INTRODUCTION

Patients with Barrett’s esophagus (BE) have a 30- to 40-fold increased risk of developing esophageal adenocarcinoma (EAC), with dramatically increased incidence in the recent decades (1, 2). It has been suggested that it may be due to the increasing incidence of BE and obesity in Western populations (3-5). Recent studies have demonstrated that obesity is an independent risk factor for EAC (5, 6). Furthermore, the link between EAC and obesity is stronger than in most other cancers (3). In addition, visceral obesity rather than BMI is associated with BE and increased EAC risk (7). The mechanisms underlying this relationship are not fully elucidated yet, but the role of adipokines as adiponectin and leptin (3, 8).

Current studies have shown the correlation between low blood adiponectin level and the increased risk of endometrial, prostate, renal cell carcinoma, breast, colon, gastric and recently esophageal cancer (9, 10). Adiponectin inhibits inflammation, promotes apoptosis, and suppresses proliferation and angiogenesis (8, 11) via multiple potential signaling pathways (12). One potential mechanism of its action is suppression of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (12). In experimental studies, adiponectin inhibited tumour growth in mice, due to the significant decrease in tumor vascularization and macrophage infiltration increase (13, 14).

Adiponectin is an adipokine that binds to 2 types of receptors, AdipoR1 and AdipoR2 (15). These receptors belong to the class of the 7-membrane receptors. The regulation of adiponectin action can occur via controlling the expression of adiponectin receptors (16, 17). Obesity decreases the expression levels of adiponectin receptors, and/or reduces postreceptor signalling, which may contribute to insulin resistance (18).

Leptin, a product of the (ob) gene (19), in vitro acts as a mitogen in many cell types. As such, it mediates the proliferative response in human prostate cancer cells in vitro via suppression of apoptosis (20, 21). In addition leptin stimulated esophageal adenocarcinoma cell lines proliferation and inhibited apoptosis. These effects were dependent on cyclooxygenase (COX)-2 and replicated by adding prostaglandin E2 (PGE2) (22, 23). Although the expression of COX-2 in BE and EAC is increased, it seems that it is not only the result of the inflammatory process, but may also depend on leptin, which stimulate the expression of COX-2 (24). Leptin acts synergistically with gastric acid to inhibit apoptosis and promote cellular proliferation in Barrett’s-derived esophageal adenocarcinoma cell lines partly through Akt activation (25). The leptin receptor (ObR) is a large single

ADIPONECIN AND LEPTIN RECEPTORS EXPRESSION IN BARRETT’S ESOPHAGUS AND NORMAL SQUAMOUS EPITHELIUM IN RELATION TO CENTRAL OBESITY STATUS
membrane spanning protein that belongs to the gp 130 family of cytokine class I receptors (26, 27). Both leptin and its receptor are highly expressed in the gastric epithelium (28). These receptors have been reported to be expressed in several malignancies, nevertheless little is known about their expression in Barrett’s esophagus.

In the present study adiponectin and leptin and their receptors in Barrett’s esophagus and adenocarcinoma patients have been evaluated and correlated with adiposity parameters.

MATERIAL AND METHODS

The study group embraced 27 patients with Barrett’s esophagus, 14 men and 13 women, aged on average 52 years. Informed consent had been obtained from all the subjects studied and the project had been approved by the local Ethics Committee (RNN/9/09/KE) for research involving human subjects according to the Helsinki agreement.

They underwent endoscopy during the surveillance program due to Barrett’s esophagus at the Department of Digestive Tract Diseases in Lodz between 2009 and 2011. Clinical features of patients studied are shown in Table 1. Twelve controls and 8 morbidly obese controls were enrolled to the study (Table 1). Controls with normal weight did not reveal any pathological changes in endoscopy and were diagnosed with functional dyspepsia. Obese controls required endoscopy prior to the planned gastric by-pass operation.

Patients underwent anthropometric assessment of central obesity parameters. Endoscopic biopsies were obtained at upper gastrointestinal endoscopy from BE (four biopsies) and from normal squamous epithelium (four biopsies) in the middle portion of the esophagus. Biopsies were taken for routine histologic examination every 2 cm in 4 esophageal quadrants and from all suspected visible lesions (29).

