Adipocytes and preadipocytes promote the proliferation of colon cancer cells in vitro

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Am J Physiol Gastrointest Liver Physiol 292: G923–G929, 2007. First published December 14, 2006; doi:10.1152/ajpgi.00145.2006.—Obesity, a risk factor for colon cancer, is associated with elevated serum levels of leptin, a protein produced by adipocytes. The aim of the present study was to clarify the effects of adipose tissue on colon cancer proliferation by using cultured cell lines. To achieve this, colon cancer cells (CACO-2, T84, and HT29) were cocultured with adipose tissue, isolated mature adipocytes, and isolated preadipocytes in a three-dimensional collagen gel culture system. The adipocytes and preadipocytes used were isolated from C57BL/6J and leptin-deficient ob/ob mice. Proliferation of the cancer cells was evaluated by nuclear bromodeoxyuridine uptake. The adipose tissue, mature adipocytes, and preadipocytes isolated from C57BL/6J mice significantly increased the proliferation of the colon cancer cells. This trophic effect of mature adipocytes on the cancer cell lines was observed only for cells from lean littermates and not for those from ob/ob mice. In contrast, the trophic effect of preadipocytes was not abolished in ob/ob mice, and this finding was supported by the result that leptin had a trophic effect on cancer cells. In conclusion, adipocytes were able to enhance the proliferation of colon cancer cells in vitro, partly via leptin, suggesting that adipose tissues, including mature adipocytes and preadipocytes, may promote the growth of colorectal cancer.

CACO-2; HT29; T84; obesity; leptin

Obesity is associated with increased risks for a number of different types of cancer (12, 13). Evidence for such risks has been most consistent for endometrial cancers, including breast cancer in postmenopausal women, esophageal cancer (21), and renal cell cancer (34). The cancer risks related to obesity include alterations of the hormonal patterns of sex hormones and insulin, distribution of body fat, and changes in adiposity at different ages (25). The increasing prevalence of obesity emphasizes the importance of further examinations of the relationship between obesity and cancer as well as the mechanisms involved in their interactions (7).

Previous studies have demonstrated a clear association between obesity and the risk of colorectal cancer (3, 4). Although the rich adipose tissue in obese patients is an important endocrine organ producing several cytokines, it is unclear whether mature adipocytes have any influence on colon cancer cells. One reason for this lack of information is the difficulties associated with culturing mature adipocytes, which contain large lipid droplets and do not attach to the surface of culture dishes owing to their buoyancy in medium (27–29). It is possible that neoplastic cells in the intestine may be affected by adipocytes, since adipocytes in the deep layer of the intestine have several effects on surrounding organs through their production of various cytokines (16). Among the adipocyte-derived cytokines, the serum level of leptin is closely related to the amount of adipose tissue in humans (26). Furthermore, a previous study has suggested a relationship between the leptin level and the levels of growth factors in colon cancer cells (10).

We hypothesized that adipocytes may be able to accelerate the growth of colon tumor cells through leptin. Therefore, the aim of the present study was to clarify this hypothesis by using colon cancer cell lines (CACO2, HT29, and T84) cultured in a three-dimensional collagen gel culture system.

MATERIALS AND METHODS

Cell Preparation

Three types of colon cancer cell lines were used in this study. The CACO-2 cell line (RCB0988) was purchased from the RIKEN Biosource Center (Tsukuba, Japan). The human colon cancer cell lines T84 (CCL 248) and HT-29 (HTB-38) were purchased from the American Type Culture Collection (Manassas, VA). The passages used were 3–16 for CACO-2 cells, 3–18 for HT-29 cells, and 3–16 for T84 cells. All cell lines were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich Japan, Tokyo, Japan) containing 4.5 g/l glucose, l-glutamine, sodium bicarbonate, 10% fetal calf serum, and 50 U/ml gentamicin at 37.0°C in a humidified atmosphere with 5% CO2.

