Oligogalacturonic Acid Inhibits Vascular Calcification by Two Mechanisms

Inhibition of Vascular Smooth Muscle Cell Osteogenic Conversion and Interaction With Collagen

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Objective—Cardiovascular diseases constitute the leading cause of mortality worldwide. Calcification of the vessel wall is associated with cardiovascular morbidity and mortality in patients having many diseases, including diabetes mellitus, atherosclerosis, and chronic kidney disease. Vascular calcification is actively regulated by inductive and inhibitory mechanisms (including vascular smooth muscle cell adaptation) and results from an active osteogenic process. During the calcification process, extracellular vesicles (also known as matrix vesicles) released by vascular smooth muscle cells interact with type I collagen and then act as nucleating foci for calcium crystallization. Our primary objective was to identify new, natural molecules that inhibit the vascular calcification process.

Approach and Results—We have found that oligogalacturonic acids (obtained by the acid hydrolysis of polygalacturonic acid) reduce in vitro inorganic phosphate–induced calcification of vascular smooth muscle cells by 80% and inorganic phosphate–induced calcification of isolated rat aortic rings by 50%. A specific oligogalacturonic acid with a degree of polymerization of 8 (DP8) was found to inhibit the expression of osteogenic markers and, thus, prevent the conversion of vascular smooth muscle cells into osteoblast-like cells. We also evidenced in biochemical and immunofluorescence assays a direct interaction between matrix vesicles and type I collagen via the GFOGER sequence (where single letter amino acid nomenclature is used, O=hydroxyproline) thought to be involved in interactions with several pairs of integrins.

Conclusions—DP8 inhibits vascular calcification development mainly by inhibition of osteogenic marker expression but also partly by masking the GFOGER sequence—thereby, preventing matrix vesicles from binding to type I collagen.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1391-1401. DOI: 10.1161/ATVBAHA.117.309513.)

Key Words: matrix vesicle ■ oligogalacturonic acid ■ type I collagen ■ vascular calcification
artery calcium have found a strong correlation between the calcium burden, the progression of arteriosclerosis, and overall survival.\textsuperscript{10} However, the relationship between calcification and atherosclerotic plaque stability (determining the risk of plaque rupture) remains subject to debate.\textsuperscript{11} Although a causal link between VC and outcomes has not yet been clearly established, it is clear that new preventive treatment strategies are needed.

Calcified deposits are found in distinct layers of blood vessels, leading to 3 types of VC: (1) calcification of the tunica media (medial calcification), (2) atherosclerotic intimal calcification, and (3) valvular calcification. The 3 types of calcification are often observed simultaneously. In recent years, it has been reported that VC is actively regulated by inductive and inhibitory mechanisms.\textsuperscript{12} The pathogenesis of medial VC is similar to that of neointimal calcification. The process is believed to mimic skeletal bone formation\textsuperscript{12} involving the surrounding vascular smooth muscle cells (VSMCs) via reprogramming and differentiation of these cells into an osteoblast-like phenotype, failure of anticalcifying mechanisms, abnormal Ca\slash inorganic phosphate (Pi) homeostasis, circulating calcioprotein particles, cell death,\textsuperscript{13} or changes of the extracellular matrix. Bone morphogenetic proteins (BMPs) are known to have an important role in the VC process.\textsuperscript{14} For instance, it has been suggested that the initial changes observed in the vessels of patients with VC consist of BMP deposition and then calcification.\textsuperscript{15} In bone, osteoblasts produce bone matrix, express osteochondrogenic genes, and release membrane-invested microparticles (known as matrix vesicles [MVs]) into the extracellular medium. The MVs initiate mineralization by interacting with the extracellular matrix (and predominately type I collagen [COL1]).\textsuperscript{16} This interaction is mediated by a variety of enzymes and factors, such as alkaline phosphatase and annexins.\textsuperscript{17} These vesicles concentrate calcium and initiate hydroxyapatite mineral crystallization.\textsuperscript{18} In cultured VSMCs, Watson et al\textsuperscript{19} measured a 3-fold increase in COL1 expression prior to calcification of the cells. MVs have also been found (using electron microscopy) in areas of VC in human vessels but also share certain components with osteoblast-derived MVs.\textsuperscript{22}

In previous work,\textsuperscript{23} we demonstrated that heterogeneous oligogalacturonic acids (hOGAs) purified from flax pectin reduced osteoclastic bone resorption in a dose-dependent manner. We also showed that hOGAs inhibited the ability of

<table>
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<th>Nonstandard Abbreviations and Acronyms</th>
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<td>BMP-2</td>
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<td>COL1</td>
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<td>DP8</td>
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Figure 1. Inhibition of inorganic phosphate-induced calcification by heterogeneous oligogalacturonic acids (hOGA). Immortalized murine aortic smooth muscle cells (MOVAS-1) were incubated with different concentrations of hOGA in the absence or the presence of 4 mmol/L inorganic phosphate (Pi) for 8 days. Calcification was assessed by von Kossa staining. **A**, Image of von Kossa staining of one representative experiment. **B**, Quantification of each well determined by Image J software (n=9). **C**, Intracellular calcium content (\(\mu g\) of Calcium/mg cell protein) measured by the o-cresolphthalein complexone (OCP) colorimetric method (n=3). Bar graphs represent the mean±SEM of independent experiments performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 vs Pi only; Mann–Whitney test. AU indicates arbitrary unit.
lyssosomal cysteine proteases to cleave COL1 via an interaction with the latter. Because VC results from the deposition of MVs on the extracellular matrix, we hypothesized that hOGAs might influence the calcification process by interacting with COL1. We, therefore, tested these molecules on VSMCs in the presence or absence of Pi. We showed that hOGAs (and more specifically an oligogalacturonic acids [OGA] with a degree of polymerization of 8 [referred to as DP8]) prevent in vitro Pi-induced VC by mainly inhibiting the osteogenic conversion of VSMCs but also by directly interacting with COL1 and, thus, preventing MV deposition.