Expression of adiponectin receptors 1 and 2 (AdipoR1, AdipoR2) and leptin receptor (ObR) in biopsies from 27 BE and normal squamous epithelium (N) in the same patients was assessed with Western-blot analysis and quantitative RT-PCR (qRT-PCR).

Anthropometry features

In all patients body mass index (BMI) value has been calculated. Waist circumference was measured at the level midway between the lowest rib margin and the iliac crest, and hip circumference was measured at the widest level over the greater trochanters. Thigh circumference was measured on the left leg directly below the gluteal fold. The mean value of 2 measurements was used in the analyses. Waist-to-hip ratio (WHR) was calculated as waist circumference divided by hip circumference, and waist-to-thigh ratio (WTR) was calculated as waist circumference divided by thigh circumference.

Quantification of serum adiponectin and leptin: blood samples have been obtained in the morning after 12 hours fasting from patients of all examined groups and controls before upper gastrointestinal endoscopy.

Blood serum was obtained after 30 minutes clotting and centrifugation at 2000 rpm for 15 minutes at 4°C. Serum was removed and stored frozen at –80°C. Leptin and adiponectin concentrations were measured with ELISA (R&D Systems, USA).

Immunoblotting

Total protein from frozen tissue samples of patients with Barrett esophagus, obese and healthy controls was extracted in RIPA protein extraction buffer (Sigma-Aldrich, St. Louis, MO, USA), supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysate was centrifuged at 14,000 × g and 4°C for 20 min, and the pellet discarded. Protein concentrations were determined by the BCA Protein Assay Kit (Pierce Thermo Scientific, Rockford, USA) according to the manufacturer’s protocol and using bovine serum albumin as a standard. Tissue homogenates were mixed in a 4:1 ratio with 4-fold concentrated NuPAGE LDS Sample Buffer (Life Technologies, Carlsbad, California) and in a 1:10 ratio with NuPAGE Reducing Agent (10X), heated for 10 min at 70°C and 20 μg of protein were loaded per one well. Protein samples were subjected to electrophoresis in 4–12% SDS-NuPAGE Gels (Life Technologies, Carlsbad, California) and in a 1:10 ratio with manufacturer’s protocol and using bovine serum albumin as a standard. Tissue homogenates were mixed in a 4:1 ratio with 4-fold concentrated NuPAGE LDS Sample Buffer (Life Technologies, Carlsbad, California) and in a 1:10 ratio with NuPAGE Reducing Agent (10X), heated for 10 min at 70°C and 20 μg of protein were loaded per one well. Protein samples were subjected to electrophoresis in 4–12% SDS-NuPAGE Gels (Life Technologies, Carlsbad, California) at 200 V, and electrophoretically transferred to a nitrocellulose membrane at 30 V for one hour. The membrane was blocked in 5% non-fat milk in TBST (20 mM Tris-HCL, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 1 hour at 4°C. Then, the membranes were incubated for 12 h at 4°C with the mouse anti- leptin receptor and anti- adiponectin primary antibody (Abbiotech, San Diego, CA, USA) at the dilution 1:400. At the end of the overnight incubation, the membrane was washed with TBST and incubated for one hour in PBST containing the goat anti-mouse IgG

Table 1. Clinical features of patients with Barrett’s esophagus and controls studied.

<table>
<thead>
<tr>
<th>Features</th>
<th>Mean values (range)</th>
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<td>Patients with</td>
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<td></td>
<td>Barrett’s esophagus</td>
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<tr>
<td>Male/Female ratio</td>
<td>14/13</td>
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<tr>
<td>Age (yrs)</td>
<td>52 (25–78)</td>
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<tr>
<td>Weight (kg)</td>
<td>78 (52–120)</td>
</tr>
<tr>
<td>BMI (body mass index kg/m²)</td>
<td>27 (18–35)</td>
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<tr>
<td>WHR (waist-to-hip ratio)</td>
<td>0.9 (0.79–1.1)</td>
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<tr>
<td>WTR (waist-to-thigh ratio)</td>
<td>1.69 (0.98–2.0)</td>
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<tr>
<td>Waist circumference (cm)</td>
<td>96 (76–123)</td>
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secondary antibodies conjugated with alkaline phosphatase (Merck Millipore, Billerica, USA). After incubation with secondary antibodies, the membrane was washed three times (3×5 min) in PBST buffer. The band was developed using BCIP/NBT Alkaline Phosphatase Substrate (Merck Millipore, Billerica, USA). Densitometric analysis of bands was performed with ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, Bethesda, MD) and the results were expressed as optical density (OD).