Mature obese ob/ob C57BL6J mice (4–6 wk of age) and their lean littermates (+/?, referred to as lean control mice) were purchased from Charles River Laboratories Japan (Yokohama, Japan). The animals were housed in temperature- and humidity-controlled rooms, kept on a 12:12-h light-dark cycle, and provided unrestricted access to food and water unless otherwise specified. Adipocytes were collected from the abdominal hypodermis of the mice. Briefly, aseptically excised adipose tissue was minced, digested with collagenase solution at 37°C for 30 min, and filtered through a 70-μm mesh sieve, as described previously (29). The filtered cells in the suspension were dispersed in minimum essential medium supplemented with 10% fetal calf serum. After centrifugation, the fat cells floating on top were collected with a Pasteur pipette. This suspension-centrifugation procedure was repeated three times to dilute the collagenase solution, and dissociated fat cells were obtained. In addition, preadipocytes were collected during the procedure as described previously (17). These preadipocytes expressed S-100 protein, a marker of preadipocytes that...
is not expressed in fibroblasts or endothelial cells. All procedures involving animals were performed in accordance with regulations laid down by the ethical guidelines of Saga Medical School.

Three-Dimensional Collagen Gel Matrix Culture System

To examine the effects of the stromal component cells, we used a three-dimensional collagen gel culture system, as described previously (20, 29, 30, 36). A schematic explanation of the procedure is shown in Fig. 1.

**Experiment 1.** Eight volumes of acid-soluble type I collagen solution (pH 3), 1 volume of 10× concentrated minimum essential medium, and 1 volume of reconstruction buffer (2.2 g of sodium bicarbonate and 4.77 g of HEPES dissolved in 100 ml of 0.05 N sodium hydroxide) were mixed. The mixture, without stromal cells, was poured into a 30-mm dish with a nitrocellulose bottom and incubated at 37°C for 30 min to allow solidification of the gel. This inner dish was placed into a 90-mm outer dish, and medium was added to both dishes. The collagen gel layer prepared in the inner dish corresponded to the lamina propria in vivo. Next, 1 ml of CACO2 cells suspended in culture medium at a concentration of 1 × 10^6 cells/ml was spread onto the reconstructed lamina propria. To examine the interaction between CACO2 cells and stromal cells, six experimental systems were prepared (experiments 2-1, 2-2, 3-1, 3-2, 4-1, and 4-2) and examined in a similar manner to experiment 1.

**Experiment 2.** Minced adipose tissues derived from lean control mice (experiment 2-1) and leptin-deficient ob/ob mice (experiment 2-2) were added to the collagen solution mixture at a concentration of 0.1 cm^3/ml. After being mixed, 1 ml of each mixture was placed in the inner dish. After gelation, the CACO2 cell suspension was spread on the gel.

**Experiment 3.** Mature adipocytes derived from lean control mice (experiment 3-1) and ob/ob mice (experiment 3-2) were added to the collagen solution mixture at a concentration of 3 × 10^5 cells/ml. After being mixed, 1 ml of each mixture was poured into the inner dish. After gelation, the CACO2 cell suspension was spread on the gel.

**Experiment 4.** Preadipocytes derived from lean control mice (experiment 4-1) and ob/ob mice (experiment 4-2) were added to the collagen solution mixture at a concentration of 1 × 10^6 cells/ml. After being mixed, 1 ml of each mixture was poured into the inner dish. After gelation, the CACO2 cell suspension was spread on the gel.

**Experiment 5.** To examine the direct effects of leptin on cancer cells, CACO2 cells were administered recombinant rat leptin (R&D Systems, Minneapolis, MN) for up to 7 days at various concentrations (10). The various concentrations of leptin were added to the dishes in...
the same manner as described for experiment 1. In additional dishes, 500 ng/ml of leptin antagonist [human leptin antagonist (quadruple mutant), recombinant protein: Biovendor, Candler, NC] was added to 500 ng/ml leptin.

**Evaluation of Cultured Cells**

The cultured cells were examined by the following three methods. First, the cells were observed under a light microscope. Second, collagen gels containing cultured cells were incubated with 1% formalin, embedded in paraffin, cut into vertical thin sections, deparaffinized, and stained with hematoxylin and eosin. Third, cell growth was examined by immunohistochemistry for bromodeoxyuridine (BrdU; Cell Proliferation Kit, Amershams, Arlington Heights, IL) uptake after a 24-h incubation with 30 mg/ml BrdU, as described previously (22, 23). To obtain the rate of nuclear BrdU uptake, 1,000 cells were counted and the percentage of BrdU-positive nuclei was calculated.

