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Expression of the obesity hormone leptin and its receptor correlates with hypoxia-inducible factor-1α in human colorectal cancer

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Background: The obesity hormone, leptin, has been found to play a role in development and proliferation of normal and malignant tissues. Leptin activity is mediated through the leptin receptor (ObR) that is often expressed in different human cancer cells. Previously, we found that the expression of leptin and ObR can be stimulated by hypoxia-mimetic agents. The aim of this study was to analyze the abundance of and relationships among leptin, ObR and hypoxia-inducible factor-1α (HIF-1α, transcriptional regulator) in human colorectal cancer.

Materials and methods: We investigated the expression of leptin, ObR and HIF-1α in colorectal cancer specimens from 135 patients who underwent curative resection.

Results: Immunoreactivity for leptin, ObR and HIF-1α protein was observed in 69 of 135 (51.1%), 129 of 135 (95.5%) and 88 of 135 (65.2%) of colorectal cancers, respectively.

Statistically significant positive correlations were noted between leptin and HIF-1α ($P = 0.005$, $r = 0.243$), ObR and HIF-1α ($P < 0.001$, $r = 0.325$) as well as leptin and ObR ($P < 0.001$, $r = 0.426$) in the group of all patients as well as in various subgroups depending on clinicopathological features.

Conclusions: The results indicate that the leptin system is overexpressed in human colorectal cancer and this overexpression appears to be associated with the abundance of HIF-1α.

Key words: colorectal cancer, hypoxia-inducible factor-1α, leptin, leptin receptor, obesity

introduction

Leptin (obesity protein) is a 16 kDa cytokine produced mainly by adipose tissue. The levels of circulating leptin in humans are proportional to body mass index and are significantly elevated in obese individuals [1]. Leptin has been first described as a hormone regulating energy balance in the brain. In addition, leptin is known to influence various reproductive and immune processes in peripheral organs [2, 3]. Later studies documented that the hormone can also influence malignant progression [4–7]. Numerous studies demonstrated that different tumor tissues and cell lines respond to leptin and express functional leptin receptors (ObRs) [6–12]. Importantly, in breast, endometrial and gastric cancers, the expression of ObR has been found significantly elevated relative to noncancer control cases [8–13] The activation of ObR and subsequent induction of multiple signaling cascades, such as the JAK2/STAT3, ERK1/2, PKC-alpha and Akt/GSK3 pathways, has been shown to stimulate cell growth, transformation and survival [7–16]. Leptin can also promote invasiveness, migration and neoangiogenesis, all of which can promote tumor spreading [17–19]. Interestingly, we and others observed that different cancer tissues (e.g. breast, endometrial) coexpress leptin and ObR [8–20], indicating that leptin might affect tumor cells in an autocrine manner. The mechanism of this concomitant overexpression is not clear, but our data obtained in breast cancer cell lines indicate the involvement of obesity-related stimuli, including development of hypoxic conditions [8].

Hypoxia is a characteristic feature of cancers, especially fast developing tumors, and can also be induced by excess of surrounding adipose tissue. Lack of proper oxygenation stimulates the expression of various genes, e.g. those encoding for glycolytic enzymes, growth factors, their receptors and vasoactive peptides [21, 22]. The expression of numerous hypoxia-responsive genes is regulated by hypoxia-inducible factor-1 (HIF-1). HIF-1 is a basic helix–loop–helix transcription factor of heterodimeric structure that contains constitutively expressed HIF-1β and hypoxia-induced HIF-1α subunits [23]. HIF-1α activates transcription through binding to specific hypoxia-responsive

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element (HRE) present in promoter region of target genes [24]. Noteworthy, the leptin gene promoter contains eight HRE with the minimal core sequence 5′-RCGTG-3' that can recruit HIF-1. The leptin promoter has been shown to be induced by hypoxia or hypoxia-mimetic agents in BeWo cells [25, 26]. Our preliminary data indicate that similar mechanism might operate in breast cancer cells (S. Cascio, V. Bartella, A. Auriemma et al., unpublished data).