**Materials and Methods**

**Materials and Methods** are available in the online-only Data Supplement.

**Results**

**hOGAs Inhibit Pi-Induced Calcification**

Pi-induced calcification was assessed by Von Kossa staining and by the o-cresolphthalein method after 8 days of treatment of immortalized murine aortic smooth muscle cells (MOVAS-1) with various concentrations of hOGAs in the absence or presence of Pi (2–4 mmol/L). As previously described in VSMCs, our results showed that 4 mmol/L Pi significantly promoted calcification in MOVAS-1 cells (P<0.001 versus nontreated cells) as assessed by von Kossa staining (Figure 1A and 1B) and the o-cresolphthalein method (Figure 1C). Addition of hOGAs induced a dose-dependent decrease in Pi-induced calcification (by ≤80% at 90 μmol/L; P<0.001 versus Pi alone; Figure 1B). These results were confirmed by measuring the intracellular calcium content with the o-cresolphthalein method (Figure 1C). Overall, these results showed that hOGAs inhibit Pi-induced calcification in MOVAS-1 cells.

**DP8 Is the Only hOGA That Inhibits Pi-Induced Calcification**

Because the hOGA fraction is heterogeneous, we next sought to identify which OGA was specifically responsible for this inhibition. Hence, MOVAS-1 cells were treated in the presence or absence of 4 mmol/L Pi with 60 μmol/L of 2 groups of OGAs presenting different DPs, referred to as group 1 (DP2≤DP≤DP6) and group 2 (DP≥DP7). In the absence of Pi, neither of the 2 groups differed from a control experiment with regard to quantified von Kossa staining (Figure 2A). However, in the presence of Pi, the inhibition of calcification (P<0.01 versus Pi) was observed with group 2 but not with group 1 (Figure 2A).

Figure 2. Effect of various oligogalacturonic acid degree of polymerization (DP) on inorganic phosphate-induced calcification. A, Effect of a mixture of DP. Immortalized murine aortic smooth muscle cells (MOVAS-1) were incubated with 60 μmol/L of various mixtures of DP (DP2≤DP≤DP6 and DP≥DP7) in the absence or the presence of 4 mmol/L inorganic phosphate (Pi) for 8 days. (Continued)
To determine which DP in group 2 (DP ≥ DP7) was involved in this inhibition, we tested DP7, DP8, and DP9 separately at concentrations of 6, 30, and 60 μmol/L. Only DP8 clearly inhibited Pi-induced mineralization in a dose-dependent manner, as assessed by the intracellular calcium content (Figure 2B). The degree of inhibition reached 75% at 30 μmol/L and 80% at 60 μmol/L. As a consequence, a DP8 concentration of 60 μmol/L was, therefore, used in all subsequent experiments. Finally, we confirmed the DP8 inhibition on primary human VSMC (Figure 2C) on which it reached ≤70% at 60 μmol/L (P<0.01 versus Pi).

DP8 Inhibits the Calcification of Aortic Rings
Next, we assessed DP8’s ability to inhibit the calcification of vascular tissue taken from an animal model. Aortic rings were, therefore, prepared from isolated rat aorta and incubated with 4 mmol/L Pi for 7 days in the presence or absence of 60 μmol/L DP8. Mineralization of the aorta rings in the presence of Pi was observed after 7 days and was quantified by Alizarin red staining (Figure 3A) and an o-cresolphthalein assay (Figure 3B). Addition of 60 μmol/L DP8 significantly decreased (by ≤50%) calcification of the aortic rings (Figure 3A and 3B; P<0.01 versus Pi alone). No effect was observed for DP8 in the absence of Pi. These results indicate that DP8 inhibit Pi-induced calcification of aortic rings, albeit to a lesser extent than in in vitro studies of cell cultures.

DP8 Inhibits the Expression of Osteoblast Differentiation Markers
To probe the molecular mechanism involved in DP8’s inhibitory effect, we first looked at whether OGAs could inhibit the Pi-induced differentiation of VSMCs into osteoblast-like cells. We, therefore, used reverse transcriptase quantitative polymerase chain reaction and Western blots to quantify the expression of several markers involved in the differentiation of VSMCs into osteoblast-like cells (Figure 4). After 4 days of treatment of MOVAS-1 cells with 4 mmol/L Pi, the mRNA expression levels of matrix Gla protein (MGP; Figure 4A), osteopontin (Figure 4B), osteocalcin (Figure 4C), and BMP-2 (Figure 4D) had increased by a factor of 4, 2, 1.2, and 1.3, respectively (P<0.05 versus control for all except P<0.1 versus control for osteocalcin). In the presence of 60 μmol/L DP8, Pi’s effects on MGP, osteopontin, osteocalcin, and BMP-2 mRNA expression were significantly less intense.