Real time-PCR analysis

Samples were obtained fresh from esophageal biopsy immediately frozen and stored in 1.5 ml Eppendorf’s tubes at –70°C until RNA extraction. In order to isolate mRNA, samples (size 1×1 mm to 3×3 mm, no more than 30 mg weight) were cut using chirurgical scalpel on sterile Petri dish. Afterwards, samples were homogenized via sonification on ice (4×15 s), and centrifuged (10,000 × g, at 4°C); supernatant was discarded. RNA isolation was performed using QIAshredders and RNeasy Mini Kit (Qiagen, Hiden, Germany) according to the manufacturer’s instructions. Concentration of RNA obtained was measured using BioPhotometer (Eppendorf, Hamburg, Germany).

Reversed transcription utilizing Im-Prom-II™ Reverse Transcription System (Promega, Madison, WI, USA), was performed in order to obtain cDNA for further analysis. Gene expression was measured using real time PCR and TaqMan® Gene Expression Assays Systems (Applied Biosystems) for leptin (Hs00174497_m1) and adiponectin receptors (Hs01115874_m1 for receptor type 1 and Hs01047564_g1 for receptor type 2). Relative gene expression was normalized to GAPDH transcripts and calculated as a fold change compared to control. The results were expressed as gene copy number/GAPDH mRNA ratio 2–ΔΔCt.

Statistical analysis

To determine the differences between groups Student’s t-test was used. Variables exhibiting a heavily skewed distribution were compared between groups using Mann-Whitney test. Data were analyzed by Wilcoxon matched pair test. All statistical analyses were performed using STATISTICA for Windows.
release 6.0 software. \( P<0.05 \) was used as the definition of statistical significance.

RESULTS

AdipoR1 and ObR protein levels were similar in BE mucosa and squamous epithelium in the same patients in Western-blot analysis (2303 vs. 2448 OB units; 106927 vs. 103390, respectively; \( p=0.05 \)) (Fig. 1). We have confirmed these results with RT-PCR analysis which revealed that mean expression of AdipoR1 and AdipoR2 genes were also similar in metaplastic and squamous epithelium in Barrett’s esophagus patients (0.11±0.08 vs. 0.19±0.24, \( p=0.78 \); 0.24±0.36 vs. 0.33±0.49, \( p=0.5375 \), respectively). Mean expression of ObR gene was also similar in metaplastic and squamous epithelium in Barrett’s esophagus patients (0.71±0.8 vs. 1.33±2.95, \( p=1.0 \)).

Using linear correlation analysis we found that there is a positive correlation between AdipoR1 gene expression in Barrett’s mucosa compared to normal mucosa in the same patients (\( r=0.5; p=0.008 \) and also between ObR gene expression in BE and N (\( r=0.8; p=0.001 \)) (Fig. 2).

This association between AdipoR1 protein level in Barrett’s mucosa compared to normal mucosa in the same patients and also between ObR protein level in BE and N was also seen using Western-blot analysis, however increasing number of cases would help achieve statistical significance (\( r=0.73, p=0.06 \) for AdipoR1; \( r=0.75, p=0.77 \) for ObR).

On the other hand AdipoR1 and AdipoR2 proteins expression were significantly higher in BE patients, both in BE mucosa and in squamous epithelium in BE patients, compared to controls (895 and 76646, respectively, \( p<0.05 \)) (Fig. 1). Leptin receptor protein level was also significantly higher in BE patients compared to obese controls (70254, \( p<0.05 \)) (Fig. 1).

