups. Paired *t*-test was used to compare the MR image-derived parameters within groups at the two time points. All probabilities are two-sided, with statistical significance taken as a value of $P < 0.05$. All values are expressed as mean ± SD.

Results

Magnetic resonance imaging

When comparing 'atherosclerosis control' vs. 'end of treatment' studies within groups, there was observed a mean reduction in TVA of $12.6 \pm 9.8\%$ (from 21.9 ± 7.5 to 18.7 ± 5 mm^2; $P < 0.05$) (mean ± SD) in the S18886 group, whereas in the control group there was a slight increase of $3.6 \pm 8.1\%$ (from 19.3 ± 7.4 to 19.9 ± 7.8 mm^2; $P = 0.11$). The LA increased from 6.97 ± 2.1 to 6.98 ± 1.4 mm^2 ($P = 0.97$) in the S18886 group and $1.7 \pm 23.8\%$ (from 6.1 ± 4.1 to 6.3 ± 4.6 mm^2; $P = 0.95$) in the control group. The VWA (plaque burden) was reduced by

Table 1 Assessment of atherosclerosis burden by MRI and atherosclerotic plaque composition by immunohistochemistry

	S18886	Control
MRI		
Change in TVA (%)	-12.6 ± 9.8^a	3.6 ± 8.12
Change in LA (%)	5.7 ± 25.5	-1.7 ± 23.84
Change in VWA (%)	-18.5 ± 15.3^a	5.7 ± 9.7
Immunohistochemistry		
RAM-11	1.1 ± 1.3^a	6.6 ± 5.2
Caspase-3	9.3 ± 4.3^a	19.7 ± 6.4
MMP-1	3.2 ± 2.8^a	11.5 ± 6.7
Endothelin-1	2.9 ± 4.1^a	6.2 ± 6.8
α-Actin	8.7 ± 6.9^a	1.6 ± 1.5

All values are expressed as mean $\pm$ SD. Units in the immunostaining are % of TVA.
[a] $P < 0.05$.

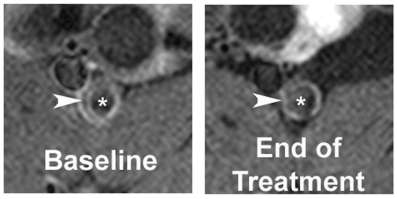

Figure 2 Representative cross sectional MR images of the abdominal aorta showing regression in the S18886 group. The panel on the left shows a section obtained at the time of randomization (baseline) showing a rim of low SI (white arrowheads) and right panel shows the same image at the end of treatment. Asterisk indicates lumen of abdominal aorta.

$18.5 \pm 15.3\%$ (from 14.6 ± 6.1 to $11.7 \pm 4.6\ mm^2$; $P < 0.05$) in the group receiving the study drug, and increased $5.7 \pm 9.6\%$ (from 13.0 ± 4.1 to $13.64 \pm 3.8\ mm^2$; $P = 0.06$) in the control group (*Table 1*). When the changes in VWA and TVA were compared among groups, there was a statistically significant difference ($P < 0.001$). The comparison of changes in LA among groups was not statistically significant. Interestingly, the qualitative analysis of plaque composition with multicontrast MRI revealed that overall the atherosclerotic lesions of the S18886 group presented lower content of lipids [low signal intensity (SI) in T2W, high SI in T1W] and higher content of fibrotic material (high SI in T2W and T1W) than the control group (*Figures 2* and *3*).

Histopathology

The histopathology analysis showed that the mean difference between groups for TVA was $4.4 \pm 0.36\ mm^2$. For VWA, LA, and MWT, the mean difference was 3.35 ± 0.43, 1.05 ± 0.21, and $0.13 \pm 0.011\ mm^2$, respectively. In line with the MRI findings, the histopathology demonstrated more fibrous rich plaques in the treatment group than in the control group and more lipid rich plaques in the control group than in the group receiving the study drug.

