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Leptin synergistically enhances the anti-apoptotic and growth-promoting effects of acid in OE33 oesophageal adenocarcinoma cells in culture☆

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Abstract

Obesity and gastro-oesophageal reflux are the main predisposing factors for oesophageal adenocarcinoma. We have examined the effects of transient acid exposure and leptin on OE33 oesophageal adenocarcinoma cells. Leptin and acid individually stimulated proliferation and inhibited apoptosis and the combination was synergistic. Leptin receptor protein levels were unchanged by acid exposure. The COX-2 inhibitor NS 398 blocked the effects of acid and leptin but while both acid and leptin individually significantly increased PGE2 production and COX-2 mRNA levels, the combination was not more effective than either stimulant alone. Leptin synergistically enhanced acid-stimulated EGFR and ERK phosphorylation but did not further increase JNK or p38 MAP kinase phosphorylation. Specific EGFR and ERK inhibitors reduced the effects of leptin and acid alone and in combination. The combination of increased circulating leptin levels in obesity and transient reflux of gastric acid may promote oesophageal carcinogenesis by increasing proliferation and inhibiting apoptosis.

Keywords: Apoptosis; Akt; Barrett's oesophagus; Cyclo-oxygenase; Epidermal growth factor receptor; Leptin; Mitogen-activated protein kinase; Oesophageal cancer

1. Introduction

Oesophageal adenocarcinoma (OAC) is an increasing problem in the developed world (Buttar and Wang, 2004). Although the incidence of squamous cancer of the oesophagus is relatively static, the age-standardised incidence of OAC has increased significantly over the last 30 years (Jemal et al., 2006). The reasons for this increase as well as the molecular pathology of OAC are not fully understood, but because of the generally poor prognosis for this cancer improved understanding of the causes is essential to target preventative and treatment strategies.

Oesophageal adenocarcinoma arises in most, if not all cases, from metaplastic columnar oesophageal epithelium (Barrett's oesophagus (BO)) and all the factors which drive the transformation remain to be elucidated. Increased proliferation and reduced apoptosis of the Barrett's epithelium are believed to be important in the progression of OAC; the increased cell division and reduced cell death probably allow accumulation and perpetuation of genetic abnormalities leading to malignant transformation. Increased proliferation in BO is associated with an increased risk of progression to OAC (Buttar and Wang, 2004). Because of this it is important to determine the factors which drive proliferation and inhibit apoptosis in OAC and BO.

The two main epidemiological risk factors for OAC are obesity (Lagergren et al., 1999b) and symptomatic gastro-oesophageal reflux (Lagergren et al., 1999a). These factors appear to be independent of each other. Experimental studies

Abbreviations: BO, Barrett's oesophagus; COX, cyclo-oxygenase; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; OAC, oesophageal adenocarcinoma; ELISA, enzyme-linked immunosorbent assay; EGFR, epidermal growth factor receptor; ERK, p42/44 extracellular signal regulated kinase; FBS, foetal bovine serum; G-Gly, glycine-extended gastrin; JAK, Janus tyrosine kinase; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-y-l]-2,5-diphenyltetrazolium bromide; NSAIDs, non-steroidal anti-inflammatory drugs; p38 MAP kinase, p38 mitogen-activated protein kinase; PBS, phosphate buffered saline pH 7.4; PGE2, prostaglandin E2; PI3-kinase, phosphatidylinositol 3′-kinase

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have shown than transient exposure of Barrett's oesophageal cells and oesophageal adenocarcinoma cells to acid stimulates proliferation and inhibits apoptosis: as yet the exact mechanisms underlying these effects are unclear but growth promotion and inhibition of apoptosis by acid have been described using transformed and non-transformed cell lines and biopsy explants (Souza et al., 2002; Fitzgerald et al., 1996; Kaur and Triadafilopoulos, 2002; Sarosi et al., 2005). Activation of cellular signalling events including the mitogen-activated protein (MAP) kinase cascades, activation of protein kinase C, the Na^+/H^+ exchanger and upregulation of cyclo-oxygenase-2 (COX-2) and prostaglandin (PG) production have all been implicated in the biological responses to acid, although as yet there is no consensus about how these pathways integrate together to regulate the responses to acid (Buttar and Wang, 2004; Souza et al., 2002, 2004; Sarosi et al., 2005; Kaur and Triadafilopoulos, 2002).

