HDL Mimetics Inhibit Tumor Development in Both Induced and Spontaneous Mouse Models of Colon Cancer

Feng Su1,2, Victor Grijalva2, Kaveh Navab2, Ekambaram Ganapathy1, David Meriwether1, Satoshi Imaizumi2, Mohamad Navab2, Alan M. Fogelman3, Srinivasa T. Reddy1,2,3, and Robin Farias-Eisner1

Abstract
Recent studies suggest that high-density lipoprotein (HDL) levels are inversely related to colon cancer risk. HDL mimetics constructed from a number of peptides and proteins with varying structures possess anti-inflammatory and antioxidant properties reminiscent of HDL. In this article, we examined whether HDL mimetics, L-4F (an apolipoprotein A-I mimetic peptide) and G' (an apolipoprotein J mimetic peptide) affect tumor growth and development in mouse models of colon cancer. HDL mimetics reduced viability and proliferation of CT26 cells, a mouse colon adenocarcinoma cell line, and decreased CT26 cell–mediated tumor burden in BALB/c mice when administered subcutaneously or orally. Plasma levels of lysophosphatidic acid (LPA), a serum biomarker for colon cancer, were significantly reduced in mice that received HDL mimetics, suggesting that binding and removal of proinflammatory lipids is a potential mechanism for the inhibition of tumor development by HDL mimetics. Furthermore, L-4F significantly reduced size and number of polyps in APCmin/+ mice, a mouse model for human familial adenomatous polyposis, suggesting that HDL mimetics are effective in inhibiting the development of both induced and spontaneous cancers of the colon. Our results, for the first time, identify HDL mimetics as a novel therapeutic strategy for the treatment of colon cancer. Mol Cancer Ther; 11(6); 1311–9. ©2012 AACR.

Introduction
Colon cancer is the third most common cancer worldwide and the third leading cause of cancer death in both men and women in the United States, with approximately 150,000 new cases diagnosed and 50,000 disease related deaths every year (1). Like most cancers, early diagnosis and surgery significantly improve the chances of cure for colon cancer (2). Therefore the development of serum-based biomarkers and novel therapeutic targets for treating colorectal cancer are greatly needed.

High-density lipoprotein (HDL) is an important mediator of lipid homeostasis. HDL and HDL-associated molecules (proteins and lipids) provide a number of protective functions including anti-inflammatory, antioxidant, antimicrobial, and innate immunity (3). HDL cholesterol (HDL-C) is an accepted marker for cardiovascular risk assessment (4) and several clinical strategies for cardiovascular therapy are designed to elevate HDL-C (5, 6). Although there is significant correlation between lipid metabolism and cancer, until recently very little is known about the potential role for lipoproteins in cancer biology. There is a significant inverse association between HDL-C and the risk of incident cancer, which is independent of low-density lipoprotein cholesterol, age, body mass index, and smoking status (7). The concentrations of HDL and apolipoprotein A-I (apoA-I, the major protein component of HDL) were found to be inversely associated with the risk of colon cancer (8, 9).

Recent studies suggest that increasing the amount of circulating HDL-cholesterol alone does not reduce the risk of coronary heart disease events, coronary heart disease deaths, or total deaths (10). One method that has been reported to modify the lipid and protein cargo of HDL involves treatment with HDL mimetic peptides (11). Our previous studies showed that apoA-I is a biomarker for detection of early-stage ovarian cancer and a promising therapeutic target for the treatment of ovarian cancer (12–17). In this study, we show that 2 HDL mimetics, the apoA-I mimetic peptide L-4F (Ac-D-W-F-K-A-Y-D-K-V-A-E-K-F-K-E-F-NH2 synthesized from all L-amino acids) and the apoJ peptide termed G’ (Ac-L-V-G-R-Q-L-E-E-F-L-NH2 corresponding to amino acids 113 to 122 in apoJ (L-[113–122]), decrease tumor burden in mice injected with CT26 cells. We further show that L-4F and G’ peptides reduce plasma lysophosphatidic acid (LPA) levels in mice. Our results show that HDL mimetics L-4F and G’ mimic the functions of HDL.
may serve as therapeutic agents for the treatment of colon cancer.

Materials and Methods

Mice

The Animal Research Committee at University of California at Los Angeles approved all mouse protocols. Six-week-old BALB/c female mice and 6-week-old C57BL/6j-APCMin+/− male mice were purchased from The Jackson Laboratory.

