A novel approach to oral apoA-I mimetic therapy

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Abstract Transgenic tomato plants were constructed with an empty vector (EV) or a vector expressing an apoA-I mimetic peptide, 6F. EV or 6F tomatoes were harvested, lyophilized, ground into powder, added to Western diet (WD) at 2.2% by weight, and fed to LDL receptor-null (LDLR−/−) mice at 45 mg/kg/day 6F. After 13 weeks, the percent of the aorta with lesions was 4.1 ± 4.3%, 3.3 ± 2.4%, and 1.9 ± 1.4% for WD, WD + EV, and WD + 6F, respectively (WD + 6F vs. WD, not significant). While body weight did not differ, plasma serum amyloid A (SAA), total cholesterol, triglycerides, and lyso-phosphatidic acid (LPA) levels were less in WD + 6F mice; P < 0.0295. HDL cholesterol and paroxonase-1 activity (PON) were higher in WD + 6F mice (P = 0.0055 and P = 0.0254, respectively), but not in WD + EV mice. Plasma SAA, total cholesterol, triglycerides, LPA, and 15-hydroxyeicosatetraenoic acid (HETE) levels positively correlated with lesions (P < 0.0001); HDL cholesterol and PON were inversely correlated (P < 0.0001). After feeding WD + 6F: i) intact 6F was detected in small intestine (but not in plasma); ii) small intestine LPA was decreased compared with WD + EV (P < 0.0469); and iii) small intestine LPA 18:2 positively correlated with the percent of the aorta with lesions (P < 0.0179). These data suggest that 6F acts in the small intestine and provides a novel approach to oral apoA-I mimetic therapy.—Chattopadhyay, A., M. Navab, G. Hough, F. Gao, D. Meriwether, V. Grijalva, J. R. Springstead, M. N. Palghnachari, R. Namiri-Kalantari, F. Su, B. J. Van Lenten, A. C. Wagner, G. M. Anantharamaiah, R. Farias-Eisner, S. T. Reddy, and A. M. Fogelman. A novel approach to oral apoA-I mimetic therapy. J. Lipid Res. 2012. 54: 995–1010.

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High density lipoprotein (HDL), its main protein, apolipoprotein A-I (apoA-I), and mimetics of apoA-I have been shown in a number of laboratories to reduce inflammation in animal models of disease (1–5). The apoA-I mimetic peptide 4F showed great promise in a variety of mouse models of disease (5) leading to a phase I/II study in humans with a high risk of cardiovascular disease (6). In this study the 4F peptide synthesized from all D-amino acids (D-4F) was administered orally at doses that ranged from 0.43 to 7.14 mg/kg. The resulting plasma peptide levels were low [maximal plasma concentration (Cmax) 15.9 ± 6.5 ng/ml]. Despite these very low plasma levels, doses of 4.3 and 7.14 mg/kg significantly improved the HDL inflammatory index (HII), which is a measure of the ability of a test HDL to inhibit LDL-induced monocyte chemoattractant protein-1 (MCP-1) production by cultured human artery wall cells; doses of 0.43 and 1.43 mg/kg were not effective (6). A second clinical trial focused on achieving high plasma peptide levels using low doses (0.042–1.43 mg/kg) of the 4F peptide synthesized from all L-amino acids (L-4F) delivered by intravenous (IV) or subcutaneous (SQ) administration (7). Very high plasma levels were in fact achieved (e.g., Cmax 3,255 ± 630 ng/ml in the IV study), but there was no improvement in HII (7). To resolve this paradox, new studies were conducted in mice that led to the surprising discovery that the major site of action for the peptide may be in the intestine, even when the peptide is administered SQ (8). Moreover, the dose administered, not the plasma level, was the major

Abbreviations: apoE−/−, apoE null; Cmax, the maximal plasma concentration; DHA, docosahexaenoic acid 22:6 (n-3); EET, eicosatetraenoic acid; EPA, eicosapentaenoic acid 20:5 (n-3); EV, empty vector; 6F, the peptide D-W-L-K-A-F-Y-D-K-F-F synthesized from all D-amino acids; HETE, hydroxyeicosatetraenoic acid; HII, HDL inflammatory index; IV, intravenous; LDLR−/−, LDL receptor-null; LPA, lyso-phosphatidic acid; MCP-1, monocyte chemoattractant protein-1; PG, prostaglandin; PON, paroxonase-1; SAA, serum amyloid A; SQ, subcutaneous; TX, thromboxane; WD, Western diet; WT, wild-type.

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determinant of efficacy (8). Efficacy was the same at the same dose when the peptide was administered orally or SQ suggesting that in the compartment controlling peptide efficacy, peptide concentrations should be similar; the peptide concentration was similar only in the feces (8). In a subsequent study, this compartment was further identified as the small intestine (9). Additionally, metabolites of arachidonic and linoleic acids in the enterocytes of the small intestine were found to be ~10-fold higher than in the liver, but the percent reduction in these metabolites after oral 4F peptide administration was significantly greater in the liver compared with the small intestine strongly suggesting that the small intestine is a major site for peptide action (9). As a result of these studies (8, 9), it was concluded that doses of peptide ranging between 40 and 100 mg/kg/day would be required instead of doses of 0.42-1.43 mg/kg/day as were used in the studies of Watson et al. (7).

The 4F peptide (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH₂) has end blocking groups (Ac- and -NH₂) that stabilize the class A amphipathic helix and dramatically increase efficacy (10-13). In unpublished studies in mice, our laboratory and Animal Medicine at the David Geffen School of Medicine at UCLA; (8) have published data in light of our more recent findings. This report describes the results of our search for a peptide that does not require chemically added end groups for efficacy and which can be produced in genetically engineered plants. The search began by reviewing our previously published data in light of our more recent findings. The peptide 4F was similar in efficacy to 6F based on our in vitro assays (12). The 4F peptide was initially chosen due to its increased solubility compared with 6F because we thought that absorption of the peptide was required to achieve some critical plasma peptide level. Because this did not turn out to be the case, we turned our attention to the 6F peptide. Neither the 4F nor the 6F peptide has any sequence homology to apoA-I. The first apoA-I mimetic peptide with 18 amino acids was known as “18A” (14). The terminal charges of this peptide were modified by adding end blocking groups, which resulted in increased lipid affinity for nonoxidized lipids (10, 11). The 18A peptide is also known as “2F” because the peptide has two phenylalanine residues at positions 3, 10, 11, 14 and 17. The 18A peptide was highly effective in a mouse model of atherosclerosis (12). Because of this similarity between 4F and 6F in interacting with DMPC, which we previously demonstrated was highly effective in a mouse model of atherosclerosis when it was administered orally (19); we chose to initially focus our attention on the 6F peptide instead of the 5F peptide. As shown by the experiments reported here, the 6F peptide is efficacious without chemically added end groups, it can be expressed in genetically engineered tomatoes, and perhaps most remarkably, the 6F peptide is effective when the tomatoes are fed, even without isolation and purification of the peptide.

