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A High-Fat Diet Increases Angiogenesis, Solid Tumor Growth, and Lung Metastasis of CT26 Colon Cancer Cells in Obesity-Resistant BALB/c Mice

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We evaluated whether high-fat diet (HFD), in the absence of increased calorie intake, increases colon cancer growth and metastasis. Four-week-old male BALB/c mice were fed on an HFD (60 kcal% fat) or control diet (10 kcal% fat) for 16 wk, after which CT26 colon cancer cells were subcutaneously injected into the right flank. Solid tumor growth and the number and volume of tumor nodules in the lung were increased markedly in the HFD group with only a slight increase in body weight (5.9%). HFD feeding increased tumor tissue levels of Ki67, cyclin A, cyclin D1, CDK2, Bcl-xL, and Bcl-2; reduced p53 levels and TUNEL-positive apoptotic cells; increased the levels of CD45, CD68, CD31, VEGF, P-VEGF receptor-2, iNOS, and COX-2 as well as hemoglobin content; and increased the levels of HIF-1α, P-STAT3-Y705, P-STAT3-S727, P-IκB-α, P-p65, p65, P-c-Jun, P-Akt, P-ERK1/2, P-p38, and P-SAPK/JNK. HFD feeding increased the serum levels of EGF, insulin, IGF-I, IFN-γ, leptin, RANTES, MCP-1, IL-1ra, and SDF-1α and media conditioned by epididymal fat tissue explants from HFD-fed mice caused an increase in microvessel outgrowth from the mouse aorta and tube formation of human umbilical vein endothelial cells. These results indicate that the chronic consumption of an HFD increases colon cancer cell proliferation, tumor angiogenesis, and lung metastasis in mice in the absence of discernible weight gain. HFD feeding increases the levels of growth factors which activate transcription factors, thereby inducing the expression of many genes involved in the stimulation of inflammation, angiogenesis, and cellular proliferation.

Key words: colon cancer; high-fat diet; angiogenesis; metastasis; inflammation

INTRODUCTION

Colon cancer is the third most frequently diagnosed cancer in both men and women, and typically has a poor prognosis with a high rate of mortality [1]. Colon cancer is intrinsically linked to eating behavior (reviewed in Ref.[2]). Since the development and progression of colon cancer generally spans several decades, lifestyle interventions including dietary modifications are important adjuncts to medical treatment for effectively suppressing colon cancer development and metastasis.

Over the past few decades, the worldwide incidence of overweight and obesity has been rising at an alarming rate [3,4]. Epidemiological studies reveal that obese individuals are at increased risk of developing colon cancer [5]. In 2002, the International Agency for Research on Cancer concluded that obesity was responsible for 11% of colon cancer cases [6]. Case–control studies frequently

Additional Supporting Information may be found in the online version of this article.

Abbreviations: HFD, high-fat diet; EGF, epidermal growth factor; IGF, insulin-like growth factor; IFN, interferon; IL-1ra, interleukin-1 receptor antagonist; MCP, monocyte chemoattractant protein; RANTES, regulated upon activation normal T-cell expressed and secreted; SDF, stromal cell-derived factor; VEGF, vascular endothelial growth factor; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; VEGFR, VEGF receptor; CDK, cyclin-dependent kinase; HIF, hypoxia-inducible factor; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; CD, control diet; IHC, immunohistochemistry; TUNEL, transfer-mediated dUTP nick-end labeling; MCEFTE, media conditioned by epididymal fat tissue explants; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; BMI, body mass index.

Conflict of interest: none.

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demonstrate that total energy consumption with physical inactivity and excessive adiposity, especially if centrally distributed, is associated with a higher risk of colon cancer [7]. In addition to the increase in tumor incidence, obesity also affects tumor prognosis; and obesity is associated with approximately 14% of cancer deaths in males and up to 20% of cancer deaths in females [8]. Additionally, a growing body of evidence shows that greater pre-cancer anthropometric measures are associated with increased risk of colon cancer mortality [9].

High intakes of dietary fat are known to cause obesity in humans and rodents [10,11] and have also been implicated in colon cancer [12]. However, it remains to be determined if high fat consumption is an independent risk factor for colon cancer or if it indirectly causes colon cancer by increasing the incidence of obesity [7]. Obesity is associated with a state of a chronic low-grade inflammation (reviewed in [13]), which may represent an additional mechanism linking increased adiposity to colorectal carcinogenesis (reviewed in [14–16]). However, to the best of our knowledge, it has yet to be determined whether, in non-obese individuals, the consumption of a high-fat diet (HFD) can result in chronic inflammation which stimulates the development and metastasis of colon cancer.

In this study, we evaluated whether the chronic consumption of an HFD without decreasing the intake of protein, minerals, vitamins, and fiber increases colon cancer growth and metastasis. We used the CT26 colon cancer allograft model, in which CT26 cells are injected subcutaneously into syngeneic BALB/c mice, which are known to be obesity-resistant and in which HFD consumption has little effect on body weight [17,18]. We noted that the consumption of an HFD, without increasing energy intake, stimulated angiogenesis, solid tumor growth, and lung metastasis in BALB/c mice, accompanied by the maintenance of normal body weight.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following suppliers: epidermal growth factor (EGF), an antibody against mouse CD45 (leukocyte common antigen) and ELISA kits for mouse EGF, insulin, mouse/rat insulin-like growth factor (IGF)-I, interferon (IFN)-γ, interleukin-1 receptor antagonist (IL-1ra), leptin, monocyte chemoattractant protein (MCP)-1, regulated upon activation normal T-cell expressed and secreted (RANTES), stromal cell-derived factor (SDF)-1α, and vascular endothelial growth factor (VEGF) from R&D Systems (Minneapolis, MN); antibodies against cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), phospho-signal transducer and activator of transcription (STAT)3 (Tyr705) and phospho-STAT3 (Ser727) from BD Transduction Laboratories (Palo Alto, CA); antibodies against Akt, phospho-Akt, Bcl-xL, c-Jun, phospho-c-Jun, phospho-p38 mitogen-activated protein kinase (MAPK), p42/44 extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2, phospho-IκB-α, STAT3, stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK), phospho-SAPK/JNK, VEGF receptor (VEGFR)-2 and phospho-VEGFR-2 from Cell Signaling (Beverly, MA); antibodies against Bcl-2, cyclin-dependent kinase (CDK)2, CDK4, cyclin A, cyclin D1, endothelial cell adhesion molecule-1 (PECAM-1, CD31, an angiogenesis marker), hypoxia-inducible factor (HIF)-1α, p53, and VEGF from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against Ki67, and F4/80 from Abcam (Cambridge, MA, UK); horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG from Life Technology (Carlsbad, CA); Immobilon™ Western chemiluminescent HRP substrate from Millipore Corporation (Billerica, MA); an antibody against CD68 (myeloid cell markers, monocytes/macrophages, dendritic cells, and granulocytes) from ABBIOTEC (San Diego, CA). Unless otherwise noted, all other materials were purchased from Sigma (St. Louis, MO).