Immunohistochemistry

The RAM-11 staining revealed that the content of macrophages in the atherosclerotic plaques of the treatment group was lower than in the control group. The mean area of macrophages, expressed as a percentage of the TVA, was 1.1 ± 1.3 and $6.6 \pm 5.2\%$ ($P < 0.05$), respectively. Equally, the occurrence of apoptotic cells (caspase-3) was lower in the treatment group ($9.3 \pm 4.3\%$) than in the control ($19.7 \pm 6.4\%$; $P < 0.05$). In accordance with the MRI data, the presence of α-actin-positive areas (reflecting areas rich in VSMC) was higher in the treatment group ($8.7 \pm 6.9\%$) than in the control group ($1.6 \pm 1.5\%$; $P < 0.05$), giving a mean difference among groups of 7.0%. The immunostaining for MMP-1 showed more positive areas in the control group ($11.5 \pm 6.7\%$) than in the group receiving S18886 ($3.2 \pm 2.8\%$; $P < 0.05$), allowing for a difference of 8.3%. Endothelin-1-positive areas were $2.9 \pm 4.1\%$ in the treatment group and $6.2 \pm 6.8\%$ ($P < 0.05$) in the control group (*Figure 4*).

Discussion

We are reporting, for the first time, regression of already established atherosclerosic lesions after 6 months of treatment with a selective TP receptor inhibitor in a rabbit model of atherosclerosis while maintaining the atherogenic diet. Our results showed a reduction in plaque burden (VWA) and TVA as assessed by MRI. In addition, MRI showed a slight increase in the LA of the group receiving S18886. Importantly, there was a change in the phenotype of the atherosclerotic plaques in the treatment group. The reduction in the content of macrophages, apoptotic cells, MMP-1- and endothelin-1-positive areas, and the increase in α-actin-positive areas, suggests that selective inhibition of TxA2 pathway may confer a more stable plaque phenotype.

TxA2 is the major COX-1 (cyclooxygenase) product of arachidonic acid metabolism in platelets. Its biosynthesis is also incremented in the smooth muscle cells and macrophages of patients with atherosclerosis.[20,21] TxA2 is considered to be one of the most powerful agonists for platelet activation and thus, thrombus formation. In addition, TxA2 exerts a vasoconstrictor effect by serving as an agonist of the TP receptors on the vascular smooth muscle cell membranes. The critical effect of TxA2 on platelet activation and thrombosis has been clearly demonstrated by the clinical effectiveness of acetylsalicylic acid (ASA) in the prevention of acute coronary syndromes.[22] ASA irreversibly inactivates the enzyme cyclooxygenase COX-1 via its acetylation. The inhibition of this enzyme not only inhibits the synthesis of TxA2 by platelets but, at high concentrations, it may also inhibit the endothelial cyclooxygenase COX-2.[23] Selective inhibition of COX-2 has been recently associated with an increased risk of CAD and has resulted in the removal of one of this agents from the market.[24] TxA2 is mainly synthesized by the COX-1 enzyme in the platelets, whereas prostacyclin (PGI2) is synthesized by the COX-2 isoform.[2,23] Therefore, the availability of a selective blockade of TxA2, by blocking either its synthesis or its receptors without affecting the synthesis of PGI2, could have significant clinical implications by redirecting the arachidonic cascade towards the production of inhibitory and vasodilator prostanoids (PGI2 and prostaglandin D).[3]

S18886 is an orally active, specific, potent blocker of the receptor for TxA2 and related compounds (endoperoxides and isoprostanes), which does not affect the synthesis of PGI2. Its synthesis and biological evaluation have already been reported.[26] It is intended for use in the prevention of

thrombotic events in patients with cardiovascular diseases. The antiplatelet activity of S18886 has been previously demonstrated by our group[13] and the compound is now in phase III of clinical development. Interestingly, experimental and clinical evidence indicate that the compound has some antiatherosclerotic properties also.[14,15] Similarly, another TxA2 inhibitor has also shown favourable effects on atherosclerotic vessels.[27,28] More recently, a combined inhibitor of TxA2 synthase and receptor reduced 2 year overall mortality in diabetic patients with peripheral arterial disease.[29]