Materials and Methods

Materials

The peptide 6F (D-W-L-K-A-F-Y-D-K-F-E-K-F-K-E-F-F) was synthesized from all L-amino acids by solid phase synthesis as described (12) using Wang resin (Advanced Chem Tech, Louisville, KY) to obtain C-terminal free acid after the cleavage of the peptide from the resin except that the N-terminal acetylation step was omitted. The vector pBI121 containing a kanamycin resistant gene (NPT II), a cauliflower mosaic virus 35 S promoter (CaMV35S), and a nopaline synthase terminator was obtained from “The Arabidopsis Information Resource” (TAIR) (www.arabidopsis.org; stock number CD3-388, vector pBI121). Agrobacterium tumefaciens LBA 4404 was obtained from Invitrogen, Electromax (catalog number 18313-015). ELISA kits for determination of losophosphatidic acid (LPA) were purchased from Echelon (catalog number k-2800s). All other materials were purchased from previously described sources (8, 9).

Mice

Female wild-type (WT) C57BL/6j, female LDL receptor-null (LDLR⁻/⁻), or apoE null (apoE⁻/⁻) mice originally purchased from Jackson Laboratories on a C57BL/6j background were obtained from the breeding colony of the Department of Laboratory and Animal Medicine at the David Geffen School of
Medicine, University of California at Los Angeles. The mice used in these studies were of different ages, which is stated in each legend. The mice were maintained on a chow diet (Ralston Purina) before being switched to Western diet (WD) (Teklad, Harlan, catalog #TD88137). The addition of chemically synthesized 6F peptide to the diet was accomplished as previously described for the addition of the 4F peptide (8); preparation and addition of tomatoes with or without transgenic 6F to WD is described below in the processing and analysis of tomatoes section. For experiments in which WD with or without 2.2% by weight of powdered tomatoes was presented to the mice, the preparations, which were stored at −80°C until use, were thawed each evening, tightly compacted, and presented to each cage of four mice each night. Additional Supplementary Fig. I shows an example of the tightly compacted WD presented to the mice. All experiments involving mice were approved by the University of California at Los Angeles Animal Research Committee.

**Determination of plasma and intestinal constituents and atherosclerotic lesions**

Plasma was collected and analyzed for total cholesterol, triglycerides, serum amyloid A (SAA), HDL cholesterol, and paraoxonase-1 (PON) activity as described previously (8, 9). Tissue levels of cholesterol were measured as previously described (8). Levels of arachidonic acid and its metabolites were measured by LC-ESI/MS/MS as described previously (9). Lysophosphatidic acid was measured either by ELISA according to the manufacturer's instructions or LC-ESI/MS/MS as described previously (9). The percent of the aorta with atherosclerosis was determined by en face analysis as previously described (8, 20).

**Generation of transgenic tomato plants**

The strategy that we chose involves the use of the bacterium *Agrobacterium tumefaciens*, which carries a T1 plasmid that can be manipulated to insert a gene of interest into plant cells (21). To accomplish this we used the plant binary vector pBI121 that contains a kanamycin resistance gene (NPTII), a cauliflower mosaic virus 35S promoter (CaMV35S), the GUS gene that encodes for the marker protein β-glucuronidase, and a nopaline synthase terminator (Fig. 1). The gene encoding 6F is 54 bp long and encodes the 18 amino acids D-W-L-K-A-F-Y-D-K-F-F-E-K-F-K-E-F-F with a molecular mass of 2,435.81 Da. The expression cassette for 6F also contained the plant-derived signal peptide with 23 amino acids (M-I-M-A-S-S-K-L-S-L-A-F-L-A-L-L-S-H-A-N-S), 69 bp long (22). The codon usage table (www.kazusa.or.jp/codon) specific for *Lycopersicon esculentum* was used to design the DNA sequence: TCTAGAATGATCATGGCCCTTCTCTAAAACCTCTTTCTTTGC-TCITTTTTCTTGCTCTCTTCTCAGTACATATCGTTGGCTTT- TAACGCTTTTTATGATAAATTTTTGAAAAATTTAAAGATTTT TTGGAGACTC. The DNA was synthesized from DNA 2.0 (https://www.dna20.com). The cassette was cloned into the XbaI/Sacl site replacing the GUS gene of the plant binary vector pBI121 and a TGA stop codon was introduced before the SacI site [Arabidopsis Biological Resource Center (ABRC), http://www.arabidopsis.org] under a CaMV35S promoter (Fig. 1). The sequence was verified by DNA sequencing. The vector also contained the npt II gene for kanamycin selection of transgenic plants (Fig. 1).

Transgenic plants were generated through a core service contract with the Saint Louis Donald Danforth Plant Science Centre, MO (Mr. Kevin Lutke). Initially, a total of 1,200 tomato cotyledons (*Lycopersicon esculentum*) was transformed with and without the cassette containing the sequences for the plant-derived signal peptide and 6F. Transformations without the cassette containing the sequences for the plant-derived signal peptide and 6F are referred to as empty vector (EV), which still contains the GUS gene in the pBI121 vector that encodes for the marker protein β-glucuronidase. Copies harboring the binary vector were sequence verified in the University of California at Los Angeles GeneSeq Core and then used for plant transformation using the strategy of Frary and Earle (21).