CT26 Cell and Human Umbilical Vein Endothelial Cell (HUVEC) Culture

CT26 murine colon carcinoma cells were acquired from the American Type Culture Collection (Manassas, MA) and maintained in RPMI1640 containing 100 mL/L of fetal bovine serum (FBS) with 100,000 U/L of penicillin and 100 mg/L of streptomycin. HUVECs (Lonza, Walkersville, MD) were maintained in Medium 199 (M199) containing 200 mL/L of FBS with 100,000 U/L of penicillin, 100 mg/L of streptomycin, 1.5 μg/L of EGF, and 75 μg/L of hydrocortisone.

Ethics Statement

The Animal Use Committee of Hallym University approved all animal study protocols (Hallym 2009-123), and all experiments were conducted in accordance with the guidelines for the care and use of laboratory animals.

Animals, Experimental Diets, and CT26 Tumor Cell Injections

Pathogen-free male BALB/c mice (3 wk of age) weighing 12–13 g were purchased from Orient Bio Inc. (Gapyung, Korea). The mice were maintained in a pathogen-free animal facility for 1 wk before the experiment and then randomly divided into two dietary groups fed purified diets (Research Diets Inc., New Brunswick, NJ); HFD containing 60 kcal% as fat (D12492) or control diet (CD) with

10 kcal% as fat (D124508). The two diets contained identical quantities of protein, cellulose, soybean oil, vitamins, and minerals per kilocalorie, and only lard and carbohydrates varied between the two diets. The diets were stored at −20°C and fresh diet was provided daily to allow the mice free access to feed. Daily feed intake was monitored throughout the study.

Sixteen weeks after the beginning of feeding, CT26 cells (5×10^4 cells suspended in 0.1 mL matrigel/PBS; BD Biosciences, San Jose, CA) were subcutaneously injected into the right rear flanks of the mice. The mice were fed continuously on the same diets. The tumor volume was measured with a set of calipers and calculated using the formula $0.52 \times \text{long diameter} \times \text{short diameter}^2$ [19]. Thirty-one days after the CT26 cell injections, the mice were anesthetized via an intraperitoneal injection of 2.5% avertin (100% avertin, 1 g tribromoethyl alcohol/ml tertiary amyl alcohol) and blood was collected from the orbital venous plexus. After blood collection, all mice were sacrificed via CO_2 asphyxiation and the tumors, lungs, livers, spleens, and mesenteric and epididymal fat pads were excised from the mice and weighed. The sera were then prepared for protein arrays and ELISAs. The tumors were formalin-fixed and paraffin-embedded for immunohistological analyses or homogenized to prepare the tissue lysates for Western blot analysis. The lungs were fixed in Bouin's solution. To evaluate metastasis, lung metastatic nodules were counted and the total tumor volumes were estimated as described previously [20,21].

Hemoglobin Assay

The hemoglobin assay was conducted using Drabkin's reagent (Sigma) in accordance with the manufacturer's instructions. Tumor tissues were chopped and incubated in Drabkin's solution at room temperature for 15 min, then maintained at 4°C for 1 wk with gentle agitation (rocking). After 1 wk, the absorbance was measured at 450 nm. Hemoglobin values (mg/g of tumor) were calculated from a calibration curve prepared with a cyanmethemoglobin standard (Sigma).

Immunohistochemical (IHC) Analysis

Paraffin-embedded tumor tissues were sectioned (5-μm thick), deparaffinized, rehydrated, and incubated for 5 min in 3% H_2O_2 to deactivate endogenous peroxidase. The sections were blocked with 5% BSA followed by incubation with antibodies raised against Ki67 (1:250), cyclin A (1:250), cyclin D1 (1:250), CDK2 (1:250), CD45 (1:250), CD68 (1:200), CDK4 (1:250), CD31 (1:250), F4/80 (1:200), or VEGF (1:100) overnight at 4°C. The sections were then incubated with $LSAB^+$ system-HRP (Dako, Carpinteria, CA) in accordance with the manufacturer's instructions, and then counterstained with hematoxylin. Apoptotic cells were identified via terminal deoxynucleotidyl transfer-mediated dUTP nick-end labeling (TUNEL) staining using a Dead End Fluorometric TUNEL system (Promega Corporation, Madison, WI). Photographs were obtained using a Carl Zeiss AxioImager microscope (Carl Zeiss, Jena, Germany).

Western Blot Analysis

Tissue lysates were prepared as previously described [22] and Western blot analyses were conducted, also as described previously [23]. The relative abundance of each band was quantified using the Bio-profile Bio-1D application (Vilber-Lourmat, Marine la Vallee, France), and the expression levels were normalized to β-actin.

Protein Arrays and ELISA

The differences in the serum levels of angiogenesis-related proteins and cytokines between the two dietary groups were assessed using a mouse angiogenesis array kit (Catalog number, ARY015) and a mouse cytokine array panel A array kit (Catalog number, ARY006), respectively, in accordance with the manufacturers' instructions (R&D Systems). The levels of EGF, IGF-I, insulin, VEGF, IFN-γ, leptin, RANTES, SDF-1α, MCP-1, and IL-1ra in sera were estimated using the relevant ELISA kits in accordance with the manufacturers' instructions.

Preparation of Media Conditioned by Epididymal Fat Tissue Explants (MCEFTE), Aortic Ring Assay, and Capillary-Like Tube Formation Assay

Adipose tissue explants were obtained from epididymal fat pads of male BALB/c mice that had been fed on the CD and HFD for 16 wk. The conditioned media were prepared as described previously [24]. Briefly, 50 mg of chopped epididymal fat tissue fragments were incubated for 24 h in 2 mL of serum-free DMEM (low glucose)/M199 (1:1)/well supplemented with 50 μg/mL gentamicin and 0.5 μg/mL amphotericin B in six-well plates. Aliquots of MCEFTE were used for the aortic ring assay as previously described [25]. For tube formation assay, HUVECs (50,000 cells/well) were seeded into 24-well plates that had been pre-coated with 300 μL of growth factor reduced matrigel (MA 01730, BD Biosciences, Bedford, MA). Fresh media in the absence or presence of MCEFTE were subsequently added. Tubular structures were photographed after 3 h. The total length of formed tubes was measured for quantification of angiogenesis by the Motic Images Advanced 3.2 system (Motic, Richmond, BC, Canada).