Peptides

HDL mimetics, the apoA-I peptide L-4F (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2) and a scrambled peptide (sc-4F) containing the same amino acids as in the 4F peptides but arranged in a sequence (Ac-D-W-F-A-K-D-Y-F-K-A-F-V-E-E-F-A-K-NH2) that prevents the formation of a class A amphipathic helix, and the apo mimetic, named G+ peptide {Ac-L-V-G-R-Q-L-E-E-F-L-NH2 corresponding to amino acids 113 to 122 in apoJ (L-[113–122] apoJ)}, were synthesized from all L-amino acids. The peptides were dissolved in H2O for administration by injection. For administration of peptides in the diet, the peptides were mixed into standard mouse chow (Ralston Purina) using techniques essentially as described previously for a Western diet (18). However, the Western diet was not administered in any of the experiments reported here; the mice only received standard mouse chow with or without the peptides.

Cell culture experiments

CT26 cell line derived from N-nitroso-N-methyl urethane-induced mouse colon carcinoma of BALB/c origin was purchased from the American Type Culture Collection. CT26 cells (2,000 cells per well) were first cultured in complete medium in 96-well culture plates, and 24 hours later the medium was replaced with serum-free medium. Following an overnight incubation, the cells were either treated with vehicle (control), or treated with 10 μg/mL of L-4F or G+ peptide. The peptides were dissolved in H2O. Cells were incubated for an additional 48 hours and assayed for viability using the MTS assay kit (Promega) according to the manufacturer’s protocol. For proliferation assay, cells were labeled with bromodeoxyuridine (BrdUrd) for the last 4 hours of the 48 hours incubation. Cells were subsequently washed, fixed, and incubated with mouse anti-BrdUrd antibody for 1 hour at room temperature and detected by a peroxidase-coupled goat anti-mouse secondary antibody (Calbiochem). Absorbance was measured using dual wavelengths 450 and 540 nm.

Tumor load study

Six-week-old BALB/c female mice were given a 100 μL subcutaneous injection of 1 × 106 CT26 cells prepared as a single cell suspension in PBS, and the mice were treated with sc-4F or L-4F at 10 mg/kg administered subcutaneously daily for 15 days. The mice were sacrificed and tumor weights were measured.

Pulmonary metastasis in vivo

BALB/c mice were intravenously injected with 2 × 104 CT26 cells in 100 μL of PBS via tail vein injection and the mice were treated with L-4F or sc-4F at 10 mg/kg/d administered subcutaneously for 3 weeks, or treated with sc-4F or L-4F or G+ peptide at 100 mg/kg/d administered in a chow diet for 3 weeks. After 3 weeks treatment, the mice were sacrificed; lungs were harvested, weighed, and fixed with Bouin solution (Sigma). Tumor nodules on the lung surface were counted.

APCMin+/− mice study

Six-week-old APCMin+/− male mice on a C57BL/6J background were treated with L-4F or sc-4F at 100 mg/kg/d administered in a chow diet. After 8 weeks treatment, mice were sacrificed. The entire intestine was immediately removed, fixed in formalin and 70% ethanol. The intestine was opened and examined under a dissecting microscope to count and measure the tumors.

Immunohistochemistry staining

Tumor tissues from the lung surface were fixed and embedded with paraffin, sectioned at 5 μm thickness. Sections were deparaffinized with xylene, rehydrated with 100%, 90%, 70%, and 50% ethanol, treated with 3% H2O2 for 30 minutes at room temperature and detected by a peroxidase-coupled goat anti-mouse monoclonal CD31 antibody overnight at 4°C. The sections were incubated with corresponding biotinylated secondary antibody for 1 hour, followed by incubation with Vectastain ABC Elite reagents.

Cell-cycle analysis

CT26 cells were cultured in 6-well plates overnight and then serum starved for 48 hours. Cells were either treated with vehicle (control), or treated with 10 μg/mL of L-4F or G+ peptide, and incubated for an additional 48 hours. Cells were collected, washed with PBS, and fixed with 70% ice-cold methanol overnight at 4°C. The fixed cells were collected by centrifugation, washed with PBS, and resuspended in 0.3 mL of PBS containing 40 μg/mL RNaseA and 100 μg/mL propidium iodide, and subjected to flow cytometric cell-cycle analysis by FACSscan from BD Biosciences.

Western blot analysis

Total cell proteins were collected after treatment in cell lysis buffer containing 0.1 mol/L NaCl, 5 mmol/L EDTA, 50 mmol/L sodium orthovanadate, 1% Triton X-100, and protease inhibitor tablet in 50 mmol/L Tris buffer (pH 7.5). Twenty micrograms of total proteins were separated by SDS-PAGE and transferred onto
nitrocellulose membrane and followed by incubation with primary antibody at 4°C in 5% skim milk and 0.1% Tween-20. Anti-cyclin D1 and anti-cyclin A rabbit polyclonal antibodies were used at 1:1,000 dilution, and anti-β-actin polyclonal antibody was used at 1:2,000 dilution.