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**Fig. 1.** A schematic diagram of the full length pBI121-6F cassette. The top panel depicts the pBI121 vector, which is referred to in this manuscript as the EV. The bottom panel shows the vector for expressing the 6F peptide in which the GUS gene has been replaced at the XbaI/Sacl site by the plant-derived signal peptide (SP) and the gene encoding 6F under the CaMV35S promoter and nopaline synthase terminator (NOS term) as described in Materials and Methods. The NPT II gene conferring kanamycin resistance is under the control of nopaline synthase promoter (NOS pro).
Identification and quantification of 6F in tomatoes

The seeds from homozygous ripened tomatoes were removed and the remaining seedless tomato pulp and skin were rapidly frozen and shipped by overnight courier to the University of California at Los Angeles where they were processed in a freeze-dry lyophilizer system (VirTis, Gardener, NY) to obtain lyophilized tomato fruit tissue (pulp plus skin). For SDS-PAGE gel analysis, proteins from the lyophilized fruit were obtained by homogenization with a mortar and pestle in liquid nitrogen and homogenized in extraction buffer (50 mM Tris-Cl, 150 mM NaCl, 2% Nonidet P-40, 1% desoxycholic acid, 0.5% SDS) at pH 8.0 with complete protease inhibitor mixture (Roche Applied Science, Indianapolis). The total soluble proteins, 100 µg per lane were resolved on 4–20% gradient gels; samples of 500 µg per lane were resolved on 20% SDS-PAGE gels. Mini Protean TGX gels (Bio-Rad) were stained with Sypro Ruby (Invitrogen) or silver stain (Invitrogen). For LC-ESI-MS/MS or LC-ESI-MS analyses the 6F peptide band was in-gel digested as previously described (23). Briefly, the band of interest was excised and in-gel trypsin digested (5–10 ng/ml of Trypsin Gold, V5280, Promega) overnight at 37°C, eluted in 50% acetonitrile containing 0.1% trifluoroacetic acid followed by Zip-Tip C-18, tip size P10 (Millipore), and subjected to LC-ESI-MS/MS analysis using a 4000 QTRAP quadruple mass spectrometer (Applied Biosystems) equipped with electrospray ionization source, or analysis was performed by LC-ESI-MS on an LCQAdvantage Max ion trap mass spectrometer (ThermoElectron, Inc.) equipped with electrospray ionization source as previously described (7, 8). Quantification of 6F peptide in the lyophilized tomatoes by LC-ESI-MS/MS or LC-ESI-MS was accomplished by using a 15N-labeled chemically synthesized 6F peptide without end blocking groups. When the 6F fractions from the HPLC prior to injection into the mass spectrometer were subjected to SDS-PAGE analysis and the

Fig. 2. The 6F peptide synthesized from all L-amino acids without end blocking groups (L-6F) when fed to apoE−/− mice significantly reduced plasma SAA levels and decreased the percent of the aorta with atherosclerotic lesions. A: Groups of female apoE−/− mice (n = 20) 16–18 months of age were maintained on rodent chow that did not contain peptide (Chow) or contained 1.2 mg of the 6F peptide without end blocking groups per 4 g of chow (Chow + 6F) providing a dose of ~60 mg/kg/day peptide. The mice in both groups consumed approximately 4 g of the chow per mouse per day. The peptide constituted ~0.03% of the diet by weight. After 10 days the mice were bled and SAA levels were determined by ELISA as described in Materials and Methods. B: Groups of female apoE−/− mice 4–6 months of age were fed WD that did not contain or which contained L-6F without end blocking groups (WD + 6F) at a dose of 60 mg/kg/day of peptide. After 6 weeks the mice were bled and plasma SAA levels were determined by ELISA as described in Materials and Methods. C: Groups of female apoE−/− mice 6–8 months of age (n = 30 per group) were fed WD that did not contain peptide (WD No Peptide) or that contained L-6F without end blocking groups (WD + 6F) at a dose of 60 mg/kg/day of peptide. After seven weeks the percent of the aorta with atherosclerotic lesions was determined by en face analysis as described in Materials and Methods.
bands migrating with authentic 6F peptide were in-gel trypsin digested, on subsequent examination in the mass spectrometer, only the signature for 6F was seen (i.e., no other protein or peptide could be detected in these bands). Consequently, SDS-PAGE analysis of protein extracts of lyophilized tomatoes following LC (but without MS analysis) was used for routine quantification of 6F. For routine quantification, the gels were scanned by densitometry and the results calculated from a standard curve generated by the lanes containing chemically synthesized 6F peptide without end blocking groups.

Identification and quantification of 6F in small intestine and plasma
For analysis of the small intestine, 200 mg of small intestine (including contents) were homogenized in 10 ml of acetonitrile: water (1:1) and the homogenates were lyophilized and re suspended in 400 µl of acetonitrile:water (1:1). For analysis of plasma, 100 µl of plasma were lyophilized and brought up in 400 µl of acetonitrile:water (1:1). Samples were run on HPLC using a C-18 reversed-phase analytical column and a gradient solvent system of acetonitrile: water (30–80% in 20 min) in the presence of 0.1% trifluoroacetic acid (TFA) and monitored at 280 nm. Chemically synthesized 6F samples (5 µg in the same solvent as samples) were injected and retention times were obtained. Unknown samples (200 µl out of original 400 µl) were injected and 0.5 ml fractions were collected. Samples corresponding to the retention time of the chemically synthesized 6F were dried and analyzed by SDS gel electrophoresis as described above and the bands migrating with chemically synthesized 6F were quantified by scanning and comparison to known quantities of chemically synthesized 6F (without blocking groups) run on the same gels.

Addition of tomatoes to WD
For in vivo experiments, the lyophilized tomato fruit tissue was thoroughly ground to a fine powder in liquid nitrogen without the extraction buffer and then thoroughly mixed with increasing quantities of powdered WD to yield WD containing 2.2% lycophilized tomato powder, which was frozen and stored at −80°C until use. In some experiments, WT tomatoes were used instead of the EV tomatoes as controls. This is explicitly indicated in the legends. In these instances, the WT tomatoes were grown in St. Louis and processed identically to the EV and 6F tomatoes.

Determination of lycopene content of tomatoes
Lycopene content in the tomatoes was determined by previously described methods (24). Briefly, the ground lyophilized tomato powder was suspended in NaCl (3.42 M) and extracted using ethyl acetate and cyclohexane (1:1; v/v) by centrifuging for 5 min at 600 g. The organic layer was carefully removed and the optical density was measured at a wavelength 503 nm in triplicates in a spectrophotometer (FLUOstar Omega, BMG Labtech) as described (25). Lycopene standards from Sigma (catalog number L9879) were used for generating the standard curves.