Similarly, the mRNA expression levels of type I collagen A1 (COL1A1) and collagen A2 (COL1A2; Figure 4E and 4F, respectively) were elevated (relative to the control) in presence of Pi, albeit to a lesser extent than for the other osteogenic markers. After addition of DP8, these levels were equivalent to control levels.

Incubation with 60 μmol/L of DP8 fully reversed the effects of Pi on these osteoblastic markers, and control levels were observed (Figure 4A through 4F). Total COL1 in VSMCs was then quantified by Western blotting (Figure 4G and 4H). After 6 days of treatment with 4 mmol/L Pi, we observed 1.8-fold upregulation of COL1 expression. This upregulation was abrogated when 60 μmol/L of DP8 was present. No effect was observed with DP8 alone. These results were confirmed by immunofluorescence assays (Figure 5A). The quantification of COL1 mRNA levels (Figure 5B) showed a 2.6-fold increase in the presence of Pi (P<0.05 versus control); again, the increase was completely abrogated when 60 μmol/L of DP8 was also present.
Taken as a whole, these results suggest that addition of the DP8 fraction can prevent the Pi-induced differentiation of MOVAS-1 into osteoblastic cells.

**DP8 Inhibits MV Binding to COL1**

Because we evidenced in a previous work a direct interaction between hOGAs and COL1^{23} in osteoclasts, we wanted to determine whether a direct interaction between COL1 and DP8 or MVs might also be involved in the hOGAs’ inhibitory action on Pi-induced calcification in VSMCs. Using surface plasmon resonance (Biacore), we showed that MVs specifically bind to immobilized COL1 with a $K_d$ value of 8.53±0.95 ng/mL ($R_{max}=1580±49$ relative units [RU]; Figure 6; Figure I in the online-only Data Supplement). To characterize the macromolecular interaction between DP8 and COL1, different concentrations of different OGA solutions (hOGAs [Figure II in the online-only Data Supplement], DP2, DP7, DP8, and DP9 [Figure 7 and the Table]) were injected onto immobilized COL1 before adding MVs. DP8 specifically bound to immobilized collagen (Figure 7A and 7B), as did (to a lesser extent) DP7 and DP9 (Table). No significant binding was observed with DP2 (Figure 7B).

We then studied DP8’s ability to inhibit MV binding to COL1 in a competition assay (Figure 7C). Unlike DP2, we...
found that hOGAs and DP8 efficiently competed for MV binding to COL1 (with an inhibition of 11±5%, 52±5%, and 89±4% for 5 mmol/L DP2, hOGAs, and DP8, respectively). These data show that DP8 is a better inhibitor (IC50 of 64 nmol/L) of MV binding to COL1 than hOGAs or DP2. The degree of inhibition induced by 64 nmol/L of DP8 was 6× higher than that induced by hOGAs.

DP8’s and the hOGAs’ binding affinities (at 10 mmol/L) to immobilized MVs were further investigated (Figures III and IV in the online-only Data Supplement). DP8 did not bind to MVs, suggesting that OGAs with a high DP (and particularly DP8) can interact with COL1 but not with MVs. We then fully saturated the immobilized collagen with DP8 (R_max=313 RU) and injected MVs at a protein concentration of 60 ng/mL. No MV binding was observed (data not shown), confirming that prebinding of DP8 to COL1 blocks MVs from subsequently binding to COL1.

To confirm these results in cells, we performed an immunofluorescence assay in nonpermeabilized MOVAS-1 cells using antibodies against Annexin A6, a specific marker of MVs, and against COL1. Our labeling quantification results showed a significant increase of Annexin A6 fluorescence in calcifying conditions, suggesting an increased MVs production (Figure 8A, Pi, AnxA6, and Figure 8B). Addition of DP8 decreased partially (37%) Annexin A6 fluorescence, indicating that MV production was not normalized by DP8 (Figure 8A, Pi+DP8, AnxA6, and Figure 8B). In presence of Pi, we showed a significant increased expression of extracellular COL1 (Figure 8A, Pi, Col1, and Figure 8C) that colocalized with Annexin A6 (Figure 8A, Pi, Merge). The colabeling quantification clearly shows a colocalization between extracellular COL1 and Annexin A6 equivalent to 40% of the total cell labeling in the presence of Pi (Figure 8D), suggesting an

**Table.** The Binding of OGAs With Different DP to Type I Collagen

<table>
<thead>
<tr>
<th>OGA</th>
<th>K_D, mM</th>
<th>R_MAX, RU</th>
<th>Number of Experiments (n)</th>
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<tr>
<td>hOGAs</td>
<td>3.97±0.94</td>
<td>180.6±13.7</td>
<td>n=4</td>
</tr>
<tr>
<td>DP2</td>
<td>...*</td>
<td>10.1±0.4</td>
<td>n=3</td>
</tr>
<tr>
<td>DP7</td>
<td>0.32±0.08</td>
<td>289.2±11.8</td>
<td>n=4</td>
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<tr>
<td>DP8</td>
<td>0.15±0.02</td>
<td>312.7±10.3</td>
<td>n=5</td>
</tr>
<tr>
<td>DP9</td>
<td>0.22±0.03</td>
<td>302.6±15.4</td>
<td>n=3</td>
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*No detectable binding, so K_D cannot be calculated.

DP indicates degree of polymerization; hOGA, heterogeneous oligogalacturonic acid; and RU, relative unit.