Statistical Analysis

The results were expressed as means ± SEM, then analyzed via ANOVA. Differences among

the treatment groups were evaluated via Student's *t*-test or Duncan's multiple range test. Means were considered significantly different at $P < 0.05$. All statistical analyses were conducted using the SAS System for Windows V 9.1 (SAS Institute, Cary, NC).

RESULTS

HFD Feeding Significantly Increases the Growth of Solid Tumors and Lung Metastasis in Mice Injected With CT26 Colon Cancer Cells

Body weights were increased slightly in mice 1 wk after the initiation of HFD feeding, and these weight differences were maintained for the remaining 20 wk of the feeding period (Figure 1A). At the end of the experiment, the mean body weights were 31.8 ± 0.34 and 34.0 ± 0.49 g in the CD and HFD groups, respectively. After correction for differences in tumor weight, the change in body weight due to chronic HFD feeding was measured at only 5.9%. The ad libitum food intakes in CD and HFD groups were 4.02 ± 0.23 and 3.19 ± 0.22 g/d, respectively; Food intakes (g/d) were lower but the energy intakes (kJ/d) were slightly higher in the HFD group but this difference was not statistically significant ($P = 0.32$,

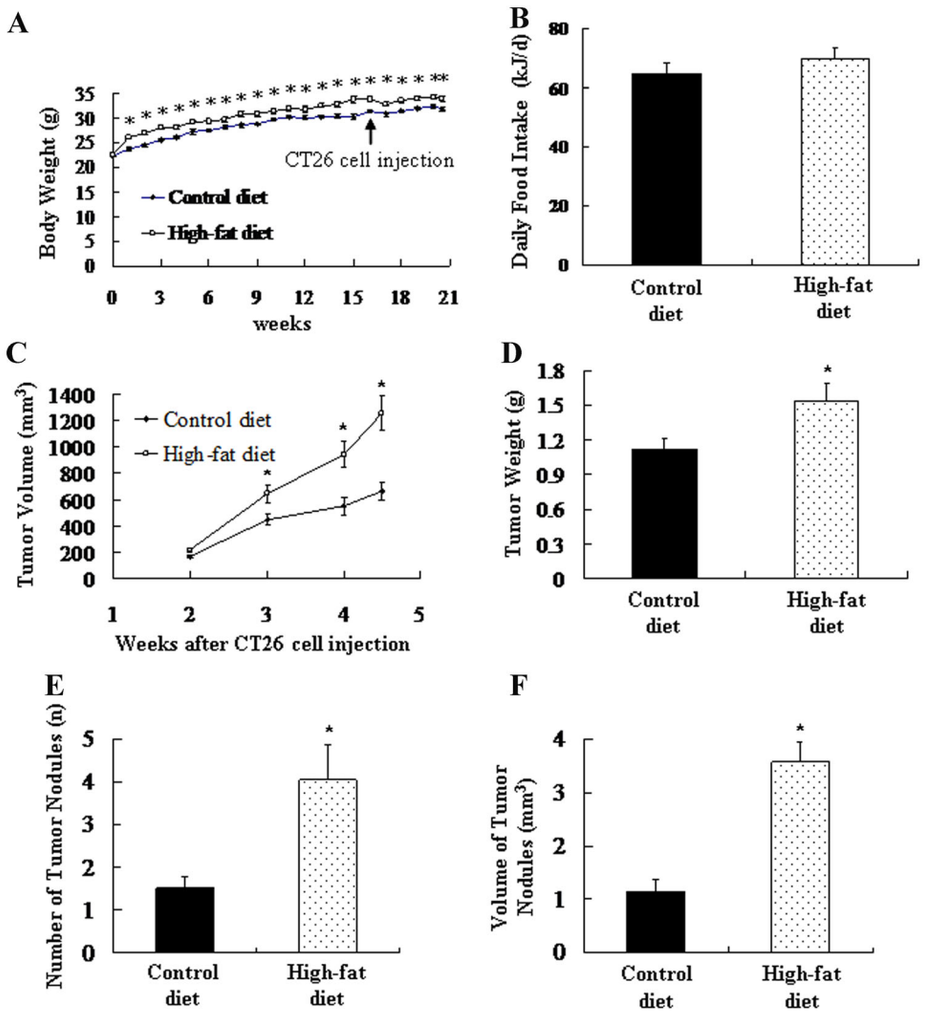

Figure 1. A high-fat diet stimulates tumor growth and lung metastasis in BALB/c mice injected with CT26 cells. Four-week old, male BALB/c mice were fed on a control diet or a high-fat diet for a period of 16 wk. After 16 wk, CT26 cells (5×10^4 cells) were injected under the dorsal skin of mice. The mice were fed continuously on the same diets. Thirty-one days after the cell injections, all mice were sacrificed and the tumors were isolated. (A) Body weights and (B) food intake of mice (kJ/d) (C) Tumor size was monitored weekly by measuring the anteroposterior diameter with calipers. Tumor volume at the implanted site was calculated via the following formula: Tumor volume (mm^3) = 0.52 × (short diameter)^2 × (long diameter). (D) The tumors were removed from mice and weighed. (E,F) The lungs were excised from mice and fixed in Bouin's solution. (E) The numbers of tumor nodules were counted and (F) the total volume of tumor nodules in the lung were estimated. Each point represents the mean ± SEM ($n = 21$). *Significantly different from the control group, $P < 0.05$.

Figure 1B). The weights of the spleen, liver, and epididymal fat pad were higher in the HFD group, but the weights of the lung and mesentery adipose tissue were not significantly different (Supplemental Table 1). The volumes and weights of tumors were significantly higher in HFD mice (Figure 1C,D) as were the numbers and volumes of tumor nodules in the lungs (Figure 1E,F).

HFD Feeding Increases Cell Proliferation and Reduces Apoptosis in Tumor Tissues in BALB/c Mice Injected With CT26 Colon Cancer Cells

The IHC assay results revealed that the expressions of Ki67, cyclin A, cyclin D1, and CDK2 were increased significantly in tumor tissues obtained from the mice fed on the HFD. The expression of CDK4 appeared to increase but did not reach statistical significance (Figure 2A,B). Small numbers of TUNEL-positive apoptotic cells were detected in the tumor tissues of control mice, and these numbers were significantly lower in the tumor tissues of mice fed on the HFD (Figure 2C). Consistent with these results, the expression of the p53 tumor suppressor protein was reduced and that of Bcl-2 and Bcl-xL was significantly increased in the tumor tissues of HFD-fed mice relative to control mice (Figure 2D).