Statistical analysis
Statistical analyses were performed by ANOVA, unpaired two-tailed t-test or by linear regression using GraphPad Software, San Diego, CA.

RESULTS

Is the 6F peptide without end blocking groups effective in vivo?
As shown in Fig. 2A, feeding apoE−/− mice the 6F peptide without end blocking groups by incorporating the peptide into mouse chow significantly decreased plasma SAA levels. Feeding apoE−/− mice the 6F peptide without end blocking groups by incorporating the peptide into WD also decreased plasma SAA levels (Fig. 2B) and decreased the percent of the aorta with atherosclerotic lesions (Fig. 2C).

Would the 6F peptide without end blocking groups be efficacious in more than one mouse model of atherosclerosis if it was mixed with homogenized tomato before incorporation into the diet?
The efficacy of apoA-I mimetic peptides is thought to be due to their ability to bind oxidized lipids (26, 27). Before proceeding with an attempt to produce the peptide in a genetically engineered plant such as a tomato, we thought it was important to determine if homogenized tomato might saturate the peptide with plant lipids rendering it ineffective. As shown in supplementary Fig. II A this was not the case for plasma SAA levels. As shown in supplementary Fig. II B, incorporating the 6F peptide without end blocking groups into WD containing 10% ripened tomato homogenate resulted in a significant decrease in plasma levels of the potent growth factor LPA in both apoE−/− and LDR−/− mice. Having assured ourselves that the presence of tomato fruit would not alter the efficacy of the 6F peptide we then set out to determine if we could genetically engineer tomatoes to express the 6F peptide.

Can the 6F peptide be expressed in the fruit of tomato plants?
As shown in Fig. 3, most of the tomato lines that were PCR positive for 6F expressed a band on SDS-PAGE gels that migrated similarly to authentic chemically synthesized 6F without end blocking groups. Other tomato lines in Fig. 3 did not (e.g., line 95 and the WT control). Tomato line 95 did show PCR evidence of gene insertion, but presumably the gene was not expressed at the protein level. Following HPLC and SDS-PAGE, the region on each lane corresponding to 6F was excised and in-gel trypsin digested and subjected to LC-ESI-MS/MS or LC-ESI-MS analysis as described in Materials and Methods. Figure 4A demonstrates that the bands migrating similarly to authentic 6F exhibited the LC-ESI-MS signature for 6F while the same region from those lanes without bands did not

Statistical analysis
Statistical analyses were performed by ANOVA, unpaired two-tailed t-test or by linear regression using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA).
6F Tomato

RT: 20.00 - 200.00

- EFF
  - singly charged
  - 21.01 33.27 41.38 68.18 77.10

- DWLKA<FYDK
  - doubly charged
  - 33.45 34.69 69.08 74.40 93.19

- FFEKF<K
  - singly charged
  - 33.22 41.26 64.46 79.34 90.17

EV Tomato

RT: 20.00 - 200.00

- EFF
  - singly charged
  - 26.16 34.72 41.53 72.03 77.02

- DWLKA<FYDK
  - doubly charged
  - 20.47 36.09 53.51 78.02 79.37

- FFEKF<K
  - singly charged
  - 25.83 35.51 47.66 75.85 85.71
Figure 4A: The 6F peptide was found to account for between 0.6 and 1.0% of the weight of the lyophilized tomatoes. Founder lines were selected and grown to collect seeds. Homozygous lines were generated as described in Materials and Methods. SDS-PAGE gels from a control homozygous line and from two homozygous lines (1A and 17A) expressing 6F are shown in supplementary Fig. III. Having been successful in producing the 6F peptide in homozygous transgenic tomatoes we next set out to determine if feeding these tomatoes compared with control tomatoes would show a beneficial effect on plasma biomarkers in short-term feeding studies.

**Would feeding WD for two weeks with tomatoes transgenic for 6F improve plasma biomarkers compared with no added tomatoes or compared with feeding WT tomatoes?**

At the start of these experiments we had a limited supply of homozygous transgenic tomatoes and an even more limited supply of homozygous EV tomatoes. Thus, we designed experiments with relatively few mice for measuring biomarkers following short-term feeding. In the first short-term experiment we used WT tomatoes as the control tomatoes. These were grown identically to the EV and transgenic 6F tomatoes in the St. Louis core facility and were processed identically as stated in Materials and Methods. Feeding ground lyophilized transgenic 6F tomatoes at 2.2% of the WD to female LDLR−/− mice 10 weeks of age (40 mg/kg/mouse/day of 6F) for two weeks significantly improved many (but not all) of the biomarkers measured (Fig. 5A–K). Not shown in Fig. 5 are data indicating no significant improvement in plasma total cholesterol, triglycerides, free arachidonic acid, or thromboxane B2 levels after feeding either the WT or transgenic 6F tomatoes.

**Would feeding WD for two weeks with tomatoes transgenic for 6F improve plasma biomarkers compared with no added tomatoes or compared with feeding EV tomatoes?**

The second experiment differed from the first experiment in the following details: i) the mice were older (4–5 months of age); ii) the control tomatoes were EV tomatoes instead of WT tomatoes; iii) not all of the biomarkers measured in the first experiment were repeated in the second. The results were similar between the two experiments as shown in supplementary Fig. IV. Feeding EV tomatoes with WD for two weeks significantly decreased SAA levels; feeding transgenic 6F tomatoes slightly decreased SAA levels beyond that of the EV tomatoes but this difference did not reach statistical significance (supplementary Fig. IVA).

Feeding WD with transgenic 6F tomatoes significantly increased PON activity; feeding EV tomatoes did not (supplementary Fig. IVB). Feeding WD with EV tomatoes significantly decreased plasma-free 5-hydroxyeicosatetraenoic acid (HETE), 15-HETE, and prostaglandin (PG)D2 levels; feeding transgenic 6F tomatoes significantly reduced these levels more than feeding WD with EV tomatoes (supplementary Fig. IVC–E). Feeding WD with transgenic 6F tomatoes significantly reduced plasma PGE2 levels, while feeding EV tomatoes did not (supplementary Fig. IVF). Feeding WD with transgenic 6F tomatoes significantly increased plasma HDL-cholesterol levels; feeding EV tomatoes did not (supplementary Fig. IVG). Not shown in supplementary Fig. IV are data indicating no significant improvement in plasma total cholesterol, triglycerides, free arachidonic acid, or thromboxane B2 levels after feeding either the EV or transgenic 6F tomatoes.