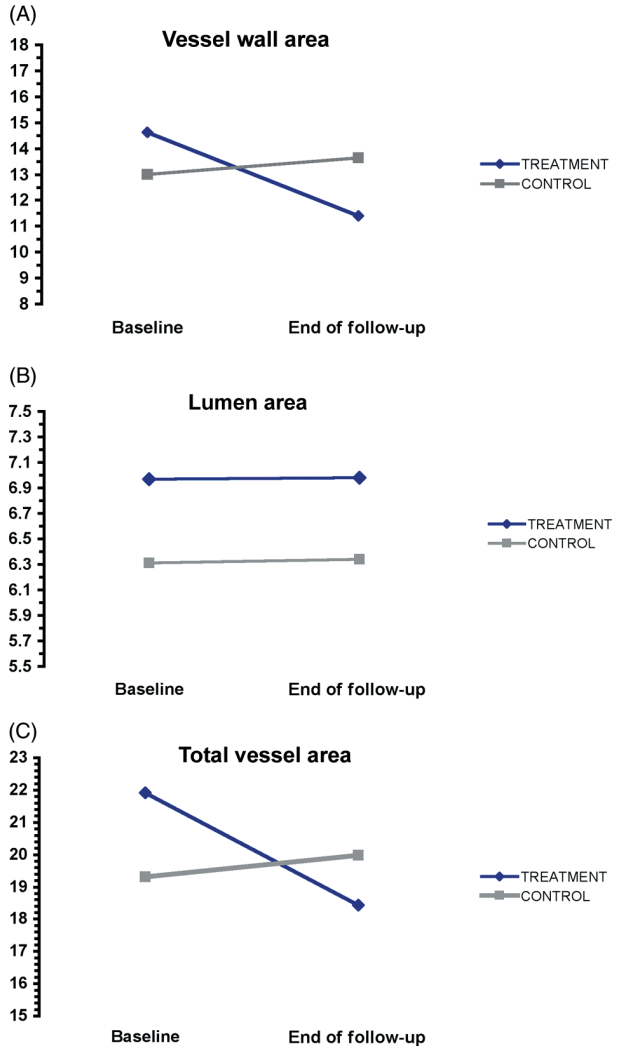

Figure 3 Graphical representation of MRI changes.

The potential mechanism of action for the observed effects of the S18886 may include a 'pleiotropic' anti-inflammatory effect (due to the reduction in the number of macrophages) which in turn abrogates the apoptotic phenomenon, rendering a beneficial effect on the endothelium[15] (due to the reduction in the endothelin-1-positive areas), and a mitogenic effect on VSMC, all of which are crucial to the progression of atherosclerosis. Indeed, two independent groups have formerly demonstrated the impact of inhibiting the thromboxane pathway in atherogenesis; Cayatte *et al.*[14] reported a reduction in the development of atherosclerosis in *apoE* deficient mice after 11 weeks of treatment with the selective TP receptor inhibitor S18886. Furthermore, a recent study using atherosclerosis-prone (*apoE*$-/-$) and TP receptor deficient (*TP*$-/-$) mice showed that these animals had less platelet reactivity, endothelial dysfunction, leukocyte–endothelial cell interaction, and importantly a reduction of 30% in atherosclerotic plaque formation.[1]