HFD Feeding Stimulates Leukocyte Infiltration and Angiogenesis in Tumor Tissues of BALB/c Mice Injected With CT26 Colon Cancer Cells

CD45- and CD68-positive cells and CD31 and VEGF expression were increased in the tumor tissues of the HFD-fed mice (Figure 3A–C). The serum levels of VEGF (Figure 3D) and hemoglobin contents (Figure 3E) in tumor tissues were increased significantly in the HFD-fed mice. Western blot analysis demonstrated that the protein levels of P-VEGFR-2, VEGFR-2, iNOS, and COX-2 were increased in the tumor tissues of mice fed on the HFD (Table 1).

HFD Feeding Increases the Activated Forms of Akt, MAPKs, and the Transcription Factors HIF-1α, STAT3, NFκB, and AP-1 in the Tumor Tissues of BALB/c Mice

Western immunoblot analyses of tumor tissue revealed that HIF-1α levels were increased significantly in the HFD-fed mice relative to controls. The levels of both P-STAT3-Y705 and P-STAT3-S727 were markedly increased in the tumor tissues obtained from the HFD group, whereas the total STAT3 levels remained unaltered. The levels of P-IκB-α, P-p65, and p65 as well as those of P-c-Jun and c-Jun were also increased markedly in the tumor tissues obtained from the HFD group (Table 1). The levels of P-Akt, P-ERK1/2, and P-SAPK/JNK were increased markedly in the HFD-fed mice. The levels of Akt, ERK1/2, and SAPK/JNK were also increased, but to a lesser degree (Table 1).

HFD Feeding Increases the Serum Levels of Growth Factors and Pro-Inflammatory Cytokines/Chemokines in BALB/c Mice Injected With CT26 Cells

In an effort to determine whether HFD feeding increases the serum levels of a variety of pro-inflammatory cytokines/chemokines and angiogenesis-related proteins, we conducted protein arrays of pooled sera ($n = 21$). The results shown in Supplemental Table 2 demonstrated that the levels of 42 proteins were increased, whereas the levels of 5 proteins (TIMP-4, MMP-3, IGFBP-3, osteopontin, and basic FGF) were decreased in the HFD-fed group. We conducted ELISA assays, the results of which confirmed the findings of the array demonstrating that the serum levels of VEGF (Figure 3D), EGF, IFN-γ, leptin, RANTES, MCP-1, IL-1ra, and SDF-1α were increased in the HFD-fed mice. Serum IGF-I and insulin levels were also increased in the HFD-fed group (Table 2).

HFD Feeding Induces Macrophage Infiltration Into Epididymal Fat Tissues and Increases Lipid Vacuoles in Tumor Tissues of BALB/c Mice Injected With CT26 Colon Cancer Cells

IHC analysis revealed that the number of F4/80-expressing mature macrophages was increased in the epididymal fat tissues from the HFD-fed mice. Hematoxylin and eosin stain results showed that tumor tissues contained more lipid vacuoles in HFD-fed mice relative to those observed in CD-fed mice. These lipid vacuoles were mostly localized in the edge of the tumor (Figure 4A–C).

Media Conditioned by Epididymal Fat Tissue Explants (MCEFTE) From HFD-Fed Mice Stimulate Capillary-Like Tube Formation

ELISA results revealed that the levels of leptin, MCP-1, and IL-6 were increased in MCEFTE from HFD-fed mice relative to those from mice fed on the CD (Figure 4D–F). Consistent with these results, MCEFTE from the HFD-fed mice caused an increase in microvessel outgrowth from the mouse aorta (Figure 4G) and capillary-like tube formation of HUVECs (Figure 4H), relative to those observed in CD-fed mice.

DISCUSSION

The relationship of the percentage of fat in the diet to the progression of colon cancer has yet to be clearly established. Obesity resulting from HFD feeding, and visceral adiposity in particular, is generally accepted as a risk factor for colorectal cancer. Epidemiological evidence indicates that obesity increases the risk of and mortality from colorectal cancer [26–28]. However, the objective of this study was to determine whether dietary fat, in the