Obesity is one of the risk factors for colon cancer, and some preliminary reports indicate that leptin may enhance colorectal cancer cell growth. For instance, coculture of colorectal cancer cells with leptin-producing adipocytes significantly increased proliferation of cancer cells [27]. Other data obtained with cellular models indicated that in colorectal cancer cells (HT-29, LoVo, Caco2, SW 480 and HCT-8/S11 lines) leptin can activate the Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide-3 kinase (PI-3K) pathways and stimulate DNA synthesis, cell proliferation, migration and invasion [7–29]. It is possible that leptin impacts on the development and progression of human colorectal cancer, as we noticed significant overexpression of the hormone in colorectal cancer tissues [30]. However, the expression of ObR and HIF-1α colorectal cancer biopsies has never been described. Consequently, here we investigated the abundance of the leptin system and HIF-1α in human colorectal cancer and we assessed correlations among these biomarkers.

methods and results

immunohistochemistry

The study included specimens of primary colorectal cancers from 135 patients who underwent curative resection. The age of patients ranged from 35 to 92 years (mean 65.8 years). Tumor samples were collected immediately after tumor removal, fixed in 10% buffered formaldehyde solution for 48 h, embedded in paraffin blocks at 56°C according to standard procedures and then stained with hematoxylin and eosin.

Leptin, ObR and HIF-1α were investigated in representative tissue sections using specific antibodies (Abs): for leptin, polyclonal Ab (pAb) A-20 (Santa Cruz Biotechnology, Santa Cruz, CA); for ObR, pAb H-300 (Santa Cruz Biotechnology); for HIF-1α, pAb (Santa Cruz Biotechnology). All primary Abs were diluted in phosphate-buffered saline with 1.5% normal blocking serum. Immunohistochemistry was carried out as described previously [8]. Ab–antigen reaction was revealed with avidin–biotin–peroxidase complex (ABC Staining System) for leptin, and EnVision system for ObR and HIF-1α, and then the slides were counterstained with hematoxylin. In negative controls, primary Abs were omitted. The immunostaining for leptin, ObR and HIF-1α was analyzed in 10 different tumor fields and the mean percentage of tumor cells with positive staining was calculated. The expression of studied proteins was classified using a three-point scale: 0, <10% positive cells; 1+, 10%–50% positive cells; 2+, >50% cells with positive staining.

statistics

The associations among leptin, ObR and HIF-1α were determined using Spearman's correlation analysis. Probabilities of $P < 0.05$ were taken as statistically significant.

leptin and ObR are frequently expressed in human colorectal cancer

The study included colorectal cancers classified histopathologically as adenocarcinoma (117 cases) and adenocarcinoma mucinosum (18 cases); 95 cases in G2 grade and 40 cases in G3 grade. For statistical analyses, because of both clinical staging and relatively small number of pT1, pT2 and pT4 cases, the samples were divided into two groups, pT1 plus pT2 (14 cases) and pT3 plus pT4 (121 cases). In all, 68 of 135 (50.4%) patients had involved lymph nodes at the time of diagnosis.

Positive leptin, ObR and HIF-1α immunostaining were detected in 69 of 135 (51.1%), 129 of 135 (95.5%) and 88 of 135 (65.2%), respectively. Immunohistochemical analysis of colorectal cancer sections revealed cytoplasmatic localization and microgranular staining for leptin and cytoplasmatic/membrane as well as occasionally nuclear staining for ObR. HIF-1α staining exhibited mainly granular cytoplasmatic pattern, with perinuclear concentration; in some cases nuclear HIF-1α immunoreactivities were noted. The expression of studied proteins was undetectable in the control samples when immunostaining was carried out with the omission of primary Abs.

expression of the leptin system correlates with HIF-1α

We found a positive correlation between leptin and ObR (for the group of all patients: $P < 0.001$, $r = 0.426$), except for poorly differentiated tumors, subgroups of mucinous cancers and tumors in pT1 + pT2 stage (Table 1). Statistically significant positive correlation between leptin and HIF-1α was noted in the group of all patients ($P = 0.005$, $r = 0.243$), and in various subgroups as follows: sets of patients with lymph node involvement, more advanced tumors (pT3 + pT4), moderately differentiated (G2) tumors, adenocarcinoma type of tumors, subgroup of women, older patients and in tumors located in rectum (Table 1).