To summarize and contrast these two experiments, in both short-term feeding experiments transgenic 6F tomatoes significantly decreased plasma SAA, free 5-HETE, 15-HETE, PGD2, and PGE2 levels and increased both plasma PON activity and HDL-cholesterol levels. Additionally, in the first experiment in which LPA levels were measured, feeding the transgenic 6F tomatoes significantly decreased plasma LPA 16:0, 18:0, 18:1, and 20:4 levels, but feeding the WT tomatoes only significantly decreased plasma LPA 20:4 levels, which were significantly decreased even further by the transgenic 6F tomatoes. In the second short-term experiment but not in the first, feeding the control EV tomatoes significantly reduced plasma SAA levels. In the first short-term experiment feeding the control WT tomatoes significantly increased PON activity, but in the second experiment feeding the control EV tomatoes did not. In the first short-term experiment feeding the control WT tomatoes did not significantly decrease plasma-free 5-HETE, 15-HETE, PGD2, or PGE2 levels, but in the second experiment feeding the control EV tomatoes significantly decreased plasma-free 5-HETE, 15-HETE, and PGD2 levels and these levels were significantly decreased even further by feeding the transgenic 6F tomatoes. In both short-term experiments feeding the control tomatoes failed to alter HDL-cholesterol levels. In both short-term experiments plasma-free arachidonic acid levels, total cholesterol levels, and triglyceride levels were not changed by feeding any of the tomatoes.

**Because control tomatoes improved some of the biomarkers in these short-term feeding experiments, could the superior performance of the transgenic 6F tomatoes be due to an induction of higher levels of antioxidants in the transgenic 6F tomatoes?**

To test this question we measured the content of the major tomato antioxidant, lycopene. As shown in Fig. 6, surprisingly, the homozygous transgenic 6F tomato lines 1A...
Fig. 5. Feeding transgenic 6F tomatoes to LDLR−/− mice for two weeks improved a number of plasma biomarkers. Female LDLR−/− mice 10 weeks of age were housed four in each cage and each cage was given each night compacted WD containing no lyophilized tomatoes (n = 20), compacted WD containing 2.2% by weight ground lyophilized WT tomatoes (n = 8), or compacted WD containing 2.2% by weight ground lyophilized transgenic 6F (Tg6F) tomatoes. The mice in all cages ate all of the diet each night. The mice receiving the Tg6F tomatoes received
and 17A (the latter was used in the two short-term feeding experiments described above) had slightly but significantly less lycopene content compared with WT tomatoes or compared with EV tomato lines 108 and 110 (the latter was used in the second short-term feeding experiment).

**Would feeding WD for 13 weeks with tomatoes transgenic for 6F improve plasma biomarkers and aortic atherosclerosis compared with no added tomatoes or compared with feeding empty vector tomatoes?**

As shown in **Fig. 7A–E**, after 13 weeks of feeding WD with transgenic 6F tomatoes (but not EV tomatoes) there was a significant reduction in plasma SAA, total cholesterol, and triglycerides and a significant increase in plasma PON activity and HDL-cholesterol levels. There also was a significant decrease in plasma LPA levels for LPA 18:1 (Fig. 7F), LPA 18:2 (Fig. 7G), and LPA 20:4 (Fig. 7H) in the mice fed transgenic 6F tomatoes (but not EV tomatoes). However, there was no significant difference in levels of LPA 16:0 or LPA 18:0 (data not shown). As shown in Fig. 7I, there was also no difference in body weight between the three groups. While the EV tomatoes did not affect parameters mentioned above, feeding both the EV and the transgenic 6F tomatoes significantly reduced plasma levels of free arachidonic acid, 5-HETE, and 15-HETE and increased plasma levels of free docosahexaenoic acid 22:6 (n-3) (DHA) and eicosapentaenoic acid 20:5 (n-3) (EPA) (**Fig. 8A–E**). Plasma levels of free 12-HETE, 20-HETE, PGD2, PGE2, thromboxane (TX)B2, 14,15-eicosatrienoic acid (EET), and 8-isoPGF2α were not significantly improved by feeding either EV or transgenic 6F tomatoes (data not shown). As shown in **Fig. 9**, feeding WD with transgenic 6F tomatoes significantly reduced the percent of the aorta with atherosclerosis as determined by en face analysis compared with WD alone or WD + EV tomatoes; the latter was not significantly different from WD alone.

**Which biomarkers correlated with the percent of the aorta with atherosclerotic lesions?**

Using linear regression of individual data for all mice regardless of treatment revealed a significant positive correlation between the percent of the aorta with atherosclerotic lesions and plasma SAA ($r^2 = 0.5358, P < 0.0001$), total cholesterol ($r^2 = 0.5937, P < 0.0001$), triglycerides ($r^2 = 0.3425, P < 0.0001$), and free 15-HETE ($r^2 = 0.2666, P < 0.0001$). There was also a very weak but significant positive correlation between lesions and plasma-free PGD2 levels ($r^2 = 0.06078, P = 0.046$). There was a significant inverse correlation between the percent of the aorta with atherosclerosis and LPA 18:1 ($r^2 = 0.5948, P < 0.0001$). There was a very weak but significant inverse correlation between the percent of the aorta with atherosclerotic lesions and plasma levels of free EPA ($r^2 = 0.09596, P = 0.0107$). There was no significant correlation between the percent of the aorta with atherosclerotic lesions and body weight, plasma-free arachidonic acid, 5-HETE, 12-HETE, 20-HETE, PGE2, TXB2, 14,15-EET, DHA, or 8-isoPGF2α (data not shown). As shown in supplementary Fig. VA and B, there was no correlation between the plasma levels of LPA 16:0 or LPA 18:0 and the percent of the aorta with lesions. In contrast, as shown in supplementary Fig. VC–E, there was a significant correlation between the levels of LPA 18:1, LPA 18:2, and LPA 20:4 levels with the percent of the aorta with lesions. These correlations were for all mice with all treatments. The correlation between some of these plasma biomarkers and the percent of the aorta with lesions for mice that received WD with transgenic 6F tomatoes is shown in **Fig. 10A–E**.