MV, suggesting that OGAs with a high DP (and particularly DP8) can interact with COL1 but not with MVs. We then fully saturated the immobilized collagen with DP8 (R_MAX=313 RU) and injected MVs at a protein concentration of 60 ng/mL. No MV binding was observed (data not shown), confirming that prebinding of DP8 to COL1 blocks MVs from subsequently binding to COL1.

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**Figure 5.** Oligogalacturonic acid with a degree of polymerization of 8 (DP8) inhibits inorganic phosphate (Pi)–induced upregulation of intracellular type I collagen protein expression. Immortalized murine aortic smooth muscle cells (MOVAS-1) were permeabilized and incubated with 60 μmol/L DP8 in the absence (left) or the presence (right) of 4 mmol/L Pi for 5 days. After treatment, cells were stained with fluorescein isothiocyanate (FITC)–labeled antitype I collagen antibody and DAPI to label the nuclei. **A,** Magnification ×60. **B,** The fluorescence intensity of collagen was measured using ImageJ software by the following calculation: corrected total fluorescence=Integrated density–(Area of selected cell×Mean fluorescence of background readings). Bar graphs represent the mean±SEM of 5 independent experiments performed in triplicate. *P<0.05, Mann–Whitney test.
interaction between MVs and COL1. In calcifying condition, DP8 reduced only partially extracellular COL1 expression (23%; Figure 8C) but drastically reduced (≥80%) the colocalization of Annexin A6 and extracellular COL1 (Figure 8A, P<0.05, Merge, and Figure 8D), suggesting the disruption of the interaction MV–collagen.

The GFOGER Sequence Is Involved in the MVs’ Interaction With Collagen, and DP8 Prevents MVs From Interacting With the GFOGER Sequence

MVs are deposited at sites of pathological VC. Analysis of their composition (using protein mass spectrometry) has identified as many as 80 different proteins. These include plasma membrane proteins and, more specifically, pairs of integrins (α1, α2, αV, β1, etc.), some of which act as collagen receptors. Recent results have shown that α1β1, α2β1, and α11β1 integrins recognize a triple-helical GFOGER (Gly-Phe-Hyp-Gly-Glu-Arg amino acid) sequence present on several collagens (including COL1). To determine whether the GFOGER sequence is the binding site for MVs, we chemically synthesized a soluble GFOGER peptide and evaluated its role in the interaction between COL1 and MVs.

We first performed a competition assay in which soluble GFOGER peptide could compete for MV binding to immobilized COL1. Addition of GFOGER peptide to MV solutions prior to injection decreased the equilibrium responses, which suggested that the GFOGER sequence is involved in the MV–COL1 interaction (Figure 9A). By fitting these data, we determined a relative IC50 value of 0.62 mmol/L. The degree of inhibition never exceeded 77%, indicating that the GFOGER peptide cannot completely abolish MV binding to immobilized COL1.

To further analyze the interaction between the GFOGER peptide and MVs, we used surface plasmon resonance to characterize MV binding to an immobilized GFOGER sequence (Figure V in the online-only Data Supplement). MVs specifically bound to the immobilized peptide with a Kd of 5.65±0.74 ng/mL and an Rmax of 206±6 RU.

To determine whether inhibition of the MV–COL1 interaction by DP8 involves the GFOGER sequence, we tested DP8’s ability to inhibit MV binding to immobilized GFOGER peptides. Various concentrations of DP8 were added to MVs prior to injection (Figure 9B). DP8 displayed an IC50 of 15.8 mmol/L. The maximum degree of inhibition was 57±4%, which is lower than that obtained with DP8 (89±4%; Figure 6). This discrepancy indicates that the inhibitory effect of DP8 involves binding sites other than GFOGER. Nevertheless, the present study is the first to have identified the main protein site involved in DP8’s inhibition of MV binding to COL1.

Discussion

VC is characterized by the deposition of hydroxyapatite in the medial or intimal layers of the vessel wall. In the vessel wall, VSMCs play a key role in the initiation and regulation of VC, leading to an osteocyte/chondrocyte phenotypic change characterized by increased expression of bone-related proteins and the release of MVs. Electron microscopy experiments have shown that these MVs localize close to COL1 fibrils and form a nidus for mineralization. There are no preventive treatments for VC. We studied the MOVAS-1 cell line, which is a well-established, relevant model that closely displays the same time course of calcification and significant increases in the mRNA expression of key VC-associated genes as the one observed in primary murine aortic VSMCs. Growing evidence show that extracellular Pi induces calcification by stimulating (via the PTH1 transporter) the transcription of genes coding for proteins involved in osteoblast function and bone formation.