We also found statistically significant linkage of ObR and HIF-1α in the group of all patients ($P < 0.001$, $r = 0.325$) and some subgroups characterized by various clinicopathological features, except for poorly differentiated tumors, subgroups of mucinous cancers and tumors located in the rectum (Table 1).

discussion

Recent data indicated that leptin system might be involved in development and progression of various human malignancies [4–13]. For instance, we observed abundant expression of leptin and ObR in primary and metastatic breast cancer and in endometrial cancer tissue. In these studies, the leptin/ObR system was overexpressed in cancer relative to non-tumor tissues [8–13]. Ishikawa et al. [31] indicated that coexpression of leptin and ObR could play a role in gastric cancer progression via auto- and paracrine mechanisms.

Despite some indications from cellular models, the impact of the leptin system on human colorectal cancer development is not yet clear. Studies on the correlations between serum leptin levels and cancer produced contradictory results [32–35]. However, we found significant leptin overexpression in colorectal cancer biopsies, while relatively low levels of the hormone were identified in normal colorectal epithelium and colorectal adenomas [30]. Thus, it possible that local, rather than endocrine, leptin could exert a crucial role in cancer development and progression.

Here, we provided evidence for statistically significant coexpression of leptin and ObR, indicating that leptin might activate colorectal cancer cells through an autocrine mechanism, similar to that observed in breast and endometrial cancers [8–20]. In addition, we found a positive correlation between leptin and HIF-1α as well as ObR and HIF-1α, which indicates that the expression of the leptin

Table 1. Relationships among leptin, ObR and HIF-1α in colorectal cancer (Spearman's correlation test)

Groups of patients		Leptin–HIF-1α		ObR–HIF-1α		Leptin–ObR	
		P	*r*	*P*	*r*	*P*	*r*
All patients	$n = 135$	0.005	0.243	<0.001	0.325	<0.001	0.426
N	pN (−), $n = 67$	0.119	0.192	0.013	0.311	<0.001	0.511
	pN (+), $n = 68$	0.012	0.303	0.006	0.345	0.003	0.341
T	pT1 + 2, $n = 14$	0.280	0.311	0.010	0.736	0.071	0.516
	pT3 + 4, $n = 121$	0.010	0.234	0.001	0.310	<0.001	0.422
G	G2, $n = 95$	0.033	0.219	0.001	0.342	<0.001	0.467
	G3, $n = 40$	0.553	0.097	0.195	0.224	0.153	0.227
HP	Adenocarcinoma, $n = 117$	0.009	0.240	<0.001	0.338	<0.001	0.425
	Carcinoma mucinosum, $n = 18$	0.584	0.138	0.810	0.068	0.119	0.360
Sex	Male, $n = 72$	0.137	0.177	0.004	0.336	<0.001	0.410
	Female, $n = 63$	0.005	0.349	0.024	0.301	<0.001	0.450
Age	≤60, $n = 44$	0.079	0.268	0.008	0.407	0.001	0.472
	>60, $n = 91$	0.029	0.229	0.006	0.295	<0.001	0.408
Location	Rectum, $n = 62$	0.026	0.283	0.164	0.189	<0.001	0.425
	Colon, $n = 73$	0.093	0.198	<0.001	0.428	<0.001	0.430

Analysis of leptin, ObR and HIF-1α in all patients, or in patients divided into subgroups according to N, lymph node involvement; pT, depth of intramural growth; G, grading of cell differentiation; HP, histopathologic type; sex; age and tumor location.
ObR, leptin receptor; HIF-1α, hypoxia-inducible factor-1α.

system could, at least in part, be HIF-1α mediated. Interestingly, significant relationships between leptin and HIF-1α and ObR and HIF-1α were seen in more advanced subgroups of cancer cases, which could indicate the involvement of the leptin system in some steps of colorectal cancer progression related to hypoxia. On the other hand, lack of correlations among studied proteins in poorly differentiated tumors might indicate leptin-independent growth during the process of dedifferentiation. We noted concomitant expression of the leptin system and HIF-1α in endometrial cancer, but the markers were not correlated with histological grade, and extent of tumor growth (pT) [13].

In conclusion, our current and previous observations as well as the results from other laboratories indicate that HIF-1α may play a role in the progression of various human malignancies, including colorectal cancer via up-regulation of the leptin system.

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