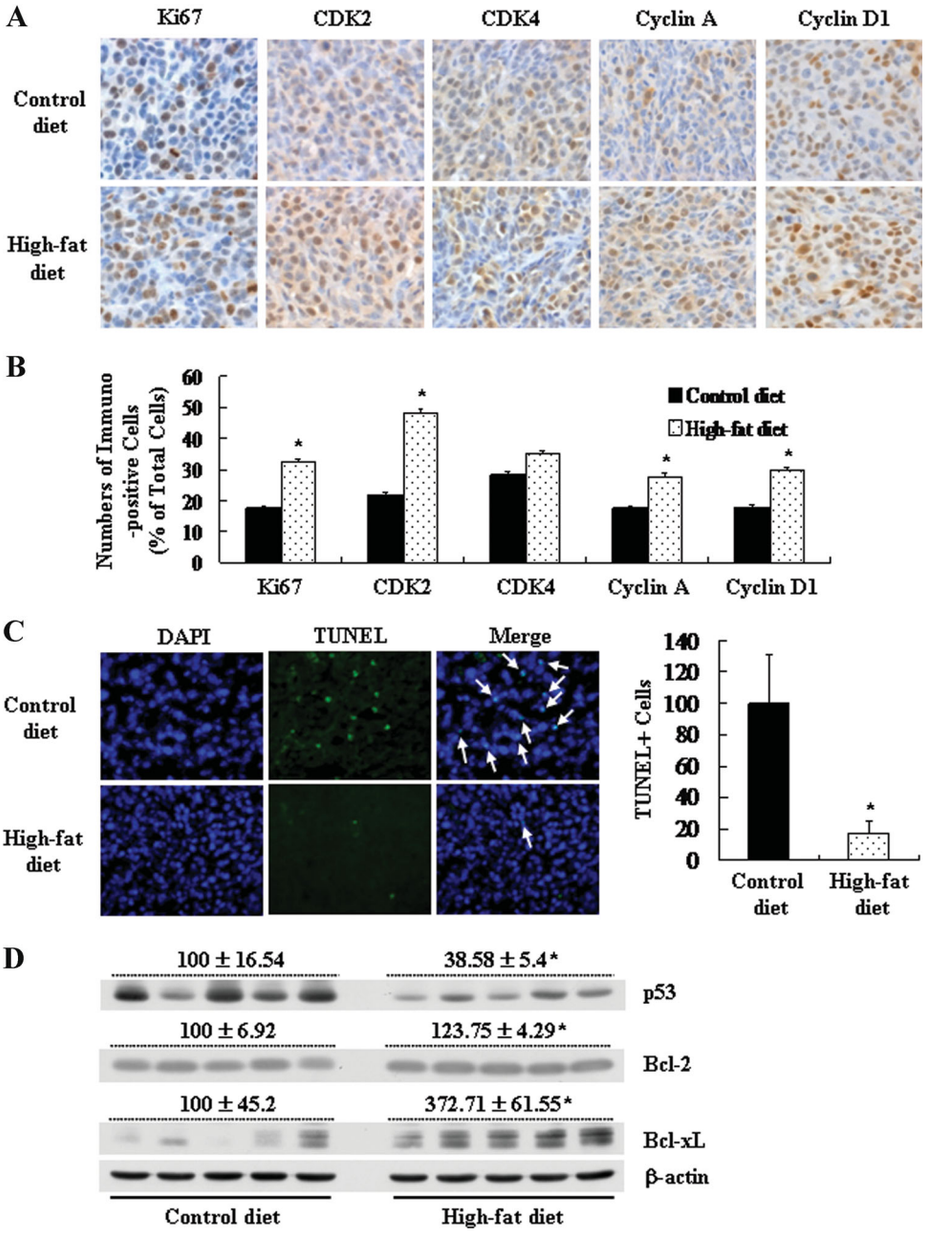

Figure 2. Effect of a high-fat diet on the expression of proteins involved in the regulation of cell cycle progression and apoptosis in CT26 tumors of BALB/c mice. Mice were fed, injected with CT26 cells, and then sacrificed as described in Figure 1. (A) Tumor sections were stained with an antibody raised against Ki67, CDK2, CDK4, cyclin A, or cyclin D1 and counterstained with hematoxylin. Representative images of the immunohistochemical analysis are shown. (B) The Ki67-, CDK2-, CDK4-, cyclin A-, and cyclin D1-positive cells were counted. Each bar represents the means ± SEM ($n = 9$). (C) TUNEL stainings of the primary tumor. (D) Tumor lysates were analyzed via Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots are shown. The relative abundance of each band to its own β-actin was quantified and the control levels were set at 100%. The adjusted mean ± SEM is shown above each blot. *Significantly different from the control group, $P < 0.05$.

absence of increased caloric intake or substantial increases in weight gain, stimulates the promotion and progression of colon tumors. BALB/c mice were used in the present study, because it has been reported that the mice are resistant to HFD-induced obesity [17]. When BALB/c mice were fed on the 60 kcal%-fat diet, they consumed less food, such that their intake of protein, vitamins, minerals, fiber, and kilocalories did not differ from those of the mice fed on the 10 kcal%-fat diet. The

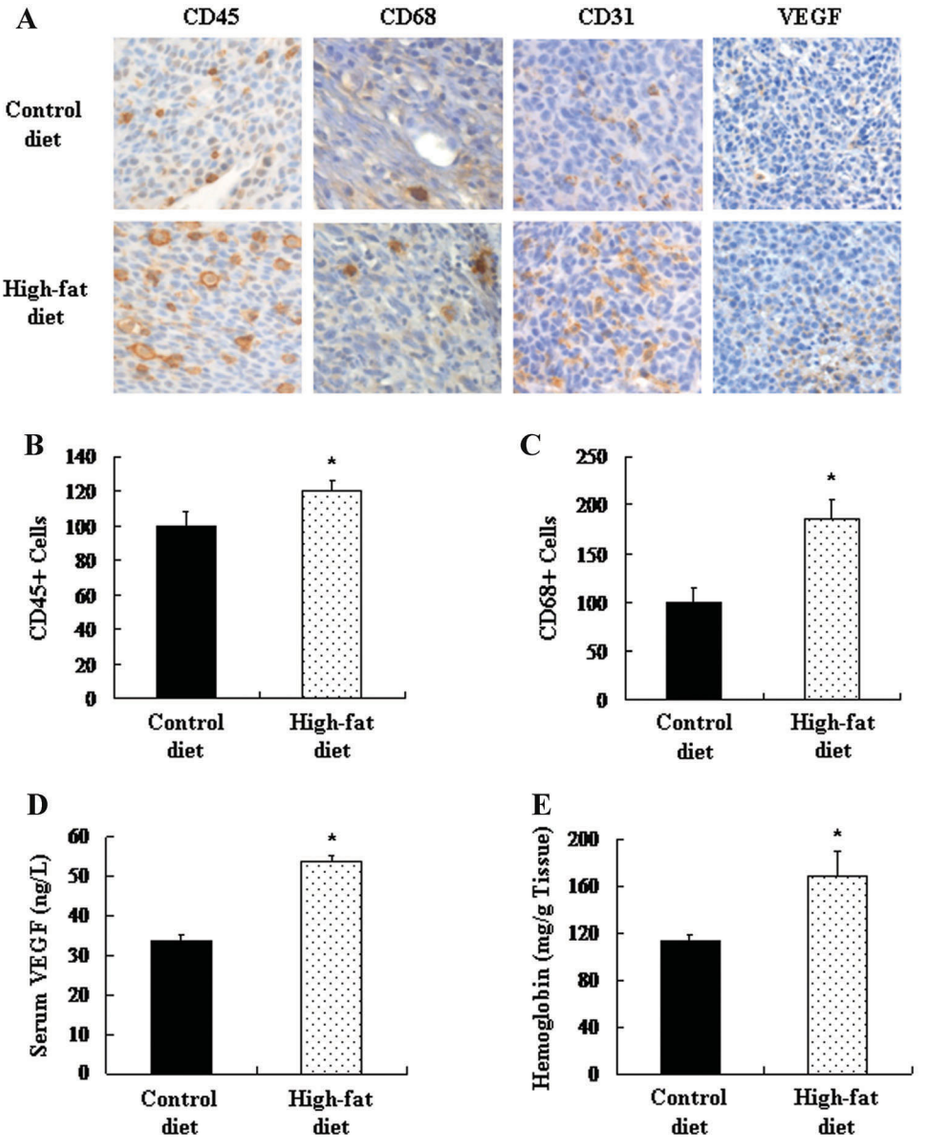

Figure 3. A high-fat diet stimulates leukocyte infiltration and angiogenesis in tumor tissues of BALB/c mice. Mice were fed, injected with CT26 cells, and sacrificed as described in Figure 1. (A) Tumor sections were stained with an antibody against CD45, CD68, CD31, or VEGF. Representative images of immunohistochemical analysis are shown ($n = 9$). (B,C) The CD45- and CD68-positive cells were counted. (D) VEGF levels in sera were measured with an ELISA kit ($n = 10$). (E) Hemoglobin content in the tumor specimens was measured using Drabkin's reagent. Each bar represents the mean ± SEM ($n = 21$). *Significantly different from the control group, $P < 0.05$.