**Western Blotting**

Confluent monolayers of cells from six-well plates (IWAKI, Chiba, Japan) were washed in ice-cold PBS and scraped into 100 μl of 4× sample buffer [125 mmol/l Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 2%, β-mercaptoethanol, 20% glycerol, and 1 mg of bromophenol blue]. Aliquots (30 μl) of the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 2% low-fat milk powder in PBS containing 1% Triton for 1 h at room temperature, washed for 3 × 10 min in wash buffer (PBS containing 1% Triton and 0.2% low-fat milk powder), and then incubated in a primary antibody diluted 1:1,000 in wash buffer overnight at 4°C. Next, the membranes were washed for 3 × 10 min in wash buffer and incubated in a horseradish peroxidase-conjugated secondary antibody diluted 1:200 in wash buffer for 1 h at room temperature. After a final three washes for 10 min each in wash buffer, the membranes were incubated in ECL reagents (Amersham Biosciences, Buckinghamshire, UK) for 1 min, and the chemiluminescence generated was analyzed using a LAS1000 chemiluminescence detector (Fuji Photo Film, Tokyo, Japan).

For the Ob receptor Western blot analyses, M-18 goat polyclonal anti-Ob receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) raised against the intracellular portion of mouse Ob-Rα and reactive against both isoforms of the human leptin receptor (Ob-Rα and Ob-Rβ) were used as the primary antibody. After the primary antibody incubation, extra steps were added in which the membranes were incubated with biotin-labeled rabbit anti-goat antibodies (Dako Japan, Kyoto, Japan) diluted 1:2,000 in wash buffer for 1 h, washed for 3 × 5 min in wash buffer, and incubated with streptavidin-horseradish peroxidase (Dako) diluted 1:100 in wash buffer for 60 min before the detection of horseradish peroxidase as described above. Equal protein loading was further confirmed by probing the same blots with an anti-β-actin antibody (Santa Cruz Biotechnology).

**Statistical Analysis**

The results are expressed as means ± SE. Data were evaluated by analysis of variance in which multiple comparisons were performed by the least-significant difference method. Differences were considered significant if the probability of the difference occurring by chance was less than 5 in 100 (P < 0.05).

**RESULTS**

**Experiment 1: Stromal Cell-Free System**

Experiment 1 was conducted as a control for subsequent experiments. The CACO2 cells formed only a thin epidermal layer on the stromal cell-free collagen gel. Specifically, the CACO2 cells formed a stratified layer of one or two cells that were poor in cytoplasm (Fig. 2; experiment 1). The proliferation of CACO2 cells in these control experiments was evaluated after 1 wk by the BrdU uptake method (Fig. 3).

**Experiment 2: Stromal Adipose Tissue-Containing Systems**

The effects of stromal adipose tissues containing mature adipocytes and preadipocytes on the proliferation of CACO2 cells were examined in experiments 2-1 and 2-2. In the presence of adipose tissues from both lean and leptin-deficient obese ob/ob mice, the CACO2 cells clearly formed high cell density layers and were rich in cytoplasm compared with the control experiments (Fig. 2: experiment 2-1: lean littermates; experiment 2-2: obese ob/ob mice). BrdU uptake by the CACO2 cells was increased in the cocultures with both types of adipose tissue (Fig. 3; P < 0.05).

**Experiment 3: Mature Stromal Adipocyte-Containing Systems**

The effects of purified stromal mature adipocytes on the proliferation of CACO2 cells were evaluated in experiments 3-1 and 3-2. Mature adipocytes isolated from lean control mice had a proliferative effect on CACO2 cells, although the effect was lower than that induced by the stromal adipose tissue containing mature adipocytes and preadipocytes (Fig. 2; experiment 3-1). Mature adipocytes isolated from ob/ob mice, which did not contain leptin, had no influence on the CACO2 cell proliferation (Fig. 2; experiment 3-2). These results were supported by the BrdU uptake results (Fig. 3). Specifically, increased BrdU uptake by CACO2 cells was induced by mature adipocytes from lean littermates (P < 0.05), but not by those from ob/ob mice.

**Experiment 4: Stromal Preadipocyte-Containing Systems**

Stromal preadipocytes isolated from lean littermates enhanced the proliferation of CACO2 cells (Fig. 2; experiment 4-1). This effect was also observed for preadipocytes isolated from ob/ob mice (Fig. 2; experiment 4-2). The proliferative effects of the preadipocytes were the same as those of the adipose tissues in experiment 2. Regarding BrdU uptake, the preadipocytes from lean littermates and ob/ob mice had the same effects on CACO2 cells as the corresponding adipose tissues.