**ELISA analysis**
Interleukin (IL)-6 concentrations were measured in plasma by a competition ELISA according to the manufacturer’s protocol (Invitrogen).

**LPA binding affinity and serum LPA levels**
LPA (20:4) was purchased from Avanti Polar Lipids. LPA levels were determined as described previously (19).

**Statistical analyses**
The data are shown as means ± SD for each group. We carried out statistical analyses by unpaired t test. All results were considered statistically significant at P < 0.05.

**Results**

**HDL mimetic L-4F inhibits tumor development following CT26 cell injection in BALB/c mice**
CT26 is a colon adenocarcinoma cell line that develops metastatic pulmonary tumors when introduced intravenously into immunocompetent BALB/c mice (20–22). CT26 cell line has been widely used as a syngeneic tumor model to study therapeutic applications for cancer in mouse models and therefore we chose CT26 cells for the colon cancer study in our HDL mimetic studies. We first examined the effect of L-4F and sc-4F (a scrambled peptide containing the same amino acids as in the 4F peptide but arranged in a sequence that prevents the formation of a class A amphipathic helix) administered subcutaneously at 10 mg/kg/d for 3 weeks on lung tumor formation in BALB/c mice injected with 2 × 10⁴ CT26 cells via tail vein. The lung weights (Fig. 1A) and the tumor numbers counted on the lung surface (Fig. 1B) in BALB/c mice treated with L-4F (n = 11 per group) were significantly reduced compared with mice treated with sc-4F (280 vs. 225 mg, P < 0.01; 33 vs. 18, P < 0.001). Representative
photographs of lung tumors from the 2 groups are shown in Fig. 1C. We next examined whether L-4F treatment effects the development of tumors in the flanks of BALB/c mice. Six-week-old BALB/c female mice were injected with $1 \times 10^6$ CT26 cells subcutaneously in the flank. The mice were treated with either sc-4F ($n = 9$) or L-4F ($n = 8$) at 10 mg/kg administered subcutaneously daily for 15 days at a site distant from the site where the CT26 cells were injected. The flank tumor weights were significantly larger in BALB/c mice treated with sc-4F compared with mice treated with L-4F (778 vs. 389 mg, $P < 0.05$; Fig. 1D). We also measured IL-6 levels in plasma from the experiment shown in Fig. 1A. IL-6 was significantly decreased in mice with L-4F treatment compared with control group (Fig. 1F).

Tumor development following CT26 cell injection is significantly decreased in mice that were treated with L-4F administered in mouse chow

We recently reported that 4F is effective in animal models of atherosclerosis whether administered subcutaneously or orally (18). To determine whether L-4F could reduce tumor development when administered orally, BALB/c mice were injected with $2 \times 10^4$ CT26 cells via tail vein and treated with L-4F ($n = 9$) or sc-4F ($n = 12$) at 100 mg/kg/d administered in the chow diet for 3 weeks. The lung weights (Fig. 2A) and the tumor numbers (Fig. 2B) in BALB/c mice treated with sc-4F were significantly larger compared with mice treated with L-4F (296 vs. 238 mg, $P < 0.05$; 21 vs. 12, $P < 0.0001$). We previously reported that L-4F inhibits angiogenesis in vivo (23). Immunohistochemical staining of tumor sections from this experiment showed a significant decrease in CD31 expression in tumors derived from mice treated with L-4F compared with control mice (Fig. 2C). Furthermore, plasma LPA levels were significantly reduced in mice receiving L-4F peptide compared with their corresponding control mice, $P < 0.01$ (Fig. 2D).

Tumor numbers and sizes in the intestinal tract are significantly decreased in C57BL/6J-Apc$^{Min^{-1}}$ mice treated with L-4F administered in mouse chow

We next examined whether HDL mimetics could effect the development of colon tumors in a spontaneous model of colon cancer. APC$^{Min^{-1}}$ mouse is an established mouse model for colon cancer and mirrors the development of familial adenomatous polyposis in humans (24, 25). Six-week-old C57BL/6J-Apc$^{Min^{-1}}$ male mice were treated with L-4F ($n = 5$) or sc-4F ($n = 6$) at 100 mg/kg/d administered in mouse chow for 8 weeks. The tumor numbers and sizes in the intestinal tract from mice treated
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with L-4F were significantly reduced compared with mice treated with sc-4F (100% vs. 60%, \( P < 0.05 \); 1–3 mm: 56.5 vs. 36.8, \( P < 0.05 \); >3 mm: 12.8 vs. 5, \( P < 0.05 \); Fig. 3A and B). Plasma LPA levels from this experiment were significantly reduced in mice receiving L-4F peptide compared with control mice, \( P < 0.01 \) (Fig. 3C).