Our present results showed that hOGAs induce a dose-dependent inhibition of Pi-induced calcification in MOVAS-1 cells. This inhibitory effect was dependent on the OGA’s DP. We showed that DP8 and, to a lesser extent, DP7 and DP9 (data not shown) were responsible for this inhibition. This observation is consistent with our previous studies in which the inhibitory effect of OGA on bone resorption was found to be dependent on the OGAs’ DP; a DP of 5 being required for a significant inhibitory effect. We noted that the effect of DP8 on VC inhibition was preventive but not curative because the inhibition of calcification was only observed when DP8 was present concomitantly with Pi. Interestingly, this inhibition was confirmed in primary human VSMCs, leading us to think that our results could have a clinical relevance. hOGAs and DP8 inhibit the mRNA upregulation of MGP, osteopontin, osteocalcin, BMP-2, and the α1 and α2 chains of COL1 and inhibit the increased protein expression of COL1. It has been suggested that the initial trigger for VC is the deposition of BPs in the patient’s vessels. The observed inhibition of the expression of calcification inhibitors (MGP and osteopontin) and osteogenic markers (osteocalcin and BMP-2) by DP8, therefore, indicates that Pi-induced mineralization is inhibited early in the process. Furthermore, the inhibition of BMP-2 mRNA transcription might be one component in the inhibitory mechanism induced by DP8 because BMP-2 is known to induce the calcification of human VSMCs and to have a crucial role in Pi uptake and phenotypic modulation. The VC inhibition was also confirmed in experiments on isolated rat aortic rings, in which DP8 normalized the intracellular calcium and reduced the collagen protein expression.
expression (data not shown) induced by Pi. However, only a 50% inhibition of calcification (relative to Pi conditions) was observed, which suggests that other tissue factors are involved and that DP8 partially inhibits the calcification. This observation is consistent with many other demonstrations in the literature showing that the in vivo effects of inhibitors are often less intense than those observed in vitro.32 Taken as a whole, these results suggest that the decrease of VSMC and aortic ring calcification induced by DP8 is mainly related to the inhibition of osteogenic marker expression.

One of the earliest phase of VC is the secretion of MVs, which then seed calcium phosphate (hydroxyapatite) crystals.26 MVs released by VSMCs are located close to collagen fibrils.20 They interact with COL1 and serve as nucleating foci for calcium mineralization. They are structurally similar to osteoblast-derived MVs, and it has been assumed that they have a similar origin.16 Recent research has identified VSMC-derived MVs as exosomes.22 An interaction between MVs and COL1 has already been partly characterized in bone17 and in VSMCs17 under calcifying conditions. In particular, this interaction requires annexin activity.17 However, although several underlying mechanisms have been postulated, the exact nature of this interaction and the specific sequence of COL1 involved has not previously been reported. Fetuin-A, MGP, and S100A9 (all of which are known to be present in MVs) have emerged as putative regulators of this interaction.21 Our present results evidenced the direct binding of MVs (produced by MOVAS-1 cells in calcifying condition) to COL1 with a $K_d$ of 8.53±0.95 ng/mL. This value cannot be converted into nmol/L units because of the heterogeneity of the proteins contained in MVs. Consequently, we are unable to draw any conclusions with regard to the MVs’ binding affinity for COL1. However, it is noteworthy that after sample injection, the MVs did not dissociate from COL1, indicating that the binding was strong. We then sought to determine whether a direct interaction between DP8 and COL1 might also be involved in this inhibition by preventing MV deposition. Our surface plasmon resonance results clearly showed that DP8 binds to COL1 but not to a noncollagenic substrate, suggesting a specific effect. It is also noteworthy that other DPs displayed a low binding affinity for COL1. This result was expected because in a previous study, the injection of a high concentration of COL1 (in the mg/mL range) was required to observe its binding to an OGA-coated sensor chip. The observed inhibitory properties of DP8 raise
the question of whether the prevention of MV–COL1 interaction is mainly because of MV–DP8 binding, DP8–COL1 binding, or a combination of the 2. Our results clearly demonstrated that DP8 is unable to interact with MVs; hence, and despite its low affinity for COL1, DP8 is able to act as a coating that prevents MVs from binding to COL1. We showed that the presence of DP8 was associated with a significantly weaker interaction between MVs and COL1. These results were confirmed by a set of immunofluorescence experiments in non-permeabilized MOVAS-1 cells that showed in presence of Pi a significant increase of Annexin A6 expression (a specific marker of MVs) that colocalized with extracellular COL1, thus, suggesting an interaction between MVs and extracellular COL1. Addition of DP8 did not normalize Annexin A6 expression (and, thus, the production of MVs) but reduced the colabeling with extracellular COL1 ≤80%, result that was similar than the one shown by the surface plasmon resonance/Biacore assay. This 80% reduction of colabeling cannot be solely explained by the decreased production of MVs (only 37% reduction of Annexin A6 labeling) or by the decreased expression of extracellular COL1 (23%). Thus, these data strongly suggest that DP8 prevents MVs–COL1 interaction. Moreover, because the quantification of the intracellular COL1 and the COL1A1/A2 mRNA expression showed a level equivalent to normal expression after addition of DP8, the reduced but not normalized extracellular COL1 measured here may be because of an interaction with DP8 that prevents its enzymatic degradation as demonstrated in our previous study.23

To understand how DP8 can prevent the interaction between MVs and COL1, we first sought to characterize this interaction. Proteomic analysis of MVs revealed the presence of plasma membrane proteins, including aminopeptidase N and integrins (α3, αV, and β1). Integrins are important receptors that mediate both cell–cell contact and cell–extracellular matrix interactions.
recognized. Integrins α1β1 and α2β1 are the main integrin collagen receptors. Knight et al.34 showed that the GFOGER sequence constitutes COL1’s binding site for MVs. Our hypothesis was confirmed: MVs bind specifically to GFOGER and display a 1.5-fold greater affinity for GFOGER peptide than for COL1. These results demonstrate that the GFOGER sequence constitutes a preferential binding site for MVs on COL1. In the last step in our study, we demonstrated that DP8 inhibits MV binding to the GFOGER sequence. Although we did not observe a direct interaction between DP8 and MVs, the presence of DP8 masked the GFOGER sequence and prevented MV deposition and VC. These data are consistent with our previous results, which showed that the inhibition of COL1 expression is enough to prevent calcification, independently of Pi or Ca²⁺ levels.35