The mean serum adiponectin level was 6325.3 (range 815–17136)/ml in BE patients. The mean serum leptin level was 15.3 (range 2.64–52.54) ng/ml in BE patients. The expression of AdipoR1, R2 and ObR was not correlated significantly with circulating serum adipokines level (respectively: CC= –0.122; 0.342; 0.155; \( r=0.571; 0.13; 0.58 \)).

\[ \text{Fig. 2. Correlation between adiponectin (A) and leptin (B) receptors genes expression in Barrett’s esophagus and normal mucosa. AdipoR1 – adiponectin receptor 1; ObR – leptin receptor; BE – Barrett’s esophagus.} \]
There was the trend to the negative correlation (almost statistically significant) between AdipoR1 expression in BE mucosa samples and mean weight and BMI (CC= −0.37, r = 0.075 and CC= −0.36, r = 0.081, resp.).

**DISCUSSION**

Mean protein levels of receptors for adiponectin and leptin as well as their genes expression were similar in BE compared to normal squamous epithelium from the same patients. Till now, only in one study the expression of adiponectin receptors was assessed in patients with Barrett’s esophagus. Konturek et al. showed a significant downregulation of both receptors in BE mucosa compared to normal squamous esophageal mucosa in the same patients (17). Authors demonstrated this mechanism in 15 obese patients (17). However, obesity alone may be associated with downregulation of adiponectin receptors as stated in previous studies (18). This was confirmed by our observation of low adiponectin receptor protein level in morbidly obese controls.

Similarly to our results, Fritz et al. have shown that leptin receptors were expressed with similar density and staining pattern in normal esophageal mucosa, inflamed mucosa and Barrett’s mucosa (19). Thus, Fritz et al. suggested that differential leptin receptor expression is not a major factor associated with the development of esophageal pathology (19). However, authors did not compare this receptor expression to healthy controls. We have confirmed that leptin receptor protein level was significantly higher in BE patients compared to controls.

In the literature, expression of adipokines receptors were assessed in many cancers tissue and compared to adjacent tissue, providing often inconsistent results. No difference in the expression of adiponectin receptors between colon cancer and normal counterparts (30) as well as between renal cell carcinoma and adjacent healthy kidney tissue (31) was reported. In Otani et al. study the expression level of both adiponectin receptors tended to be decreased in cancer tissue as compared to normal sites, however, only for AdipoR2 the difference was statistically significant (32). On the other hand, some studies showed higher levels of adiponectin receptors in cancer versus nontumor tissue (33-35).

It is unknown if squamous epithelium in BE patients could have some special, maybe neoplastic, properties, as it shows the similarity to metaplastic epithelium, at least in adipokynes receptors expression. Previous studies have shown that there are some properties of squamous epithelium in BE patients which allow them to heal through metaplasia, not squamous cells regeneration, as they heal in GERD (36, 37). This could be also explain by the “field cancerization” effect, which means the existence of genetic alterations in histologically normal tissue surrounding malignant tumors (38, 39).

Carcinogenic field effect was detected for more than 50% of 23 genes analyzed in histologic normal squamous esophageal epithelia from patients with Barrett’s esophagus (38). Genetic changes present in normal appearing cells could be a marker for identification of individuals at risk of developing cancer for surveillance and primary endoscopic or chemo-prevention (39).

In current study, we have revealed higher adiponectin receptors expression in BE patients compared to healthy controls. High expression of adiponectin receptors in cancer tissue may promote the anticarcinogenic effect of adiponectin (40). Serum hipoaiponectinaemia in BE was shown in previous studies (12, 41, 42). The possible mechanism of adiponectin receptors upregulation could follow low serum levels in BE patients. However, similarly to previous reports (19, 43), we did not find the relationship between receptors expression and adipokines serum levels.

Earlier we have demonstrated that serum leptin concentration was slightly higher in BE patients compared to GERD and to the controls (42). The exact role of leptin in clinical background is still controversial and its effect in clinical setting could be more complicated. Similarly to our findings, in Francois et al. study (19) plasma leptin level did not differ significantly in controls, GERD and Barrett’s patients. In study of Kendall et al. (44) high serum leptin was associated with an increased risk of BE, but only in men. This was not connected to the higher body mass or GERD among patients. Still there are no studies fully confirming, that high serum leptin is a risk factor for BE development. Furthermore, despite of leptin proliferative effects, it could play protective role for the gastrointestinal mucosa. Recent findings have suggested that the physiological role of leptin may depend on the context of other host or environmental factors (19). Leptin may have autocrine and paracrine (rather than endocrine) interactions with its receptor, as has been suggested in gastric, breast and colorectal cancers (10, 45, 46).