**L-4F alters CT26 cell viability, proliferation, cell cycle, and expression of cell-cycle–related proteins in vitro**

To examine the mechanisms by which HDL mimetic, L-4F, inhibits CT26 cell–mediated tumor development in mice, the effect of L-4F on CT26 cell viability was determined *in vitro*. Cell viability was reduced by more than 25% (\( P < 0.001 \)) in CT26 cells that were treated with L-4F (10 \( \mu \)g/mL) when compared with control (Fig. 4A). Moreover, L-4F significantly inhibited proliferation of CT26 cells (\( P < 0.001 \)) as measured by BrdUrd incorporation (Fig. 4B). To investigate whether L-4F inhibited cell proliferation through changes in cell-cycle progression, the effect of L-4F on the cell-cycle profile was assessed in CT26 cells. Cell-cycle analysis showed that L-4F treatment for 48 hours induced an increase in G0/G1 phase and arrest in S phase (Fig. 4C). Moreover, Western blot analysis showed that expression of the cell-cycle proteins cyclin D1 and cyclin A were significantly lower in cells treated with L-4F (Fig. 4D).

**HDL mimetic L-4F inhibits LPA-induced viability of CT26 cells**

LPA has been identified as an important mediator of tumor development, progression, and metastases in humans (26, 27). We have previously shown that apoA-I mimetic peptides inhibit LPA-induced viability of ID8 cells and reduce serum LPA levels in mice injected with ID8 cells (17). L-4F binds LPA (17), as expected, LPA (10–20 \( \mu \)mol/L) significantly improved CT26 cell growth, and L-4F significantly reduced LPA-induced viability at all doses tested, \( P < 0.001 \) (Fig. 5A). We measured LPA levels in culture medium by liquid chromatography–mass spectrometry and found that LPA 16:0 and 18:0 were significantly decreased with L-4F treatment compared with the control medium. LPA 20:4 and 18:1 were not detectable in cell culture medium (Fig. 5B).

**HDL mimetic G+ peptide (L-[113–122]apoJ) inhibits CT26 cell growth and CT26-mediated tumor development**

G+ (L-[113–122]apoJ) peptide was used to repeat the studies *in vivo* and *in vitro*. Pulmonary tumor development following CT26 cell injection was significantly decreased in mice treated with G+ peptide at 100 mg/kg/d administered in mouse chow for 3 weeks (Lung weights were 296 vs. 250 mg, \( P < 0.05 \); tumor numbers were 21 vs. 10, \( P < 0.0001 \); Fig. 6A and B). Cell viability was approximately 40% lower in CT26 cells treated with G+ peptide (10 \( \mu \)g/mL) when compared with no treatment (Fig. 6C). In the mouse experiment shown in Fig. 6A and B, plasma LPA levels were significantly reduced in mice receiving G+ peptide compared with their corresponding control mice \( P < 0.05 \) (Fig. 6D). Western blot showed the expression of cyclin D1 and cyclin A was lower with G+ peptide treatment compared with no treatment (Fig. 6E).

**Discussion**

There is a significant correlation between lipid metabolism and cancer, and inflammatory oxidative stress has long been thought to be associated with the pathophysiology of cancer (28–30). Lipid oxidation and resulting oxidized lipid-mediated inflammation seem to be common to the etiology of a number of inflammatory diseases (31, 32) implicating a role for lipoproteins in the development and progression of several diseases, including cancer.
HDL is recognized as an integral part of the innate immune system. HDL is a complex macromolecule whose functional repertoire includes antioxidant, anti-inflammatory, and antimicrobial activities. Unlike LDL, HDL is a heterogeneous mixture of proteins and lipids, which determine structural and functional integrity of HDL. Several protein/enzyme constituents of HDL including phospholipid transfer protein, cholesterol ester transfer protein, and lecithin cholesterol acyl transferase are important for its formation and maturation, whereas other protein/enzyme constituents such as apolipoprotein A-I (apoA-I), apoJ, and paraoxonase-1 (PON1) confer functional properties on HDL (33). Over the last decade, HDL mimetics have shown extraordinary therapeutic promise in preclinical studies in a number of inflammatory diseases (34–40).

