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Leptin promotes the growth of Colon 38 cancer cells and interferes with the cytotoxic effect of fluorouracil *in vitro*

Leptyna nasila wzrost raka Colon 38 i moduluje cytotoksyczne działanie fluorouracylu *in vitro*

Gabriela Mełeń-Mucha, Hanna Ławnicka

Department of Immunoendocrinology, Katedra of Endocrinology, Medical University, Łódź

Abstract

Introduction: Epidemiological studies underline the fact that obesity represents a significant risk factor for the development of several cancers, one of which is cancer of the colon. Moreover, multiple recent data indicate that some adipose tissue-derived hormones may influence the growth of malignant cells. Leptin, the product of the *ob* gene, is one of these. However, the evidence from research is still contradictory regarding the role of leptin in colon cancer. The aim of our study was to examine the direct effect of leptin at various concentrations (from 10^{-5} to 10^{-12} M) when applied alone or jointly with fluorouracil (the classical cytotoxic drug for colon cancer) at two concentrations (0.25 mg/ml and 2.5 mg/ml) on the growth of murine Colon 38 cancer cells *in vitro*.

Material and methods: Colon 38 cancer cells were preincubated in RPMI 1640 medium supplemented with foetal calf serum for 24 hours. The cells were then cultured for a further 72 hours in the presence of various concentrations of the substances under examination, applied either alone or jointly. The growth of the Colon 38 cell line was assessed by a colorimetric kit based on the modified Mosmann method.

Results: We found that leptin increased the growth of murine Colon 38 cancer at concentrations of 10^{-6}, 10^{-7} M and 10^{-10}, 10^{-11}, 10^{-12} M. Its stimulatory effect was fairly slight, with an increase in cancer growth of 5% to 15% as compared to controls. As we expected, fluorouracil at both the concentrations examined inhibited the growth of Colon 38 cancer maximally up to 28% (2.5 mg/ml) and 34% (0.25 mg/ml) of controls, with a stronger effect obtained from higher doses. Leptin did not modulate the cytotoxic effect of fluorouracil applied at the higher concentration (2.5 mg/ml) but, unexpectedly, at concentrations of 10^{-9} and 10^{-10} M it heightened the cytotoxic effect of fluorouracil given at a lower concentration (0.25 mg/ml).

Conclusions: These data indicate that leptin is involved in the regulation of colon cancer growth and it may even heighten the cytotoxic effect of fluorouracil.

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Key words: *leptin, fluorouracil, colon cancer*

Streszczenie

Wstęp: Z badań epidemiologicznych wynika, że otyłość jest istotnym czynnikiem ryzyka rozwoju różnych nowotworów, między innymi również raka jelita grubego. Ponadto, w wielu badaniach wykazano, że niektóre hormony wywodzące się z tkanki tłuszczowej wpływają na wzrost komórek nowotworowych oraz modulują inne procesy związane z karcynogenezą, jak np. angiogenezę. Leptyna, produkt genu *ob* jest jednym z nich. Celem badań była ocena bezpośredniego wpływu różnych stężeń leptyny zastosowanej osobno lub razem z fluorouracylem na wzrost mysiej linii raka jelita grubego Colon 38.

Materiał i metody: Komórki raka Colon 38 były preinkubowane w medium RPMI 1640 z dodatkiem płodowej surowicy cielęcej przez 24 godz. Następnie, komórki te były hodowane przez kolejne 72 godz. w obecności różnych stężeń badanych substancji. Wzrost raka Colon 38 oceniano, opierając się na zmodyfikowanej kolorymetrycznej metodzie Mosmanna.