**Were small intestine lipid levels altered by the treatments?**

We were able to measure tissue cholesterol and LPA levels in the duodenum, jejunum, and ileum in a random subset of the mice described in Figs. 7–9. As shown in supplementary Fig. VIA–C, addition of both the EV and the transgenic 6F tomatoes to the WD significantly reduced tissue cholesterol levels in the duodenum and jejunum, but not in the ileum. Addition of EV tomatoes to WD modestly but significantly reduced jejunum cholesterol levels more than did the addition of transgenic 6F tomatoes (supplementary Fig. VIB); cholesterol levels in the duodenum were not different between mice fed EV tomatoes or transgenic 6F tomatoes (supplementary Fig. VIA). As shown in supplementary Fig. VIE, jejunum cholesterol levels were very weakly but significantly correlated with the percent of the aorta with lesions in these mice, but there was no significant correlation of lesions with tissue cholesterol levels in either the duodenum (supplementary Fig. VID) or ileum (supplementary Fig. VIF). In contrast to these results for small intestine cholesterol levels, as shown in **Fig. 11A–F**, adding transgenic 6F tomatoes to
Fig. 7. Feeding transgenic 6F tomatoes to LDLR<sup>−/−</sup> mice for 13 weeks improved a number of plasma biomarkers. Female LDLR<sup>−/−</sup> mice 7–9 months of age were housed four in each cage and each cage was given each night compacted WD containing no lyophilized tomatoes (n = 28), compacted WD containing 2.2% by weight ground lyophilized EV tomatoes (from line 110) (n = 20), or compacted WD containing 2.2% by weight ground lyophilized transgenic 6F (Tg6F) tomatoes (from line 17A) to provide 900 mg of 6F per mouse per day (45 mg/kg/day). The mice in all cages ate all of the diet each night. After 13 weeks the mice were bled and the following measurements were made as described in Materials and Methods: SAA (A); total cholesterol (B); triglycerides (C); PON (D); HDL cholesterol (E); LPA 18:1 (F); LPA 18:2 (G); LPA 20:4 (H); and body weight (I).

WD compared with adding EV tomatoes to WD significantly reduced levels of LPA 18:2 and LPA 20:4 in the duodenum, jejunum, and ileum. As shown in Fig. 12A–F, except for LPA 20:4 in the duodenum, which approached significance (but did not reach it), the levels of LPA 18:2 and LPA 20:4 in the duodenum, jejunum, and ileum significantly correlated with the percent of the aorta with atherosclerotic lesions in these mice.

Where does the peptide act?

The data presented above suggests that the peptide in the transgenic 6F tomatoes is acting in the small intestine.
Methods. Intact 6F peptide was found in the small intestine of each of these six mice in microgram quantities (mean ± SD was 15.6 ± 7.4 μg 6F per 200 mg small intestine). In these studies the lower limit of detection for 6F peptide in the plasma was ~100 ng/ml. No 6F peptide was detected in the plasma of any of the six mice. Thus ~2 h after eating the transgenic 6F tomatoes, intact 6F peptide was found in the small intestine, but not in the plasma. These data are consistent with the peptide acting in the small intestine.

If this were the case, we might expect to find intact peptide in the small intestine of mice eating WD with transgenic 6F tomatoes, but little to no intact peptide in the plasma. The mice described in supplementary Fig. VII were fasted overnight for 20 h and then fed WD with lyophilized transgenic 6F tomato powder containing 900 μg of 6F in 2 g of diet. Over a period of 30–90 min each of six mice ate all of the 2 g of diet. Approximately 2 h after the mice finished eating they were bled and their small intestines were harvested and analyzed as described in Materials and Methods. Intact 6F peptide was found in the small intestine of each of these six mice in microgram quantities (mean ± SD was 15.6 ± 7.4 μg 6F per 200 mg small intestine). In these studies the lower limit of detection for 6F peptide in the plasma was ~100 ng/ml. No 6F peptide was detected in the plasma of any of the six mice. Thus ~2 h after eating the transgenic 6F tomatoes, intact 6F peptide was found in the small intestine, but not in the plasma. These data are consistent with the peptide acting in the small intestine.

Fig. 8. Feeding EV and transgenic 6F tomatoes decreased some biomarkers and increased others. The plasma from the mice described in Fig. 7 was analyzed as described in Materials and Methods for: free arachidonic acid (A); free 5-HETE (B); free 15-HETE (C); free DHA (D); and free EPA (E).
DISCUSSION

In our original manuscript on apoA-I mimetic therapy, we concluded that D-4F but not L-4F would be effective orally (28). This conclusion was based on experiments in which LDLR<sup>−/−</sup> mice were administered either L-4F or D-4F by stomach tube in a single dose of 5 mg/kg/mouse. Four hours after this single dose, the inflammatory properties of HDL and LDL as determined in a cell-based assay were dramatically and significantly improved in the case of D-4F but not L-4F. Using <sup>125</sup>I-peptides, we also found that after oral administration of L-4F there was virtually no intact peptide in plasma, but after administration of D-4F there was intact peptide identified in the plasma. Because L-4F was ineffective and D-4F was effective in this study it was assumed that it was necessary for intact peptide to gain access to the plasma to be effective (28). Supporting this assumption was the finding that when given by injection at a dose of 10 mg/kg/day to cholesterol-fed rabbits, the efficacy of L-4F and D-4F was identical (29). A phase I/II study in humans was undertaken in which D-4F was administered orally in doses ranging from 0.43 to 7.14 mg/kg. Maximum plasma peptide levels were low (Cmax 15.9 ± 6.5 ng/ml) but doses of 4.3 and 7.14 mg/kg significantly improved the HII, while doses of 0.43 and 1.43 mg/kg were not effective (6). Subsequently, in preclinical studies it was found that D-4F had delayed clearance from tissues, particularly liver and kidney making its use in humans problematic; this was not the case for L-4F (7). Because it was known that L-4F and D-4F were equally efficacious when given by injection (29), and it was thought that plasma levels of the peptide would be the critical success factor for its efficacy, studies in humans were designed to achieve high plasma levels with low doses of L-4F administered IV or SQ (7). Doses of 0.042–1.43 mg/kg of L-4F produced high plasma levels of peptide (e.g., Cmax 3,255 ± 630 ng/ml in the IV study), but surprisingly there was no improvement in HII (7). After this disappointing result, we returned to mouse models to understand this paradox and unexpectedly found that: i) plasma levels did not predict efficacy, the dose administered predicted efficacy; and ii) while the concentration of peptide differed by orders of magnitude in plasma and liver depending on the route of administration, the concentration of peptide in the feces (8) and small intestine (9) was similar at similar doses regardless of whether the peptide was administered orally or SQ. To explain equal efficacy at each dose administered regardless of the route of administration there should be equal concentrations of peptide in at least one compartment containing a major site of action. In two separate studies (8, 9) the intestine was found to be that compartment.