We have recently shown that L-4F and L-5F, 2 apoA-I mimetic peptides, reduced viability and proliferation of mouse ovarian cancer cells (ID-8 cells) and cis-platinum–resistant human ovarian cancer cells, and decreased ID-8 cell–mediated tumor burden in C57BL/6J mice when administered subcutaneously or orally (17). We further showed that apoA-I mimetic peptides inhibit tumorigenesis by (i) inhibiting angiogenesis.
(23) and (ii) inducing expression and activity of MnSOD (41). Because angiogenesis and redox pathways are common features of many cancers, we examined the effect of 2 HDL mimetics, apoA-I mimic peptide L-4F and an apoJ mimic peptide G’ (42), in the development and progression of colon cancer. Consistent with our hypothesis, our results showed that HDL mimetics inhibit the development of colon cancer generated by injecting CT26 cells into immunocompetent BALB/c mice. Furthermore, we show here for the first time using the mouse model of FAP (APC<sub>Mim−/−</sub>) that oral administration of HDL mimetics is able to suppress the spontaneous development of colon cancer in a mouse model.

There have been 2 sets of clinical trials using the 4F peptides. Bloedon and colleagues (43) found that administration of doses of 4F orally of 4.3 and 7.14 mg/kg significantly improved HDL anti-inflammatory properties despite very low plasma levels (8–16 ng/mL). Bloedon and colleagues (43) also found that administering doses of peptide of 0.43 and 1.43 mg/kg were not effective. Watson and colleagues (44) targeted plasma levels and L-4F was administered daily by either intravenous infusion for 7 days or subcutaneously for 28 days in patients with coronary heart disease. Using a dose of 0.43 mg/kg, Watson and colleagues (44) achieved very high plasma levels but did not achieve any improvement in HDL anti-inflammatory properties. It was concluded that the doses needed for improving HDL function in humans maybe much higher than those used by Watson and colleagues (44) and at least as high as those used by Bloedon and colleagues (43). Recently, Navab and colleagues (45) reported that the dose of the HDL mimetic peptide 4F that was administered, and not the plasma level achieved, determines efficacy and the intestine may be a major site of action for the peptide regardless of the route of administration. Our results show that the HDL mimetics are effective whether given orally or subcutaneously in mouse models at doses greater than those used by Bloedon and colleagues (43). Given our results with HDL mimetics in mouse colon cancer models and the results of Navab and colleagues (45) indicating that dose determines efficacy and not plasma levels, it will be important to test the high doses used here in any future clinical trials.

One of the downstream targets for the general mechanism of antitumorigenic activity of HDL mimetics seems to be angiogenesis, as seen by the reduction in CD31 staining in treated tumors. LPA plays an important role in inflammation, angiogenesis, and cancer, and has become a promising target for therapy (46). Moreover, consistent with our previous findings (17, 23) and current findings, the binding and removal of proinflammatory/proangiogenic lipids such as LPA may be a major part of the mechanism of action for the HDL mimetics.

In conclusion, our results show that HDL mimetics inhibit both induced and spontaneous colon cancer development in mice. The binding and removal of protumorigenic lipids by HDL mimetic peptides likely alters the proliferation capacity of the tumor cells as well as

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Figure 6. G’ (L-[113–122]apoJ) peptide has effects similar to L-4F in vivo and in vitro. Lung tumors were established in BALB/c as described in Materials and Methods. Mice were sacrificed 3 weeks after CT26 cells were injected into the tail vein. Lungs were harvested and weighed. Lung tumors were counted. A, the data shown are lung weights for mice receiving sc-4F (n = 12), G’ peptide (n = 12) at 100 mg/kg/d (2 mg/mouse/d) administered in mouse chow. P < 0.05. B, the data shown are the tumor numbers on the lung surface from 2 group mice of A. P < 0.0001. C, cells were assayed for viability using the MTS assay. P < 0.05. D, serum LPA levels from the mice described in A and B were determined as described in Materials and Methods. E, the expression of cyclin D1 and cyclin A by Western blot. w/sc-4F, mice treated with sc-4F; w/G’, mice treated with G’ peptide.
angiogenesis associated with the tumors. Identifying the target lipid(s) is an important next step in delineating the specific mechanism of action for these HDL mimetics. Future studies to determine the clinical efficacy of HDL mimetics seem warranted to evaluate these new antitumorogenic agents.

Disclosure of Potential Conflicts of Interest
A.M. Fogelman, S.T. Reddy, and M. Navab have an ownership interest in Bruin Pharma and A.M. Fogelman is also an officer of Bruin Pharma.

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References
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