Gabriela Mełeń-Mucha, M.D., Ph.D
Department of Immunoendocrinology,
Medical University of Łódź
Sterlinga 3, 91–425 Łódź
phone/fax: 042 636 54 27
e-mail: g.m-mucha@wp.pl

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Wyniki: Wykazano, że leptyna stymuluje wzrost raka Colon 38 w stężeniach 10^{-6}, 10^{-7} M oraz 10^{-10}, 10^{-11}, 10^{-12} M. Jej działanie było słabe, choć istotne statystycznie z nasileniem wzrostu tego nowotworu o 5–15% w porównaniu z grupą kontrolną. Jak oczekiwano, fluorouracyl w obu stężeniach (0,25 mg/ml i 2,5 mg/ml) hamował wzrost tego nowotworu maksymalnie do 28% (2,5 mg/ml) i 34% (0,25 mg/ml) grupy kontrolnej. Leptyna nie zmieniała cytotoksycznego działania fluorouracylu zastosowanego w wyższym stężeniu (2,5 mg/ml), lecz niespodziewanie w stężeniach 10^{-9} i 10^{-10} M nasiliła cytotoksyczny wpływ fluorouracylu zastosowanego w niższym stężeniu (0,25 mg/ml).
Wnioski: Uzyskane wyniki wskazują, że leptyna jest regulatorem wzrostu raka jelita grubego, a w wybranych warunkach nasila cytotoksyczne działanie fluorouracylu.

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Słowa kluczowe: *leptyna, fluorouracyl, rak jelita grubego*

Introduction

Colon cancer is a growing problem in our ageing society because of its high incidence, morbidity and mortality. It is usually diagnosed late, at the advanced stages when surgery is ineffective or even contraindicated. Thus most diagnosed colon cancers have to be treated with chemotherapy. Great strides have been made in the treatment modalities for the advanced stages of this cancer in the last ten years in association with the introduction of five new drugs (irinotecan — 1996, oxaliplatin — 2002, capecitabine, an oral formulation of fluorouracil — 1998; bevacizumab — monoclonal antibody-targeting vascular endothelial growth factor — 2004; cetuximab — monoclonal antibody targeting epithelial growth factor receptor — 2004). However, the progress achieved in prolonging average survival is minimal (approaching a doubling), cure rates remain unchanged and only drug costs have increased dramatically [1]. The treatment of this cancer still remains palliative [1]. Thus the search for new therapeutic options for colon cancer continues. What we have learned from recent observation is that the growth of this cancer can be modulated not only by means of chemotherapy but also through biotherapy such as cetuximab or bevacizumab [2, 3], which have recently been approved by the Food and Drug Administration for use in patients.

A further molecular target for colon cancer appears to be leptin, since epidemiological studies indicate that obesity significantly increases the risk of some gastrointestinal cancers, including colon cancer [4]. In accordance with this hypothesis some authors suggest that both hormonal and biochemical factors observed in obese people, such as elevated levels of leptin, insulin and insulin-like growth factors, decreased levels of ghrelin or elevated levels of triglycerides, low-density lipoproteins or prothrombotic and proinflammatory states, may be implicated in colon carcinogenesis [5–7]. Leptin, a product of the obese *(ob)* gene is a 16 kDa cytokine that was discovered in 1994 as a regulator of body weight and energy balance [8]. This protein hormone is produced predominantly by white adipocytes and its circulating concentration in humans is proportional to the total adipose tissue mass, in other words increased in obese and decreased in lean subjects [9]. There are several reports indicating that leptin promotes the growth of various cancer cell lines, including colon cancer cells, by means of stimulation of cancer cell growth, migration and invasion and through regulation of neoangiogenesis [10, 11]. Some authors treat an elevated leptin level as an independent factor for colon cancer development [12]. In support of these data, the expression of leptin receptors (ObR) in colon cancer tissues and cell lines has been confirmed by immunodetection [13]. However, the results are not sufficiently concordant and the role of leptin in colon carcinogenesis remains a subject of debate [14]. It has so far been observed that leptin increases the growth not only of normal colonic epithelium but also of colon cancer cell lines such as HT-29, LoVo and Coca-2 [12–15]. These stimulatory effects have varied considerably among various colon cancer cell lines in comparison to controls. However, *in vivo* leptin did not promote the growth of colon cancer xenografted in nude mice (Ki-67 index in tumour tissues was even inhibited) and did not stimulate intestinal tumourigenesis in ApcMin/+ mice [14]. Moreover, even in *in vitro* study leptin was mitogen only in Apc-deficient (ApcMin/+ IMCE cells) colonic epithelial cells but not in those expressing wild-type Apc (Apc+/+ YAMC cells) [15].