Administering L-4F orally (incorporated into mouse chow) at a dose of 10 mg/kg/day to female apoE<sup>−/−</sup> mice starting at 9.5 months of age and continuing for six months together with adding a low dose of statin in the drinking water did not significantly change aortic atherosclerosis (30). In contrast, if the L-4F was administered with niclosamide which binds to L-4F and protects it against trypsin degradation in the intestine, the peptide not only inhibited lesion progression, but it also actually induced lesion regression in these old mice (30). In a subsequent study, we reasoned that if instead of administering the peptide with niclosamide, we simply increased the dose by 10-fold, enough L-4F might survive degradation after oral administration to be effective. Indeed this was the case. Administering L-4F in mouse chow at a dose of 100 mg/kg/day significantly decreased plasma LPA levels and significantly decreased tumor burden in a mouse model of ovarian cancer (26).

These studies suggested that oral apoA-I mimetic therapy using peptides synthesized from all L-amino acids might be feasible if we used high doses of the peptide. Unfortunately, producing sufficient peptide to make therapy in humans practical was not likely because the 4F peptide requires end blocking groups that can only be added through chemical synthesis. Using mouse models, we explored the possibility of using 4F peptide without end blocking groups but found the activity of the peptide to be dramatically reduced (data not shown). This led us to seek alternative peptides synthesized from all L-amino acids that might be effective without end blocking groups. As described here, 6F was found to be such a peptide.

Based on our previous work (8, 9, 26) we chose to test peptide doses of 40–100 mg/kg/day. Adding the 6F peptide synthesized from all L-amino acids without end blocking groups to diets of apoE<sup>−/−</sup> or LDLR<sup>−/−</sup> mice (chow or WD) at a dose of 60 mg/kg/day resulted in significantly decreased plasma SAA (Fig. 2A, B). In addition, the percent of the aorta with atherosclerosis also significantly decreased (Fig. 2C). We chose to first test transgenic expression of the 6F peptide in tomatoes because we reasoned that the peptide should be expressed in a plant that could be eaten without cooking to avoid denaturing the peptide. Adding the 6F peptide to homogenized tomatoes did not lead to a loss of efficacy (supplementary Fig. II). The 6F peptide was successfully expressed in tomato plants...
we certainly cannot exclude that a portion (if not all) of the beneficial effects of expressing the 6F peptide in tomatoes is due to an increase in an as yet unidentified nonlycopene, non6F component of these tomatoes.

Longer feeding experiments of 13 weeks using EV tomatoes as the control revealed that only the 6F transgenic tomatoes significantly decreased plasma SAA, total cholesterol, triglycerides, and LPA levels, and increased plasma HDL cholesterol and PON activity (Fig. 7), and decreased the percent of the aorta with lesions (Fig. 9); all without changing body weight (Fig. 7I). However, the EV tomatoes did decrease plasma levels of free arachidonic acid and some of its metabolites and increased DHA and EPA (Fig. 8) suggesting that there was likely a benefit from the antioxidant content of the control tomatoes. The increase in DHA and EPA plasma levels may have been due to decreased oxidation of these highly unsaturated fatty acids on feeding the control tomatoes. The further increase in plasma DHA and EPA levels on feeding the transgenic

and was found in ripened tomato fruit (Figs. 3, 4 and supplementary Fig. III). Feeding ground lyophilized tomatoes containing 6F to LDLR−/− mice on WD for two weeks favorably altered some plasma biomarkers (Fig. 5 and supplementary Fig. IV), but did not alter plasma total cholesterol or triglyceride levels. In some of these experiments feeding the ground lyophilized control tomatoes decreased some of the biomarkers raising the possibility that the superior effects of the 6F transgenic tomatoes might be due to increased antioxidant content. This did not turn out to be the case for the major antioxidant in tomatoes, lycopene (Fig. 6). Because the addition to the diet of chemically synthesized L-6F without blocking groups produced biologic results similar to those achieved with the addition of lyophilized transgenic 6F tomatoes, it is likely that at least some of these effects were due to the presence of the peptide in the tomatoes. Based on the data in Fig. 6, it is also likely that the beneficial effects of the transgenic 6F tomatoes were not due to their lycopene content. However,

we certainly cannot exclude that a portion (if not all) of the beneficial effects of expressing the 6F peptide in tomatoes is due to an increase in an as yet unidentified nonlycopene, non6F component of these tomatoes.