The present study is the first to have highlighted the role of carbohydrate compounds in the inhibition of VC (see the Graphic Abstract). Oligogalacturonans are mainly present in smooth pectin regions of the apple cell wall matrix. Apple consumption reportedly has beneficial effects on lipid metabolism36 and vascular function,37 and it has been suggested that these beneficial effects are related (at least in part) to the presence of flavonoids.38 Our results suggest that the effects on vascular function may be because of the presence of pectin and open up new research opportunities. DP8 could potentially be used as active treatment against VC. Because all the experiments have been performed only on cellular and aortic ring models, the present study presents some limitations, and thus, it is difficult to predict an efficacy of DP8 inhibition on animal model or in humans. However, before being administered in humans, an in vivo study of a calcifying animal model should be performed. Above all, it will be important to identify the best administration route for DP8. It will also certainly be necessary to chemically modify the structure of DP8 so that the compound resists enzymatic digestion. Finally, we have also to check the potential action of DP8 on the osteoblastic mineralization.

Acknowledgments

We thank Dr Priscillia Gross for excellent technical assistance. This work was funded by the Institut National de la Santé et de la Recherche Médicale (INSERM) and via fellowships from the Conseil Régional de Picardie (CRP) and the Fonds européen de développement économique et régional (FEDER).

Sources of Funding

This work was funded by the Institut National de la Santé et de la Recherche Médicale (INSERM) and via fellowships from the Conseil Régional de Picardie (CRP) and the European Fund for Regional Development (ERDF). The research was also funded by grants from the Centre National de la Recherche Scientifique (CNRS), and the European Union (cofunding of equipment as part of the Contrat Projet État-Région [CPER] 2007 to 2013).

Disclosures

None.

References

Hodroge et al. A New Approach to Vascular Calcification Inhibition


Highlights

- Oligogalacturonic acid with a degree of polymerization of 8 (DP8) prevents Pi-induced calcification of vascular smooth muscle cells and of isolated rat aortic rings.

- Matrix vesicles produced in calcifying conditions interact with the GFOGER (Gly-Phe-Hyp-Gly-Glu-Arg) peptide sequence (involved in integrin recognition) of type I collagen.

- DP8 inhibits osteogenic differentiation of vascular smooth muscle cells.

- DP8 masks the GFOGER sequence and prevents matrix vesicles binding to type I collagen.
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Arterioscler Thromb Vasc Biol. 2017;37:1391-1401; originally published online May 18, 2017; doi: 10.1161/ATVBAHA.117.309513

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Material and Methods

Reagents. Dulbecco’s Modified Eagle Medium (DMEM 6546), penicillin, streptomycin and geneticin (G418) were purchased from Sigma-Aldrich. Fetal Calf Serum (FCS) and trypsin were obtained from Eurobio.

Antibodies. Anti-mouse antibodies against annexin A2 (1/1000; ab41803), annexin A6 (1/50; ab199422) and type I collagen (1/100; ab6308) were obtained from Abcam (Paris, France), and rabbit anti-type I collagen antibody (1/1000; 600-401-103-0.1) was obtained from Rockland (Pennsylvania, USA). Antibody directed against mouse β-actin (1/2000; A5441) was purchased from Sigma-Aldrich (St-Louis, MO, USA). The tail tendon COL1 was purchased from Aviva Systems Biology (USA).

Heterogeneous oligogalacturonic acids (hOGA) preparation and DP8 purification.

hOGA was prepared by acid hydrolysis of polygalacturonic acid (10 g/L, pH 3.4, 100°C, 30 h). hOGA were precipitated by adding a seven-fold volume of isopropanol at 4°C overnight after elimination of low molecular weight polysaccharide by lowering the pH to 2.

A HPLC system was used for OGA DP8 purification, injections (1 mL) of a 20 mg/mL hOGA solution were performed on a 21×250 mm Nucleosil 100-5 SB column (Macherey Nagel). Oligogalacturonic acids were eluted with a linear ammonium acetate gradient (15 mL/min) from 50 mM to 0.8 M. Each oligogalacturonic acid peak was monitored by an evaporative light scattering detector (Alltech 3300 ELSD) and OGA DP8 was collected and lyophilized.

Cell culture and treatments. Murine aorta smooth muscle cells were obtained from ATCC (MOVAS-1 CRL-2797™, LGC) and maintained in DMEM 6546 supplemented with 10% Fetal Calf Serum (FCS, Eurobio), 100 IU/mL penicillin, 100 µg/mL streptomycin, 4 mM glutamine, and 200 µg/ml geneticin (G418, Sigma) at 37°C under 5% CO₂ humidified atmosphere.

For experiments, cells were maintained in the same medium supplemented with only 1% Fetal Calf Serum and with 4 mM glutamine. Calcification was induced with 0.9 mM phosphate of Na₂HPO₄/NaH₂PO₄ mixture to reach a final concentration of 4 mM Pi.

MOVAS cells were incubated with hOGA and DP in the absence or presence of 4 mM Pi in the supplemented DMEM at 37°C. After 8 days, the intracellular calcium content was measured by the o-cresolphthalein complexone (OCP) colorimetric method or by Von Kossa staining as previously described. Calcium deposits were observed using a Photometrics CH250 CCD camera (Arizona, USA) and quantified using ImageJ Software (NIH: http://rsbweb.nih.gov/ij/).