difference between the mean body weight of the control mice and HFD-fed mice was only 1.7 g after subtracting the difference in tumor weight, which corresponds to only 5.9% of body weight. The weights of epididymal fat pads were increased but those of mesenteric fat tissues were not increased as the result of HFD feeding. In the present study, we demonstrate that the chronic feeding of mice with an HFD without a substantial increase in body weight leads to: (1) increases in angiogenesis, solid tumor growth, and lung metastasis; (2) increased infiltration of leukocytes into the tumor; (3) increases in phosphorylated Akt, ERK1/2, SAPK/JNK, and p38 MAPK in tumor tissues; (4) activation of a variety of transcription factors in tumor tissues; and (5) increases in inflammatory markers in tumor tissues (COX-2, iNOS), conditioned media by adipose tissue explants (leptin, MCP-1, IL-6), and sera (cytokines/chemokines). These results indicate that chronic

Table 1. A High-Fat Diet Increases the Active Forms of the Transcription Factors HIF-1α, STAT3, NFκB, AP-1, and Activated Akt and MAPKs in the Tumors of BALB/c Mice

	Control diet	High-fat diet
P-VEGFR-2	100 ± 12.21	371.41 ± 37.94*
VEGFR-2	100 ± 18.57	192.55 ± 22.29*
iNOS	100 ± 25.05	470.6 ± 66.13*
COX-2	100 ± 41.31	543.84 ± 89.7*
HIF-1α	100 ± 25.92	463.93 ± 81.68*
P-STAT3-Y705	100 ± 64.51	866.39 ± 191.8*
P-STAT3-S727	100 ± 21.46	415.81 ± 41.43*
STAT3	100 ± 3.45	109.61 ± 4.89
P-IκB-α	100 ± 28.72	279.59 ± 21.77*
P-p65	100 ± 43.88	523.18 ± 83.1*
P65	100 ± 33.55	353.48 ± 23.17*
P-c-Jun	100 ± 14.98	276.18 ± 16.86*
c-Jun	100 ± 16.57	187.79 ± 6.65*
P-Akt	100 ± 10.95	193.71 ± 11.47*
Akt	100 ± 16.5	168.07 ± 1.69*
P-ERK1/2	100 ± 14.78	171.06 ± 11.06*
ERK1/2	100 ± 6.39	117.59 ± 2.75*
P-p38	100 ± 26.59	428.9 ± 41.27*
P38	100 ± 16.76	176.18 ± 4.51*
P-SAPK/JNK	100 ± 22.34	275.5 ± 20.26*
SAPK/JNK	100 ± 13.74	156.8 ± 11.12*

Mice were fed, injected with CT26 cells, and sacrificed as described in Figure 1. Tumor lysates were analyzed via Western blotting with the indicated antibodies. The relative abundance of each band to its own β-actin was quantified and the control levels were set at 100%. The adjusted mean ± SEM is shown.
*Significantly different from the control group, $P < 0.05$.

consumption of HFD may stimulate colon cancer progression even in individuals who maintain healthy body weights, which is mediated via the induction of chronic inflammation.

The ideal animal model for studying colon tumor growth and metastasis should closely mimic all features of human colon cancers, be practical, and be optimal with respect to ethical considerations. To date, no model has been established which meets all these conditions. In this study, we subcutaneously injected CT-26 cells into syngeneic BALB/c mice. The subcutaneous route was chosen because variations between animals are small, due to the fact that it causes minimal trauma to the mice. Additionally, the injected CT-26 cells grow into solid tumors that spontaneously metastasize to the lung, while the primary tumor still grows in situ. Furthermore, unlike immunodeficient nude mice, BALB/c mice are immune-competent; thus, this animal model is useful in the study of tumor growth and progression, in which the contribution of inflammatory/immune system is an important consideration. However, it does not mimic the original anatomic site of colorectal cancer; thus, the results observed with this animal model may not be particularly representative of human colon cancer progression.

Table 2. A High-Fat Diet Increases the Serum Levels of EGF, IGF-I, Insulin, Leptin, MCP-1, IL-1ra, IFN-γ, SDF-1α, and RANTES in BALB/c Mice Injected With CT26 Cells

	Control diet	High-fat diet
EGF (ng/L)	22.31 ± 3.23	45.24 ± 9.98*
IGF-I (μg/L)	169.21 ± 2.98	205.52 ± 2.91*
Insulin (μg/L)	0.83 ± 0.13	1.88 ± 0.2*
Leptin (μg/L)	2.03 ± 0.16	12.75 ± 1.05*
MCP-1 (ng/L)	80.76 ± 7.73	184.03 ± 17.83*
IL-1ra (ng/L)	23.67 ± 1.79	45.07 ± 8.94*
IFN-γ (ng/L)	12.16 ± 0.16	16.57 ± 1.03*
SDF-1α (μg/L)	3.4 ± 0.19	3.96 ± 0.21*
RANTES (ng/L)	31.3 ± 2.31	47.1 ± 1.26*

Mice were treated as described in Figure 1. Blood samples were collected from the mice and the sera were prepared. The levels of cytokines in sera were measured using an ELISA kit. Each bar represents the mean ± SEM ($n = 10$).
*Significantly different from the control group, $P < 0.05$.

In the present study, the two diets contained identical quantities (5.5 kcal%) of soybean oil per kilocalorie, and the lard content was 4.4 and 54.4 kcal% in the 10 and 60 kcal% diets, respectively. Lard contains high concentrations of saturated and monounsaturated fatty acids (MUFAs). With respect to dietary fatty acids, there is growing evidence that long-chain n-3 polyunsaturated fatty acids (PUFAs) found in fish oil reduce colon cancer incidence (reviewed in [29]), whereas diets rich in n-6 PUFAs enhance the development of colon tumors [30,31]. Diets containing high n-9 MUFAs were shown to either inhibit [32] or promote colon cancer risk [33]. However, epidemiological evidence regarding the role of foods containing animal fat is still limited [34,35]. As the HFD in the present study contained high amounts of animal fat, which is common in human HFD, the present results indicate that HFD containing animal fat should be avoided in order to prevent colon cancer, even in individuals who do not consume extra energy and maintain a body mass index (BMI) within the normal range.