Longer feeding experiments of 13 weeks using EV tomatoes as the control revealed that only the 6F transgenic tomatoes significantly decreased plasma SAA, total cholesterol, triglycerides, and LPA levels, and increased plasma HDL cholesterol and PON activity (Fig. 7), and decreased the percent of the aorta with lesions (Fig. 9); all without changing body weight (Fig. 7I). However, the EV tomatoes did decrease plasma levels of free arachidonic acid and some of its metabolites and increased DHA and EPA (Fig. 8) suggesting that there was likely a benefit from the antioxidant content of the control tomatoes. The increase in DHA and EPA plasma levels may have been due to decreased oxidation of these highly unsaturated fatty acids on feeding the control tomatoes. The further increase in plasma DHA and EPA levels on feeding the transgenic

Fig. 10. The percent of the aorta with atherosclerotic lesions in mice receiving WD and transgenic 6F tomatoes was positively and significantly correlated with plasma total cholesterol and triglycerides, and was significantly and inversely correlated with PON activity and HDL cholesterol; there was no correlation with body weight. Linear regression of data from individual mice described in Figs. 7–9 that received WD and transgenic 6F tomatoes is shown for the percent of the aorta with atherosclerotic lesions and plasma total cholesterol (A); plasma triglycerides (B); plasma PON activity (C); plasma HDL-cholesterol levels (D); and body weight (E).
Fig. 11. Addition of transgenic 6F tomatoes (Tg6F) to the WD significantly reduced the levels of LPA in the small intestine, while addition of the EV tomatoes did not. The levels of LPA 18:2 and LPA 20:4 were determined by LC-ESI-MS/MS in a random subset of the mice described in Figs. 7–9 as described in Materials and Methods. A: LPA 18:2 in the duodenum. B: LPA 20:4 in the duodenum. C: LPA 18:2 in the jejunum. D: LPA 20:4 in the jejunum. E: LPA 18:2 in the ileum. F: LPA 20:4 in the ileum.

6F tomatoes likely represents a further reduction in the WD-induced oxidative stress beyond that achieved by the antioxidants contained in the control tomatoes.

Based on correlations between the percent of atherosclerosis and the various biomarkers measured, it seems likely that the mechanism of action of the transgenic 6F tomatoes involves alteration in lipid metabolism in the intestine that favorably alters plasma total cholesterol, triglycerides, LPA levels, HDL cholesterol, and PON activity, which result in decreased systemic inflammation (SAA levels) and atherosclerosis without changing body weight. It has been reported that LPA can alter the secretion of apoB containing lipoproteins from hepatocytes (31) and LPA 20:4 promotes atherosclerosis in mouse models (32). As shown in Fig. 11, feeding transgenic 6F tomatoes significantly reduced LPA 18:2 and LPA 20:4 levels in the duodenum, jejunum, and ileum. The levels of these LPA species were significantly correlated with the percent of the aorta with atherosclerosis while the levels of saturated LPA species did not. This needs to be the subject of future research.

As shown in supplementary Fig. VI, feeding both EV and transgenic 6F tomatoes significantly decreased the levels of cholesterol in the duodenum and jejunum, but neither reduced cholesterol levels in the ileum. As shown in Fig. 7B, only the transgenic 6F tomatoes significantly decreased plasma cholesterol levels. Additionally and in contrast to the case for plasma cholesterol levels (Fig. 10A), the levels of cholesterol in the small intestine were either very weakly correlated with the percent of the aorta with lesions (supplementary Fig. VIE) or were not correlated with the percent of the aorta with lesions (supplementary Fig. VID, F).

It is possible that the transgenic 6F tomatoes decreased the absorption of cholesterol or triglycerides in the 13 week feeding studies. However if this were the case, it is not clear why plasma total cholesterol and triglycerides were not significantly decreased in the two week feeding studies. All of these questions need to be rigorously tested in future studies. Additionally, the nature of the changes...
in plasma lipids induced by feeding transgenic 6F tomatoes needs to be further defined by additional future studies using FPLC or electrophoresis of the lipoproteins.

The studies reported here used only female mice, thus results in male mice need to be determined in future studies. We have routinely used female mice in our studies because: i) in general they develop worse atherosclerosis; and ii) in studies of the 4F peptide there were no major differences seen in responsiveness between male and female mice (33). Nonetheless, one must be aware of the potential of gender differences and this issue will need to be addressed by future studies. Similarly, the studies reported here relied on en face analysis of atherosclerotic lesions and did not provide information on lesion composition and maturity. These are all issues that need to be addressed in future studies.

In preliminary unpublished studies in a mouse model of ovarian cancer, we found that adding the lyophilized transgenic 6F tomatoes to chow gave results similar to those previously reported for L-4F (26). If these studies are confirmed they will indicate that the efficacy of the transgenic 6F tomatoes does not require either hyperlipidemia or a WD.

To our knowledge this is the first report of transgenically expressing a peptide in a fruit that when fed to mice results in the anti-inflammatory properties described here. These studies leave many questions unanswered. How does the 4F peptide without blocking groups compare with the 6F peptide without blocking groups in terms of binding oxidized lipids, susceptibility to degradation in the intestine, CD spectra, and ability to prevent LDL oxidation by artery wall cells? The data in supplementary Fig. VII indicate that ~2 h after the mice finished eating 900 μg of 6F contained in transgenic tomatoes, intact 6F peptide was identified in the small intestine in microgram quantities (15.6 ± 7.4 μg 6F per 200 mg small intestine), but no peptide was detected in the plasma with methods that would have detected 100 ng/ml. Is the 6F peptide protected from trypsin degradation by being expressed in the tomato fruit similar to the case with niclosamide? How does the peptide interact with the small intestine? Is it absorbed into the enterocytes or does it only interact with the luminal surface of these cells? Does the peptide act directly on the enterocytes or does it alter the microbiome, which in turn alters the function of the small intestine? Does the peptide alter transintestinal cholesterol efflux (34, 35)? Is the peptide acting similar to apoA-I in the small intestine (36)? Does the peptide increase apoA-I synthesis in the intestine? Does the peptide alter ABCA1 expression in the intestine? How does the 6F peptide reduce intestinal levels of the potent growth promoter LPA? How does the peptide increase PON activity? Does the peptide reduce oxidized lipid content in the intestine? What is the mechanism by which plasma levels of LPA and SAA are reduced? Regardless of whether this approach eventually turns out to be a practical therapeutic strategy, the answers to these questions will likely yield important and fundamental knowledge on the role of the intestine in diet-induced inflammation and atherosclerosis and perhaps in some cancers.

Fig. 12. The levels of LPA in the small intestine significantly correlated with the percent of the aorta with atherosclerotic lesions. The levels of LPA in the small intestine of the mice described in Fig. 11 were plotted against the percent of the aorta with lesions for each mouse, and linear regression was performed as described in Materials and Methods. A: LPA 18:2 duodenum. B: LPA 20:4 duodenum. C: LPA 18:2 jejunum. D: LPA 20:4 jejunum. E: LPA 18:2 ileum. F: LPA 20:4 ileum.