Thus the aim of our study was to examine the influence of leptin, applied alone or in combination with fluorouracil, which is the drug of choice for colon cancer, on the growth of murine Colon 38 cancer cells.

Materials and methods

Murine Colon 38 cancer cells were used in the study. The cells were cultured in a culture flask (Nunc Eas Y flask 25 cm^2, NUNC) in the presence of RPMI 1640 medium (Sigma), supplemented with 25 mM Hepes buffer (Sigma), 4 mM L-glutamine (Sigma), 100 U/ml

penicillin and 100 μg/ml streptomycin solution (Sigma), 2 g/l sodium bicarbonate (Sigma) and 5% foetal calf serum (FCS, Biochrom) (complete medium). The cells were routinely cultured in a humidified incubator at 37°C with 5% carbon dioxide. Before confluency the cells were harvested every 3–4 days in the presence of preheated (37°C) trypsin-EDTA at a concentration of 0.05% and 0.02% respectively in Hanks-balanced salt solution (Trypsin-EDTA, Sigma). Thereafter the cells were collected, rinsed three times in culture medium, centrifuged and seeded in a culture flask (2×10^5 cells/5 ml medium) for the four subsequent days.

After one of the subsequent trypsinisation procedures the cells were suspended in the complete medium in a concentration of 4×10^5 cells/ml. Next 50 μl aliquots of the suspension (20×10^3 cells) were seeded into each well of the culture plate (96 Cell Culture Cluster Dish, Nunclon MicroWell Plates, NUNC) and preincubated for 24 hours. The cells were then cultured for a further 72 hours in the presence of various concentrations of the examined substances: mouse leptin [Leptin (11–130) amide (Mouse), BACHEM] in final concentrations of 10^{-5}–10^{-12} M, and Fluorouracil (FU, Roche) in final concentrations of 0.25 mg/ml or 2.5 mg/ml, applied either alone or jointly. The control groups were incubated in the complete medium only. Two separate cultures were developed and the cell growth was assessed by the modified colorimetric Mosmann method, using the EZ4Y kit (Easy for You, the 4th Generation Non-Radioactive Cell Proliferation & Cytotoxicity Assay, Biomedica Gruppe, Austria, Bellco Biomedica, Poland). This method is based on the transformation of tetrazolium salt into coloured soluble formazan via mitochondrial enzymes, which correlates well with cell proliferation and viability. The intensity of the reaction was estimated by the measurement of optical density (OD), using an ELISA reader ($\lambda = 450$ nm). The data were statistically analysed by ANOVA and the significance of differences between means was determined by LSD (least significant differences) and presented as a percentage of the OD of the control group. $P < 0.05$ was considered the borderline of statistical significance.

Results

In our experimental model leptin enhanced the growth of Colon 38 cancer cells in 6 out of 9 of the concentrations examined: 10^{-6}, 10^{-7} M and 10^{-10}, 10^{-11}, 10^{-12} M (Fig. 1, 2). The other concentrations of leptin were insufficient to increase Colon 38 cancer growth effectively. The growth effect of leptin was somewhat weak and the maximal stimulation of growth was observed for leptin concentrations of 10^{-11} and 10^{-12} M, reaching 115% compared with controls (Fig. 2). Moreover, we did not observe the dose-response effect. As we expected, fluorouracil at both concentrations inhibited the growth of Colon 38 cancer cells and had a greater effect at higher concentrations (Fig. 1). Its inhibitory effect was strong, and it inhibited the growth of Colon 38 cancer maximally up to 28% (2.5 mg/ml) (Fig. 1) and 34% (0.25 mg/ml)