**Experiment 5: Direct Effect of Leptin on CACO2 Cells**

The results of leptin administration to CACO2 cells are shown in Fig. 4. Although leptin at doses of 5 and 50 ng/ml had no effect on BrdU uptake by CACO2 cells, leptin doses above 500 ng/ml significantly enhanced BrdU uptake by CACO2 cells (P < 0.05 for each). This trophic effect of leptin on CACO2 cells was completely reversed by a leptin antagonist, as shown in Fig. 5.

**Effects of Adipose Tissue and Mature Adipocytes on BrdU Uptake by HT29 and T84 Cells**

The results for cocultures of adipose tissue and mature adipocytes with the above cells are shown in Fig. 6 (A: HT29 cells; B: T84 cells). In both cell lines, no proliferative effects of mature adipocytes isolated from ob/ob mice were observed.
Proliferative effects of the adipose tissues, containing both preadipocytes and mature adipocytes, were observed on both cell lines, and the effects of the tissue from the \textit{ob/ob} mice were similar to those of the tissue from lean littermates ($P < 0.05$ for each).

Western Blot Analysis for Short and Long Isoforms of the Leptin Receptor

The results of immunoblotting analysis for leptin receptors in the cultured cell lines are shown in Fig. 7. All three colon...
cancer cell lines showed a band at ~100 kDa on the immuno- 
blots, consistent with the presence of the short isoform (Ob-Ra) 
of the leptin receptor. In addition, all three cell lines showed a 
band at ~130 kDa, consistent with the long isoform (Ob-Rb) 
of the receptor. Simultaneous loading controls using an anti-
β-actin antibody showed almost equal loading for all cell lines. 
These results indicate that both the short and long isoforms of 
the leptin receptor are expressed in colon cancer cells 
(CACO2, HT29, and T84).

**DISCUSSION**

Obesity, which is progressively increasing worldwide, is 
closely associated with increased morbidity and mortality
caused by several common diseases in the Western world, including diabetes, hypertension, cardiovascular disease, and cancer (13, 18). The overwhelming majority of epidemiological studies have reported a direct association between obesity and colorectal cancer (5). In contrast to these epidemiological studies, there is little available information regarding the potential roles of adipocytes in colon cancer growth using in vitro studies. We hypothesized that adipocytes may act as growth factors for colon cancer cells, since adipocytes have been observed to have several effects on breast, laryngeal, squamous cell carcinoma, and prostate cancer cells (11, 15, 31, 32, 35).

The results of the present study demonstrate that adipocytes promote the growth of several colon cancer cell lines, namely, CACO2, T84, and HT29, in the context of mesenchymal-cancer cell interactions, suggesting that adipose tissue could promote colon cancer cell growth. Adipose tissue contains mature adipocytes and immature preadipocytes. The present results indicate that both isolated mature adipocytes and preadipocytes have proliferative effects on colon cancer cell lines, although the effects of preadipocytes are more potent.

Adipose tissue possesses the characteristics of an endocrine organ and affects several other tissues, including hair follicle cells (19) and laryngeal squamous cell carcinoma cells (35), via the production of various cytokines and adipokinetins, such as leptin, TNF-α, heparin-binding EGF, IGF-II, adipin, and plasminogen activator inhibitor-I, as well as various undefined factors (14, 15). Leptin, which is secreted by adipose tissue and the gastrointestinal tract (24), regulates food intake, energy expenditure, intestinal cell apoptosis, and adaptation to starvation (1, 6, 9, 33, 37). The leptin levels in humans and animals are known to closely reflect the percentage of body fat, and obesity may be associated with hyperleptinemia (8, 18).

These findings indicate that both isolated mature adipocytes and preadipocytes promote the growth of several colon cancer cell lines. Furthermore, this trophic effect of mature adipocytes is mediated via leptin, although other factors including cytokines releasing from adipocytes are also likely to be involved in the process.

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In conclusion, the present results indicate that adipose tissue can enhance the proliferation of cultured colon cancer cell lines. Furthermore, this trophic effect of mature adipocytes is mediated via leptin, although other factors including cytokines releasing from adipocytes are also likely to be involved in the process.

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