The mechanisms linking obesity to OAC progression are likely to be multi-factorial but we have hypothesized that increased serum leptin levels may provide a direct link. Leptin is a peptide hormone secreted by adipocytes and serum levels are proportional to body fat mass (Considine et al., 1996). Effects of leptin as a regulator of appetite and body weight are well described but we have recently reported that leptin, at the concentrations seen in obese patients, is a potent growth factor for Barrett's oesophageal adenocarcinoma cells. Using the OE33 cell line we showed that leptin stimulated increased COX-2 mRNA expression and bioactivity via activation of the extracellular signal-related kinase (ERK), p38 MAP kinase and Akt pathways. Subsequently COX-2-derived PGE2 stimulated cell growth via EP-4 receptor-mediated transactivation of the epidermal growth factor receptor (EGFR) with downstream activation of c-jun-NH_2-terminal kinase (JNK) (Ogunwobi et al., 2006).

Because both acid exposure and leptin individually adversely affect Barrett's epithelial cells in a manner than would be expected to further promote oesophageal carcinogenesis, we have hypothesized that leptin and acid exposure may interact directly at the cellular level in OAC to promote growth and/or inhibit apoptosis. We have examined the mechanism of these interactions using the OE33 Barrett's oesophageal adenocarcinoma cell line. This has been used previously as an effective model for studying signalling events in Barrett's OAC and in most cases seems a reasonable model for extrapolating to non-transformed BO (Ogunwobi et al., 2006; Haigh et al., 2003; Tselepis et al., 2003). In particular we have examined the interaction at the level of early signalling events stimulated by leptin and acid responsible for transducing the growth promoting signals. We have concentrated on the MAP kinases, which have been shown to be upregulated in BO and activated by acid, COX-2 and prostaglandin production, which have been reported to be important factors in the progression of OAC and the epidermal growth factor receptor, which has also been implicated in BO and OAC (Jankowski et al., 1993; Brito et al., 1995; Wilson et al., 1998).

2. Methods

2.1. Cell culture

The OE33 Barrett's adenocarcinoma-derived cell line was cultured and subcultured as previously described (Ogunwobi et al., 2006). All cell culture media and supplements were from Invitrogen (Paisley, UK).

2.2. Exposure to acid and leptin

Serum-starved cells were exposed to serum-free DMEM at pH 4.0 for 3 min at 37 °C to stimulate transient gastro-oesophageal reflux (Souza et al., 2002). Control cells were treated with standard serum-free DMEM. After the 3 min the medium was changed to fresh serum-free medium supplemented where appropriate with leptin.

2.3. 3-[4,5-Dimethylthiazol-2-y-l]-2,5-diphenyltetrazolium bromide (MTT) assay

OE33 cells were seeded at 5×10^4 cells per well in 48-well plates in 10% FBS containing medium and allowed to attach for 24 h. Cells were then serum-starved for 24 h. Cells were treated with inhibitors, acid and/or leptin and relative cell numbers after 24 h determined using the MTT colorimetric assay as previously described (Beales, 2004; Ogunwobi et al., 2006).

2.4. Bromodeoxyuridine (BrdU) incorporation assay

OE33 cells were prepared as described previously and after serum-starving for 24 h cells were then treated with acid and/or leptin and incubated for a further 24 h. DNA synthesis and relative cell proliferation were assessed using the BrdU incorporation assay (Roche, Mannheim, Germany) (Ogunwobi et al., 2006).

2.5. Assessment of apoptosis

Cells were prepared as for the MTT assay and the serum-starved cells were treated with inhibitors, leptin and/or acid exposure and after 24 h, the cell death ELISA kit (Roche) was used to quantify intracellular nucleosomes as a measurement of apoptosis as described previously (Ogunwobi and Beales, 2006).

Caspase-3 activity was quantified in OE33 cell lysates 24 h after treatment with acid and/or leptin using the caspase-3 colorimetric assay (R&D systems, Abingdon, UK) according to the manufacturer's instructions.

2.6. Western blotting

Lysates from confluent OE33 cells in 10 cm Petri dishes were prepared and subjected to electrophoresis and western blotting as described (Ogunwobi et al., 2006). Anti-leptin receptor (anti-Ob-R) antibody (H-300: sc-8325, Santa Cruz Biotechnology, CA, USA) was used at 1:200 to detect leptin receptor proteins. Relative density of staining was quantified using the NIH Image for PC image analysis software (Scion Image, freely available from http://www.scioncorp.com).

2.7. COX-2 mRNA assay

1×10^6 cells per well were seeded into 12-well plates and cultured in complete culture medium for 48 h. After 24 h serum-starvation cells were treated with inhibitors and acid–leptin. Four hours after stimulation the media was aspirated and cells lysed with Cell Lysis Reagent (R&D Systems). COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the cell lysates were measured as described previously using Quantikine™ mRNA ELISA (R&D Systems) according to the manufacturer's instructions (Ogunwobi et al., 2006).