Hyperlipidemia Enhancement of Renal Preneoplasia but not Tumor Formation Is Due to Elevated Apoptosis in the Eker Rat

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Abstract

The influence of a high-fat diet on the appearance of renal tumors was assessed in the Eker rat model of hereditary renal carcinoma. Examination of H&E-stained sections showed a significant increase in the number of microscopic solid adenomas in the high-fat group compared with the low-fat group, whereas there was no significant difference in the number of macroscopic tumors between the two groups. Where had the tumor buds gone? Staining for apoptotic bodies occasionally revealed apoptosis in and around the microscopic adenomas. In addition, an Eker rat renal tumor-derived cell line showed apoptosis when it was cultured with high concentrations of native and acetylated low-density lipoproteins. These findings suggested that tumor buds repeatedly appeared and disappeared in Eker rats on a high-fat diet.

Introduction

It has been reported in epidemiological studies (1) that the incidence of RCC is high in patients on hemodialysis in whom serum levels of LDL and oxygen-derived free radicals are elevated. In addition, diabetes and myocardial infarction, which are related to arteriosclerosis and hyperlipidemia, are more common than normal in patients with RCC (2).

We have previously reported that human RCC cells expressed scavenger receptors and receptors for advanced glycosylation end products in vitro (3, 4) and that incubation with modified LDL induced the production of IL-6, an in vitro autocrine growth factor for RCC cells (5), thus promoting tumor cell proliferation. However, it is unclear whether or not the same phenomenon occurs in vivo. To answer this question, the present study was carried out in the Eker rat model of dominantly inherited renal carcinoma (6, 7). As expected, the number of microscopic solid adenomas was significantly higher in the high-fat diet group than in the low-fat diet group. However, contrary to our expectation, there was no significant difference in the number of macroscopic tumors between the two groups. Moreover, it was found that this might be due to apoptosis in the tumors.

Materials and Methods

Animals. Founder rats carrying the Eker mutation were kindly provided by Dr. Alfred G. Knudson (Fox Chase Cancer Center, Philadelphia, PA) in 1991. Eker rats were bred on a Long Evans background (Charles River Breeding Laboratory) by brother X sister mating and maintained pathogen free in the Animal Facility of the Cancer Institute since 1991. All animals were housed and treated in accordance with institutional guidelines. Eker rats were diagnosed as carriers by analysis of genomic DNA (8). DNAs were isolated from rat tails by proteinase K digestion followed by phenol/chloroform extraction (9). The animals were maintained on a low-fat diet (CE-2 standard diet for rats [Clea Japan Inc.] modified by removal of soybean oil and substitution of casein for fish meal) or a high-fat diet (CE-2 supplemented with 2% cholesterol and 1% cholic acid) for 48 weeks from the 6th week after birth.

Cell Line. A cultured cell line (LK9d) has been established from renal tubular tumor that arose spontaneously in Long Evans rats heterozygous (RCC+) for the Eker mutation (8). Cells were maintained in a mixture of equal volumes of DMEM and Ham’s F-12 medium supplemented with 1.6 μM Fe++, 50 nm sodium selenite, 25 μg of insulin per ml, 0.2 μM hydrocortison, 1 nm triiodothyronine, 10 μg of transferrin per ml, 10 micromunits of [Arg]vasopressin per ml, and 10 nm cholesterol, modified from Chuman et al. (10).

For examination of the effect of native and acetylated LDL, 1 × 10⁶ cells/ml of LK9d cells were plated in 24-well plates and incubated in Eagle’s MEM containing 1% FCS with various concentrations of LDL or acetylated LDL for 24 h.

Staining and Antibodies. Animals fed low- and high-fat diets were sacrificed at 48 weeks, and their kidneys were removed. Kidneys were fixed in 10% neutral buffered formalin. After being embedded in paraflin, 5-μm-thick serial sections were cut from the middle of the tissue, mounted on sialinized slides. Sections were stained with H&E and immunostained with rabbit anti-murine scavenger receptor IgG and antimouse IL-6 IgG using the avidin-biotin peroxidase complex method by using Vector stain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The polyclonal rabbit antimurine scavenger receptor IgG was generated by immunization with a synthetic peptide (MTRNLCPHEREDADC) carried the sequence of the NH2-terminal residues (11). The staining procedures were modified based on the manufacturer’s instructions. Briefly, after routine deparaffinization and washing in PBS (50 mm sodium phosphate, pH 7.4, 200 mm NaCl), samples were digested with proteinase K (Boehringer Mannheim, Indianapolis, IN) for 20 min at room temperature and washed. Slides were then put into 3% hydrogen peroxide, and treated in accordance with institutional guidelines. Eker rats were diagnosed as carriers by analysis of genomic DNA (8). DNAs were isolated from rat tails by proteinase K digestion followed by phenol/chloroform extraction (9). The animals were maintained on a low-fat diet (CE-2 standard diet for rats [Clea Japan Inc.] modified by removal of soybean oil and substitution of casein for fish meal) or a high-fat diet (CE-2 supplemented with 2% cholesterol and 1% cholic acid) for 48 weeks from the 6th week after birth.