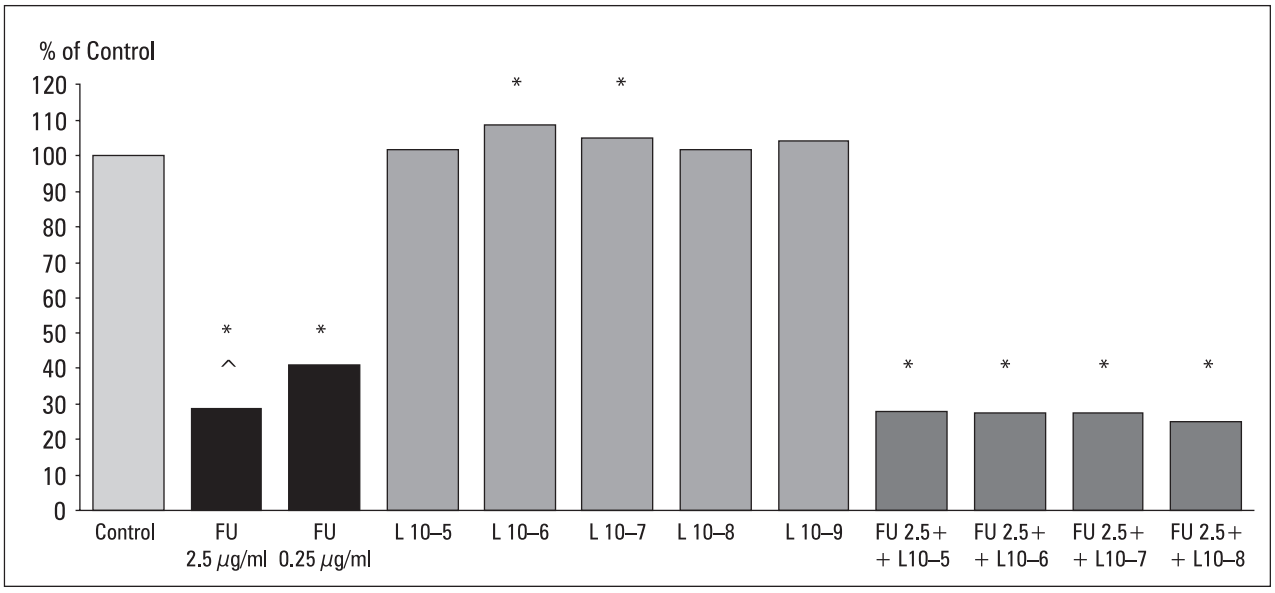

Figure 1. *The effect of leptin (L) applied alone or jointly with fluorouracil (FU) on the growth of Colon 38 cancer cells; C — Control, *p < 0.05 vs C, ^ p < 0.05 vs FU 0.25 μg/ml*

Rycina 1. *Wpływ leptyny (L) stosowanej oddzielnie lub w połączeniu z fluorouracylem (FU) na wzrost komórek raka jelita grubego Colon 38; C — Grupa kontrolna, *p < 0.05* vs. *C, ^ p < 0.05* vs. *FU 0.25 μg/ml*