Compelling evidence on the importance of endothelial dysfunction, platelet activation, and inflammation in the genesis of atherosclerosis has been accumulated over the last decades. Activated platelets may provide the reactive surface for the recruitment of monocytes and lymphocytes by releasing the content of their granules, increasing the expression of adhesive ligands (such as P-selectin), or binding molecules from the plasma milieu (such as fibrinogen). These interactions may support the adhesion of leukocytes to the vessel wall even in conditions of high flow that would otherwise not permit this. Platelets also release growth factors (platelet-derived growth factor), proinflammatory cytokines (such as CD40 ligand and IL-1), and chemokines (such as RANTES and platelet factor-4).[6] Recent data emphasize the possible influence of platelets on the cellular metabolism of lipoproteins, indicating a more direct involvement in the early changes characteristic of the atherosclerotic lesion.[30,31] All these processes favour the recruitment of monocytes to the vessel wall, which eventually undergo apoptosis[32] and perpetuate the inflammatory

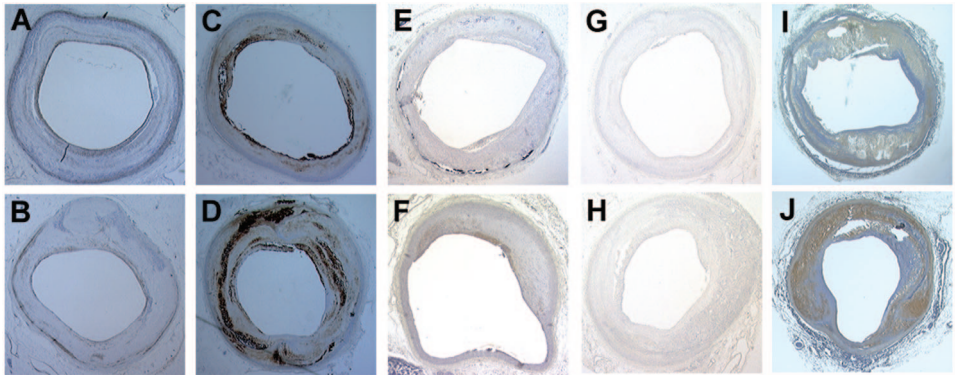

Figure 4 Representative histologic sections of the treatment (upper row) and control (lower row) groups. (*A*) and (*B*) are α-Actin (VSMC) showing more staining in the treated group; (*C*) and (*D*) RAM-11 (macrophages); (*E*) and (*F*) MMP-1 (metalloproteinases); (*G*) and (*H*) endothelin-1; (*I*) and (*J*) caspase-3 (apoptosis). (*C*)–(*J*) are evidencing more staining positive areas in the control group.

milieu within the atheromatous plaque. In addition, the interaction of activated platelets and their secreted products with endothelial cells is postulated as a trigger of the endothelial dysfunction and inflammation. It is now accepted that the higher the content of pro-inflammatory elements, such as macrophages and metalloproteases, the higher the risk of plaque disruption.

Our study is the first one reporting effect of regression of established atherosclerotic lesions. A major advantage of this study is that it demonstrates 'real' atherosclerosis regression given that by using serial MRI each animal serves as its own control. A limitation of the present study is that it does not provide a precise mechanistic explanation for the antiatherosclerotic effect of selective TP receptor inhibition. Hence, there are several questions that remain unanswered. Could the observed regression of atherosclerosis be extrapolated to other antiplatelet interventions as recently suggested[33] or is the antiatherosclerotic effect conferred by this agent explained solely by its TP receptor inhibition activity? In spite of the limitations, we believe this study opens new paths of investigation in the pathogenesis of atherosclerosis and may eventually modify the manner in which we understand the beneficial effect of 'antithrombotic' therapy.

In conclusion, based on the results of MRI, histopathology, and immunohistochemistry analysis, it can be concluded that the compound S18886 might have a potential antiatherosclerotic and plaque stabilizing effect. The possibility of combining antiplatelet activity (without inhibiting the synthesis of PGI2) with an antiatherosclerotic effect through the administration of TP inhibitors could have very important clinical implications, and therefore deserves further investigation in a clinical setting.

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