Histological Detection of Apoptotic Cells and Bodies. Formalin-fixed, paraffin-embedded kidney tissue sections and Eker rat renal tumor cell suspensions fixed in 4% formalin were stained with the Apop Tag peroxidase kit (Oncor, Gaithersburg, MD; Ref. 13). The staining procedures were modified based on the manufacturer’s instructions. Briefly, after routine deparaffinization and washing in PBS (50 mm sodium phosphate, pH 7.4, 200 mm NaCl), samples were digested with proteinase K (Boehringer Mannheim, Indianapolis, IN) for 20 min at room temperature and washed. Slides were then put into 5% H₂O₂, for 5 min and washed with PBS. After the equilibration buffer was added for 10 min, terminal deoxynucleotidyl transferase enzyme was pipetted onto the slides, which were then incubated at 37°C for 1 h in a humidified chamber. The reaction was stopped by putting slides in stop/wash buffer. After washing, antidigoxigenin-peroxidase was added to the slides. Slides were washed, stained with diaminobenzidine substrate, and counterstained with methyl green.

DNA Gel Electrophoresis. Cell pellets were washed in PBS (without Ca²⁺ and Mg²⁺), resuspended (5 × 10⁶ cells) in 0.5 ml of Tris-borate EDTA (45 mm Tris-borate buffer, 1 mm EDTA; pH 8.0) containing 0.25% NP40 (Sigma) and 0.1 mg/ml RNase A (Sigma), incubated at 37°C for 30 min, treated with 1 mg/ml proteinase K, and incubated for an additional 30 min at 37°C. After incubation, 0.1 ml of loading buffer (0.25% bromphenol blue, 0.25% xylene
cyanol FF, and 30% glycerol) was added, and 25 μl of the tube content were transferred to the gel. Horizontal 1.6% agarose gel electrophoresis was performed at 2 V/cm for 6 h, and DNA in gels was visualized under UV light after staining with 5 μg/ml of ethidium bromide (Polysciences, Inc., Warrington, PA).

Transmission Electron Microscopy. Cell pellets were fixed with 2% glutaraldehyde in PBS for 30 min at 4°C, washed in PBS, and fixed again with 1% OsO₄ in PBS, washed again, and dehydrated by being placed in increasing concentration of ethanol. Cell pellets were then embedded in EPON 812, and ultrathin sections were prepared. Sections were stained for 10 min with 2% uranyl acetate followed by staining with lead citrate. Sections were viewed and micrographs were taken using a Hitachi H-7000 electron microscope.

Results and Discussion

In heterozygous Eker rats, tumors have been reported to be more common in males than in females (6, 7). Ten and 13 male Eker rats were assigned to low-fat diet and high-fat diet groups, respectively. The animals were maintained on a low-fat diet or a high-fat diet for 48 weeks from the 6th week after birth as described in “Materials and Methods.” On completion of this diet, the body weight was 498 ± 36 g, kidney weight was 1.71 ± 0.27 g, total cholesterol was 130 ± 10 mg/dl, triglyceride was 103 ± 27 mg/dl, and LDL was 55 ± 9 mg/dl in the low-fat group. The corresponding values for the high-fat group were 490 ± 29 g, 1.75 ± 0.30 g, 289 ± 105 mg/dl, 145 ± 71 mg/dl, and 203 ± 93 mg/dl. No significant differences were noted in the body weight or kidney weight, but the total cholesterol, triglyceride, and LDL levels were significantly higher in the high-fat group than in the low-fat group. After 48 weeks, the animals were sacrificed, and the kidneys were isolated, sectioned midsagittally, and stained with H&E. It has been reported that interstitial nephritis occurred in rats on a high-fat diet (14). In the present study, degenerating and regenerating renal epithelium was also often observed in the high-fat group. In addition, the number of microscopic solid adenomas, which are not observed in normal rats, was significantly higher in the high-fat group (12 ± 5 per rat) than in the low-fat group (3 ± 1 per rat; Fig. 1). Microscopic adenomas identified by staining serial sections with H&E were also positively stained with an antibody to the N terminal peptide of murine scavenger receptor (11) and an antibody to murine IL-6 (Fig. 2). Western blot analysis showed that the antiscavenger receptor antibody recognized rat macrophage class-A scavenger receptors (data not shown). The above staining was found to be specific, because it was blocked by the NH₂-terminal peptide (MTENQRLCPHEREDADC) and by rat IL-6 (data not shown). These results suggested that the positive signals for proliferation that we detected in vitro, including modified LDL, scavenger receptor, and IL-6, play a role at the microscopic adenoma stage. We also counted the macroscopic tumors on the surface of the isolated kidneys. The number of tumors was slightly higher in the high-fat group, but it showed no significant difference between the two groups.
Fig. 3. Apoptosis in renal tumor in a Eker rat fed a high-fat diet. Kidney tissue sections were stained with the Apop Tag peroxidase kit. Nuclei including DNA fragmentation were stained brown (arrow). A, ×400. B, ×200.