The growth and metastasis of tumor cells are stimulated by interactions with non-malignant cells in the tumor microenvironment, including immune inflammatory cells, adipocytes, endothelial cells, and fibroblasts [36]. Tumor promoting inflammatory cells are macrophage subtypes, mast cells, neutrophils and T and B lymphocytes and produce growth factors (e.g., EGF and VEGF), chemokines/cytokines, and proangiogenic and/or proinvasive matrix-degrading enzymes (reviewed in [37]). In this study, CD45+ and CD68+ cells were increased significantly in tumor tissues of mice fed on the HFD (Figure 3A), thereby indicating that these increases in leukocytes in tumor tissues may have contributed to the increases in

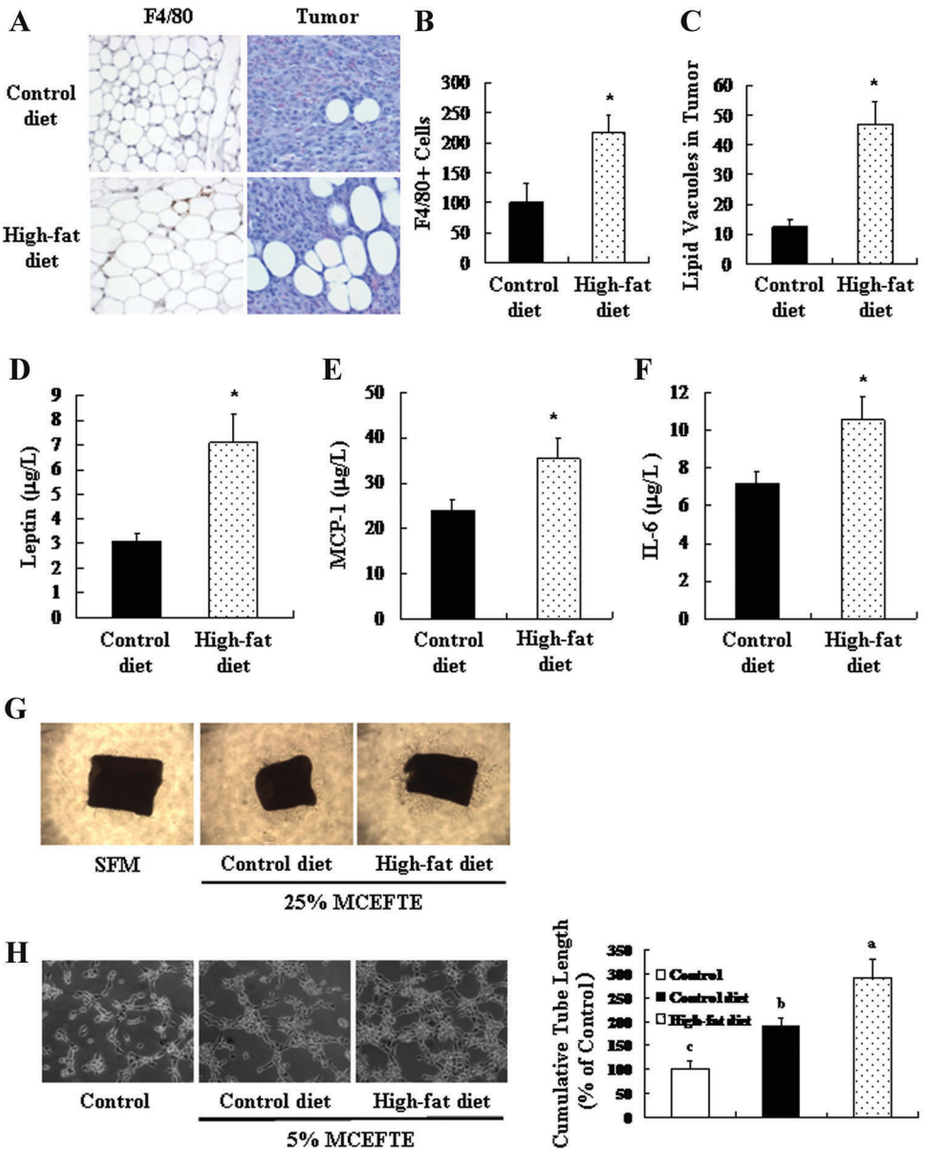

Figure 4. A high-fat diet induces macrophage infiltration into epididymal fat tissues and media conditioned by epididymal fat tissue explants (MCEFTE) from HFD-fed mice stimulate angiogenesis. (A,B) Mice were fed, injected with CT26 cells, and sacrificed as described in Figure 1. (A) Sections of epididymal fat pads and tumors were stained with an antibody raised against F4/80 and H&E, respectively. (A) Representative images are shown. (B,C) The number of F4/80-positive cells and lipid vacuoles were counted. (D–H) Four-week-old male BALB/c mice were fed on a control diet or a high-fat diet for a period of 16 wk. After 16 wk, epididymal fat tissues were removed, and the MCEFTE were prepared. The levels of leptin (D), MCP-1 (E), and IL-6 (F) in MCEFTE were measured using the respective ELISA kits. Each bar represents the mean ± SEM ($n = 10$). *Significantly different from the control group, $P < 0.05$. (G) Aortic rings from BALB/c mice were embedded in Matrigel and then treated without or with MCEFTE every 2 d. The photographs taken at 4 d, which are representative of three independent experiments, are shown. (H) HEVECs were plated at 3×10^4 cells/well in Matrigel-coated 24-well plates and then treated without or with 5% MCEFTE. After 3 h, the culture supernatants were removed and then photographed for HUVEC tube formation. Each bar represents the means ± SEM ($n = 3$). Means with different letters are significantly different, $P < 0.05$.

angiogenesis, tumor cell growth, and metastasis. Additionally, the epididymal fat tissues of HFD-fed mice contained more F4/80-positive macrophages than those observed in control mice (Figure 4A,B) and epididymal tissue explants from HFD-fed mice produced more leptin, MCP-1, and IL-6 (Figure 4D–F). Increases in the serum levels of cytokines and chemokines were also detected in the

HFD-fed mice (Supplemental Table 2 and Table 2). Furthermore, there was an increase in lipid vacuoles in tumor tissues of HFD-fed mice (Figure 4A,C). Taken together, these results indicate that increased fat cells in the tumor and epididymal fat pads may have contributed to the infiltration of leukocytes in the tumor. Within the tumor masses of mice fed on the HFD, the cross-talk between cancer cells, leukocytes, and adipocytes may have generated more growth factors, cytokines, and chemokines, thereby resulting in the stimulation of tumor growth, angiogenesis, and metastasis. These results indicate that HFD feeding may induce inflammatory changes as a consequence of small increases in fat mass in animals exhibiting minimal weight gain.