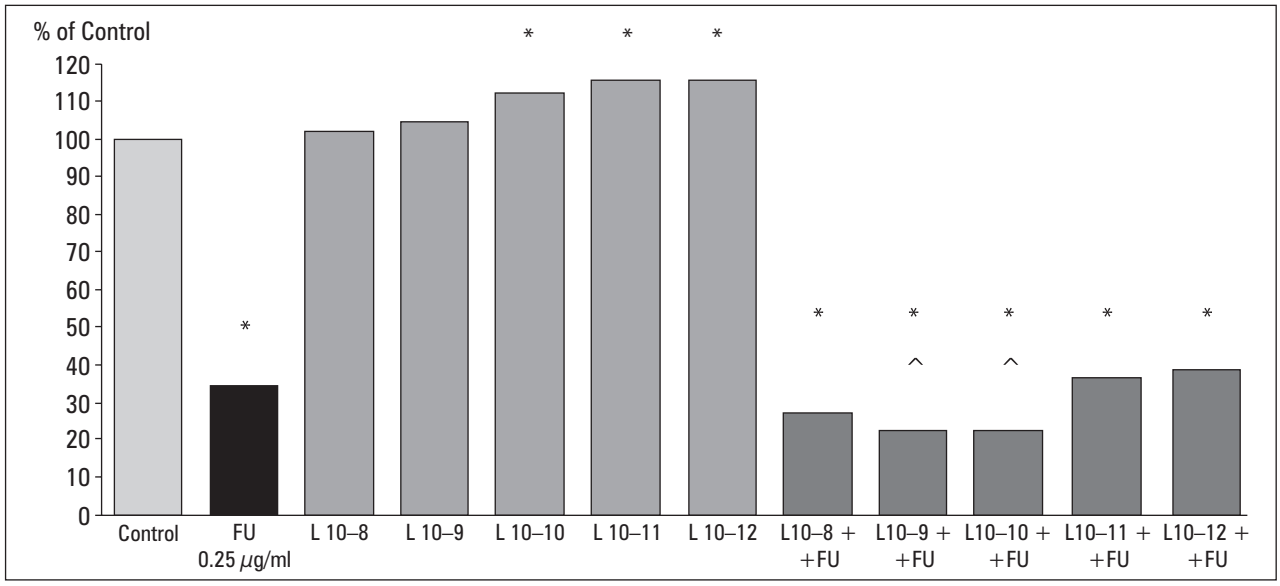

Figure 2. *The effect of leptin (L) applied alone or jointly with fluorouracil (FU) on the growth of Colon 38 cancer cells; C — Control, *p < 0.05 vs C, ^ p < 0.05 vs FU*

Rycina 2. *Wpływ leptyny (L) stosowanej oddzielnie lub w połączeniu z fluorouracylem (FU) na wzrost komórek raka jelita grubego Colon 38; C — Grupa kontrolna, *p < 0,05* vs. *C, ^ p < 0,05* vs. *FU*

(Fig. 2) of controls. Unexpectedly, leptin at the concentrations of 10^{-9} and 10^{-10} M, applied jointly with fluorouracil at lower concentrations, heightened the cytotoxic effect of fluorouracil (Fig. 2), but when fluorouracil was given at a higher concentration it did not modulate the fluorouracil effect (Fig. 1).

Discussion

These data provide the first evidence that leptin may reinforce the cytotoxic effect of fluorouracil on murine Colon 38 cancer. Moreover, in our experimental model leptin slightly but statistically significantly enhanced the growth of Colon 38 cancer at most of the examined doses. Its stimulatory effect was present in some concentrations: 10^{-6}, 10^{-7} M and 10^{-10}, 10^{-11}, 10^{-12} M but absent in the rest the concentrations examined. The explanation of this divergent and fluctuating effect of changed concentrations of leptin and the lack of effect in the intermediate concentrations (10^{-8} and 10^{-9} M) of this hormone may be the existence of two populations of leptin receptors involved in its stimulatory action on the Colon 38 cancer cells examined. So far only the long form of leptin receptor (ObR1) seems to be the functional form [16, 17], whereas shorter isoforms ubiquitously expressed on several tissues seem to be involved in intra- and transcellular leptin transport [18] or even in preventing the activation of ObR signalling in the case of soluble leptin receptor (SLR) [19].

Since in our study the growth of Colon 38 cancer was determined by the modified Mosmann method based on the MTT assay, which reflects the number of metabolically active cells, we concede that the growth stimulatory effect of leptin observed should be interpreted as the stimulation of cell proliferation or inhibition of cell apoptosis or changes in both these processes. As we know from other studies, leptin has stimulated the growth of colon cancer cell lines mainly by means of enhancement of cell proliferation [12, 14] and inhibition of apoptosis [12, 20].

Our data are in concordance with other observations indicating the stimulatory effect of leptin on colon cancer growth and are in agreement with the hypothesis that leptin is a risk factor for colon cancer. However, our observations concerning a leptin-induced heightening of the cytotoxic effect of fluorouracil are in contradiction to this suggestion. It remains an open question as to whether leptin is a favourable or unfavourable factor for colorectal carcinogenesis. Since leptin can modulate the cytotoxic effect of fluorouracil, the drug of choice for patients with advanced colon cancer, we hypothesise that leptin concentration in colon cancer patients should be taken into consideration in the adjustment of fluorouracil doses in therapeutic protocol and that doses of fluorouracil should be lower in obese people. However, this will only be demonstrably true if the synergistic effect of leptin and fluorouracil are observed for human colon cancer.

These preliminary data call for further studies to elucidate the exact role of leptin and the leptin-fluorouracil interaction in colon cancer growth and therapy.

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