Fig. 4. Dose-response effects of native LDL and acetylated LDL on Eker rat renal tumor cells. Eker rat tumor cells LK9d (8) were cultured with various concentrations of LDL (●) or acetylated LDL (○; 0–1000 μg/ml) for 24 h. Three sets of experiments were carried out. Apoptotic cells were detected by the Apop Tag peroxidase kit. Five random fields were counted, and the mean percentage of apoptotic cells was calculated. Results (mean ± SD) are from three sets of experiments. *, P < 0.01 compared to control.

Fig. 5. Ultrastructural manifestation of apoptosis of Eker rat renal tumor cells incubated with acetylated LDL. Apoptotic cells were processed for transmission electron microscopy. Cells with prominent features of apoptosis, such as condensation of nuclei and cytoplasm, were found. (Electromicrograph, original magnification ×9600.)

What had happened to the tumor buds?

We have previously suggested that apoptosis of human renal carcinoma cells can occur under certain conditions (15). Therefore, we stained each section for apoptotic bodies by using an in situ apoptosis detection kit (Apop Tag peroxidase kit; Ref. 13). Although little apoptosis was noted in the low-fat group, apoptosis was observed in and around microscopic adenomas in addition to within degenerated distal tubules in the high-fat group (Fig. 3). Thus, the reason that the number of macroscopic tumors showed no significant difference between the two groups although the number of microscopic adenomas was significantly greater in the high-fat group was presumably that the high-fat diet induced apoptosis during adenoma proliferation.

We next used a cell line derived from an Eker rat renal tumor (LK9d; Ref. 8) to investigate whether apoptosis occurred in vitro when a high concentration of LDL was added (Fig. 4). Apoptosis was uncommon when LDL was added at a concentration of 200 μg/ml (control, 8 ± 3%; LDL, 12 ± 7%) but was considerably increased by LDL at 500 (23 ± 8%) or 1000 (41 ± 15%) μg/ml. In the experiment with acetylated LDL, apoptosis was considerably increased at 200 μg/ml or higher concentrations (acetyl LDL: 200 μg/ml, 28 ± 10%; 500 μg/ml, 34 ± 12%; 1000 μg/ml, 49 ± 18%; Fig. 4). The occurrence of apoptosis was demonstrated by staining using an in situ apoptosis detection kit (data not shown) and electron microscopy (Fig. 5) and by detecting DNA ladder formation (Fig. 6). The results of the present study were in accord with the report that native and oxidized LDL induce in vitro apoptosis of mouse and human macrophages and renal mesangium cells when added at high concentrations (16, 17). In fact, the serum LDL level was over 2 mg/ml in the high-fat group, so the possibility seems strong that this in vitro phenomenon also occurred in vivo. These results suggest the possibility that a high-fat environment influences tumorigenesis at the microscopic adenoma stage in the kidneys of Eker rats, with possible repetition of active proliferation and apoptosis at this stage. Tumor buds were induced by hyperlipidemia, which might never develop into macroscopic tumors.

It has been suggested that the Fas, Fas ligand plays an important role in apoptosis of the renal tubular epithelium in nephritis (18). Therefore, it may be useful to investigate whether or not the Fas, Fas ligand was related to the apoptosis observed in the present study. Hino...
and coworkers (8) reported a second, somatic mutation (second hit) in the wild-type tuberous sclerosis (Tsc2) gene (the predisposing Eker gene; Ref. 8), even in the earliest precancerous lesion (e.g., phenotypically altered tubules), as a rate-limiting step for renal carcinogenesis in the Eker rat model of dominantly inherited cancer (7, 9, 19).

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References


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