Aortic rings. All animals were handled in accordance with French legislation (Directive 2010/63/EU of the European Parliament). They were housed in polycarbonate cages in temperature- and humidity-controlled rooms with a 12-h light/dark cycle and given free access to water and regular laboratory chow (Diet 2016, Harlan, Oxon, UK).

Thoracic aortas were isolated from Wistar wild-type male rats as previously described. Aortic rings were incubated medium (DMEM 6546-medium supplemented with 10% FCS, 50 IU/mL penicillin, 50 µg/mL streptomycin and 2 mM glutamine at 37 °C under a 5% CO₂ humidified atmosphere) with 65 µM DP8 in the absence or presence of 4 mM Pi. After 7 days of culture, with medium renewed every 2 days, one part of the aortic rings was washed twice with PBS without Ca²⁺ and Mg²⁺ before measuring their intracellular calcium content using OCP method. The other part was washed twice in phosphate buffer saline (PBS), fixed with 4% paraformaldehyde in PBS for 30 min at 4°C and stained with 2% Alizarin red in water (pH 4.1) for 15 min at room temperature.
**Matrix vesicle (MV) isolation.** Secreted MVs were isolated from cell culture supernatants as described by Chen et al. ⁴. MVs were collected in a Tris buffer saline solution (10 mM Tris, pH 7.6) containing 0.25 M sucrose and stored at -20°C until subsequent use. Protein concentration was determined with a BCA™ Protein Assay Reagent Kit (Pierce).

**Immunoblot Analysis.** Each sample was homogenized and protein concentration was determined using a BCA kit assay (Thermo Fisher Scientific, France). Proteins were precipitated and loaded onto NuPAGE® Novex® 4-12% Bis-Tris Gels (Invitrogen, Cergy-Pontoise France). Nitrocellulose membranes were blocked in 5% non-fat milk for 1 h at 37°C, incubated with primary antibodies (1:1,000) and secondary antibodies (1:7500). Protein expression were quantified using ImageJ software (NIH: http://rsbweb.nih.gov/ij/) and normalized with β-actin expression.

**Quantitative RT-PCR (q-RT-PCR).** Total RNA was isolated from cells using the Trizol method (Life technologies) and reverse transcribed into cDNA with a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Amplification was performed using SYBR Green PCRMaster Mix (Applied Biosystems) and specific primers (Table 1) with a 7500 Fast Real-time PCR system (Applied Biosystems, Life technologies). Acidic ribosomal phosphoprotein 0 (ARP0) was used as endogenous control.

**Fluorescence Microscopy.** Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde, permeabilized with 0.01% TritonX100 and stained with an antibody directed against COL1 (1/1,000). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Life technologies). Coverslips were then mounted in Mowiol (Merck) and observed under a Nikon Eclipse TE2000U microscope (Nikon) equipped with a Nikon Plan APO VC 60X / 1.40 objective under oil immersion. Images were processed and overlaid with Photoshop software (http://www.adobe.com/support/downloads/detail.jsp). For annexin A6 and type I collagen co-labeling, cells were seeded on glass coverslip and treated with PI with or without DP8 at 60µM for 8 days. Cells were labeled with primary antibodies overnight at 4°C. Cells were not permeabilized in order to label the extracted collagen and matrix vesicles. Samples were then washed with PBS and incubated for one hour at 4°C with secondary antibodies. Laser confocal microscope (LSM 780, Zeiss, Oberkochen, Germany) was used for imaging the cells. The levels of cellular fluorescence of Annexin A6 and type I collagen were measured using ImageJ (https://scientochemistryblog.com/2011/05/24/measuring-cell-fluorescence-using-imageJ). Co-labelling quantification was performed by measuring specific signal pixel numbers relative to total cell surface and expressed as a co-localization coefficient (% of total cell surface).

**GFOGER peptide synthesis.** The GFOGER peptide was synthesized on a CEM liberty1 peptide synthesizer using standard automated continuous-flow SPPS methods (double coupling) as previously described⁵. Characterization of the peptide was performed by mass spectrometry on a Q-TOF Ultima Global hybrid quadrupole/time-of-flight instrument. The predicted and observed high resolution masses (HRMS) for GFOGER (C_{29}H_{44}N_{9}O_{10}: (M+H)^+) were 678.3211 and 678.3246, respectively.