It was observed that the expression of leptin and its receptor in cancer varies with different histological origins, and even within a tumor of the same origin. In some cases of ovarian cancer, cancers of the kidney and testis both expression of leptin and its receptor were seen, suggesting autocrine action. In other cases of ovarian cancer, breast cancer, and bladder cancer, the expression of the leptin receptor was observed in the absence of expression of leptin, suggesting a paracrine or systemic effect of the cytokine on tumor cells (47). However, higher leptin receptor expression in BE patients compared to controls could suggest that leptin, maybe only partially, acts via its receptor during BE development.

In addition the correlation between of adipokines receptors expression and anthropometry and obesity status have been assessed.

Lower leptin and adiponectin receptor 1 protein levels were observed in obese controls compared to BE patients. There were no differences between morbidly obese and controls with normal weight. It could lead to hypothesis that obesity has no influence on adipokines receptors status. However, we did not assessed adipokines receptors in morbidly obese BE patients to confirmed this hypothesis. Among our BE patients, who were of normal body mass and overweight, relative downregulation of AdipoR2 was significantly correlated with longer mean waist circumference and WTR. Furthermore, there was a trend toward a lower AdipoR1 expression in obese patients although the difference was not statistically significant. Chou S. et al. (31) found increased expression of AdipoR1 in cancers strongly associated with obesity (kidney, skin, bladder cancers) compared to cancers not associated with obesity (ovarian epithelial, cervical, adenocortical carcinomas). Thus high AdipoR1 expression in Barrett’s esophagus, which leads to obesity-related esophageal adenocarcinoma, found in our study is intelligible.

Nevertheless, among BE patients we did not find the relationship between ObR receptor and obesity parameters. Similarly, Ishikawa et al. did not find the relationship of BMI with leptin and ObR expression (10). The mechanism of leptin resistance has been under recent investigation. In human obesity, leptin concentrations are elevated but there is reduced responsiveness to available leptin presumably from reduced leptin receptor expression, resulting in leptin resistance either centrally or locally at the level of the liver (10).

Moreover, leptin receptor is under influence of gastric leptin, not plasma leptin which levels reflect fat stores (19). Since gastric leptin level do not reflect BMI, rather represents local effects, it may explain why we did not find an association between leptin
receptor expression and central obesity parameters. Further studies are needed, particularly in obese BE patients, to establish the exact role of obesity in BE development.

In conclusions, we speculate that adipocytokines and their receptors could be relevant in the initial step of BE development. The correlation between the adipokines receptors expression in BE epithelium and squamous epithelium in the same patients could support the hypothesis about specific carcinogenic potential of this endoscopically normal macusa. Future studies should be performed to establish the role of squamous epithelium in patients with Barrett’s esophagus. Because of still poor prognosis in esophageal adenocarcinoma and only partially effectiveness of current targeted therapies and other treatment options, new pharmacological modalities are urgently needed (23, 48). Adiponectin or adiponectin analogues may be effective anticancer agents and may have important therapeutic implications (49). It was suggested that increasing the adiponectin level may be a new strategy for the prevention of colorectal cancer at an early step of carcinogenesis (50).

A recent study on mice has shown that the leptin receptor antagonist caused the extension of survival time in the case of triple-negative breast cancer (48). Nevertheless, the data about adipocytokines receptors in Barrett’s esophagus and esophageal adenocarcinoma in vivo are scarce in the literature. The long term and regular physical activity was proven to decrease leptin level in training persons, which may be a practical guidance for preventing cancer in patients at increased risk (51). Further elucidation of the mechanisms of action and regulation of adiponectin and leptin has the potential to create novel and powerful targets for developing intervention strategies for obesity-related disorders.

Acknowledgements: The work was supported by Medical University of Lodz; contract grant number: 503-1-002-01/503-01 and 502-03/1-002-01/502-14-043.

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Received: November 18, 2012
Accepted: March 26, 2013

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