The majority of signaling pathways relevant to the process of tumor progression converge on groups of transcription factors that ultimately regulate the expression of genes involved in the stimulation of inflammation, angiogenesis, and tumor metastasis [38]. In the tumor tissues of HFD-fed mice, the levels of HIF-1α, P-c-Jun, P-p65, p65, and P-STAT3 were markedly increased (Table 1). Increases in the levels of P-Akt, P-ERK1/2, P-p38 MAPK, and P-SAPK/JNK were also noted in the tumor tissues of HFD-fed mice (Table 1). These results demonstrate that growth factor signaling pathways may have been activated in tumor tissues, which in turn activate Akt, ERK1/2, p38 MAPK, and SAPK/JNK, thus resulting in the activation of the transcription factors NFκB, AP-1, HIF-1α, and STAT3. The activation of these transcription factors, in turn, induces alterations in the expression of a variety of genes involved in the stimulation of cancer cell proliferation (Ki67, cyclin A, cyclin D1, CDK2) and survival (Bcl-2, Bcl-xL, p53), inflammation (COX-2, iNOS, cytokines/chemokines) and angiogenesis (VEGF). It was reported that HFD-induced obesity in C57BL/6 mice leads to increased circulating levels of IGF-I [39], and free IGF-I levels have been shown to be higher in obese human subjects than in normal controls [40]. In the present study, the levels of IGF-1, insulin, and EGF were markedly increased in the sera of the mice fed on the HFD even though their body weights were only slightly increased (Table 2 and Figure 1). Future studies will be necessary to determine which growth factor signaling pathways (IGF-1, insulin, and/or EGF receptor) are activated in the tumor tissues of HFD-fed animals. Additionally, it will be necessary to determine which cytokines and chemokines and their corresponding receptors play important roles in colon tumor growth and metastasis, as well as their underlying mechanisms of action, in animals fed on an HFD.

One interesting observation in the current study is the measured change in the expression of HIF-1α as well as that of its downstream target genes Bcl-xL, p53, iNOS, COX-2, and VEGF in the tumor tissues of mice fed on an HFD. The transcription factor HIF-1 regulates the expression of a broad variety of genes involved in cancer biology, including cell survival, glucose metabolism, angiogenesis, and metastasis (reviewed in [41,42]). The results of a cohort study demonstrate that HIF-1α overexpression is associated with poor prognosis in colorectal cancer [43]. The activity of HIF-1α is tightly regulated. Under normoxic conditions, HIF-1α is hydroxylated at several prolyl residues and is subsequently degraded by the proteasome [44]. Although hypoxia is regarded as the primary stimulus that induces HIF-1 activation, a number of normoxic stimuli induce the activation of HIF-1 in a broad variety of human cancers [42]. The p38 MAPK, ERK1/2, and phosphatidyl-inositol 3-kinase pathways are frequently involved in HIF-1 regulation (reviewed in [45]). Thus, in human cancer cells, HIF-1 activity is increased as a consequence of the physiological induction of HIF-1α in response to hypoxia in the tumor microenvironment and also as a result of genetic alterations that activate tumor-promoting genes and inactivate tumor suppressor genes [42]. It may, then, be postulated that, in the tumor tissues of our HFD-fed mice, increased activation of Akt and MAPKs may have contributed to the activation of HIF-1α.

Angiogenesis is the formation of a new vascular network from pre-existing blood vessels, and is required for the rapid growth and metastasis of solid tumors and constitutes an important stage in the stimulation of cancer progression (reviewed in [46]). These new blood vessels supply tumor cells with a broad variety of substances, including oxygen, nutrients, growth factors, and cytokines [47]. Growth factors secreted by tumor cells or tumor stromal cells can bind directly to their receptors on the neighboring endothelial cells and promote angiogenesis by supporting endothelial sprouting, branching, differentiation, and survival [48]. VEGF is expressed in most human cancer types, and increased VEGF expression in tumors is frequently associated with a less favorable prognosis (reviewed in [46]). The major mediator of tumor angiogenesis is VEGF-A, specifically the circulating isoforms of VEGF-VEGF121 and VEGF165. These isoforms conduct signaling through VEGFR-2, the primary VEGF signaling receptor in vascular endothelial cells (reviewed in [49]). In the tumor tissues of the HFD-fed mice, expressions of HIF-1α, iNOS, COX-2, VEGF, CD31, and P-VEGFR-2, as well as hemoglobin content, were increased (Figure 3 and Table 1). These results demonstrate that the activation of HIF-1α within tumors resulted in the production of angiogenic factors and the subsequent activation of the VEGF/VEGFR-2

signaling pathway, thereby stimulating angiogenesis. This angiogenic stimulation contributes to tumor growth and metastasis in HFD-fed mice.

Leptin is mainly produced in adipose tissue, and an increase in the adipose tissue with weight gain increases circulating leptin (reviewed in [50]). In the present study, the number of lipid vacuoles in tumor tissues (Figure 4A) and the weights of epididymal fat pads (Supplemental Table 1) increased in the HFD group. Serum levels of leptin (Table 2) as well as leptin secretion by epididymal fat explants (Figure 4D) were markedly increased in HFD-fed mice, indicating that the increase in fat cells in tumor and adipose tissues caused the increase in serum leptin levels in HFD-fed mice. Leptin may also have been increased as a consequence of elevated insulin levels. It is well known that insulin stimulates leptin secretion [51,52]. There is an increasing amount of evidence to indicate that leptin functions as a stimulator of growth and progression of colon cancer. For example, epidemiological results demonstrated significant associations between serum leptin and colon cancer risk [53,54]. Several studies have shown leptin's ability to induce angiogenesis by stimulating increased production of VEGF [55,56]. It has been also reported that leptin is a growth factor for HT-29 human colon cancer cells [57] and promotes motility and invasiveness in LS174T and HM7 human colon cancer cells [58]. Additionally, colon tumor growth was dramatically inhibited in leptin-deficient and leptin-receptor-deficient mice despite the fact that the animals exhibit severe obesity [59]. Taken together, the present results indicate that, in our HFD-fed mice, leptin may have stimulated tumor growth and progression by paracrine and/or endocrine mechanisms.

In summary, the results of this study demonstrate that the chronic consumption of an HFD stimulates colon cancer cell growth and metastasis in mice in the absence of increased energy intake or discernible weight gain. However, these increases in tumor growth and metastasis may have been attributable to the small increase in fat mass in the tumor and adipose tissue. These effects were found to be mediated via increases in circulating growth factors and the activation of several transcription factors including HIF-1α in tumor tissues, which subsequently induce the expression of a broad variety of proteins involved in the regulation of inflammation, angiogenesis, and cancer cell proliferation and metastasis. The results of this study suggest that the chronic consumption of an HFD may stimulate colon cancer progression even in individuals who maintain a relatively healthy body weight, and further indicate that replacing dietary fat with carbohydrates may suppress colon cancer progression.

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