**Surface Plasmon Resonance measurements.** Experiments were performed on a BIAcore T100 instrument (GE Healthcare Europe, GmbH) at 25°C in a running buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl and 0.05% Tween 20 (HBS solution). i) **Type I collagen and GFOGER peptide immobilization.** Tail Tendon Type I Collagen (Aviva Systems Biology, San Diego, USA) and GFOGER peptide were covalently amine-coupled to CM3 sensor chips using the procedure previously described in ⁶. Collagen (at 200 µg/mL in 10 mM sodium acetate, pH 4) or peptide GFOGER (at 300 µg/mL in 10 mM sodium acetate, pH 4.5) were flowed over the activated hydrogel matrix for 10 min at 10 µL/min. In addition, a collagen- or peptide-free surface, used as control surface, was prepared according to the same immobilization procedure without collagen or peptide injection. All collagen- or GFOGER peptide-coated surfaces were totally regenerated by injection of 25 mM NaOH solution for 80
s at a flow rate of 10 µL/min. ii) Binding assays. OGA were dissolved and then diluted in running buffer to injection in the running buffer. For binding experiments, serial concentrations of OGA or DP8 were flowed simultaneously over the collagen and control surfaces at 30 µL/min for 700 s. MVs were diluted in running buffer from a stock suspension containing 0.4 mg/mL of proteins and serial concentrations were injected onto the collagen- or peptide-coated CM3 surfaces at 10 µL/min for 500 s. The RU value corresponding to the equilibrium plateau was taken as the equilibrium binding response (Req). All binding values were corrected for bulk refractive index effects by subtraction of RU values obtained by parallel injection on the control surface. Equilibrium dissociation constants $K_D$ were determined by nonlinear fitting of equilibrium binding response (Req) against concentration plots using a steady-state affinity model (Prism 6, GraphPad Software, Inc.). Relative IC50 were deduced from fitting competition assay data using a sigmoidal dose-response model (Prism 6, GraphPad Software, Inc). iii) Inhibition assays. Binding inhibitor molecules were added to MV solutions at a fixed concentration immediately prior to injection. Residual MV binding was expressed as a percentage of the equilibrium responses obtained by passing these mixtures over immobilized collagen or peptides divided by those obtained in the absence of competitor.

Statistical analysis. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis were performed using GraphPad Prism software (version 5.0, San Diego, CA, USA). If a significant intergroup difference was found in a Kruskal-Wallis test, a Mann Whitney test was performed. $P<0.05$ was considered significant.

References

### Supplemental Table I

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequences</th>
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</table>
| ARPO  | forward 5’ – TCCAGAGGCACCATTGAAATT – 3’  
| reverse 5’ – TCGCTGGCTCCACCTT – 3’ |
| MGP   | forward 5’ – ATGAAGAGCCTGCTCCCTCT – 3’  
| reverse 5’ – ATATTTGGCTCCTCGGCGCT – 3’ |
| OPN   | forward 5’ – AAGAAAATCTCTTCAAGCAATT – 3’  
| reverse 5’ – GTGAGATTCGTCAGATTCAT – 3’ |
| COL1A1| forward 5’ – GAGAGAGCATGACCGATGGATT – 3’  
| reverse 5’ – TGTAGGCTACGCTTCTTGGCA – 3’ |
| COL1A2| forward 5’ – CGAGACCCCTCTCCTCCTG – 3’  
| reverse 5’ – GCATCCATAGTGACATCCTT – 3’ |

**Supplemental Table I.** Primer sequences used to quantify gene expression in a RT-qPCR assay. RT-qPCR indicates reverse transcriptase quantitative polymerase chain reaction.
Supplemental Figure I. Oligogalacturonic acid inhibits vascular calcification through interaction with collagen. Surface plasmon resonance analysis of Matrix Vesicles binding to immobilized type I collagen (COL 1). Representative control-subtracted sensograms of MVs flowed over type I collagen coated-CM3 sensor chips at a series of different concentrations (expressed as protein content). MVs did not bind to the collagen-free control surface and this reference surface was used to subtract bulk shift values from all binding responses.
Supplemental Figure II. A. Overlay of sensorgrams measuring the interaction of hOGA at different concentrations with the immobilized type I collagen. The signals from the collagen-free surface were subtracted from the signals obtained with the collagen-coupled surface. B. Equilibrium responses (Req) of hOGA solutions, injected over immobilized collagen, plotted as a function of their concentration. The curve represents the fit of these data to a simple one-site Langmuir binding equation. Average and standard deviation were calculated from at least three independent measurements.
Supplemental Figure III. OGA do not bind immobilized MVs. The capacity of DP8 and hOGA to bind MVs was investigated. MVs were immobilized onto the surface using a BIAcore L1 sensor chip (Figure S3) at a level of 2135 ± 300 RU. The L1 sensor chip contains hydrophobic aliphatic chains with exposed polar head groups that are able to capture vesicles as they pass over the chip.

The surface was first cleaned by an injection of nonionic detergent, N-octyl-β-D-glucopyranoside (40 mM), at 5 µL/min for 300 s and then exposed to MV diluted in running buffer (protein concentration: 0.1 mg/mL) for 20 min at 2 µL/min. All untrapped vesicles were removed from the surface by injection of a 25 mM NaOH solution at 10 µL/min for 60 s. Complete coverage of the L1 nonspecific binding sites was confirmed by the absence of BSA binding (0.1 mg/mL in running buffer, 300 s, 10 µL/min).

Solutions of DP8 (5 mM, Figure S4) or hOGA (10 mM, data not shown) were passed over the surface onto which the MVs had been immobilized. No significant binding was observed.

Immobilization of MV on L1 sensorchip. 1: cleaning the surface by octyl glucoside. 2: MV deposition on the L1 surface. 3: removal of untrapped MV with NaOH. 4: Blocking of nonspecific binding sites by Bovine Serum Albumin injection.

Supplemental Figure IV. Representative raw SPR signal for OGA DP8 (5 mM) flowed over immobilized matrix vesicles. The bulk effect was not subtracted.
Supplemental Figure V. Equilibrium responses (Req) of MV suspensions, injected over GFOGER peptides immobilized onto CM3 sensor chip at a level of 220 ± 30 RU, plotted as a function of their protein content. MVs do not bind the peptide-free surface and the resulting bulk signals were subtracted from the data collected using the peptide coated surfaces. The curve represents the fit of these data to a simple one-site Langmuir binding equation. Average and standard deviation were calculated from at least three independent measurements.