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Review

A role of ghrelin in cancerogenesis

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Abstract

Ghrelin is a 28 amino-acid multi-functional peptide hormone, which was identified as a natural ligand of the growth hormone secretagogue receptor (GHS-R). Pituitary growth hormone-releasing activity in both animals and humans has been well documented. It has various biological functions, including regulation of appetite and body weight, control of energy homeostasis, modulation of cardiovascular and gastrointestinal system and anti-inflammatory effect.

However, both ghrelin and its receptor (GHS-R) are widely distributed in various tumors, which strongly implies their role in neoplastic cell growth trough autocrine/paracrine mechanism. Multiple studies have demonstrated the role of ghrelin in cancer cells proliferation, differentiation, invasiveness and apoptosis inhibition.

The ghrelin axis is more complex than it was originally thought and consist of several compounds that might interact with each other and affect ghrelin activities. Here, we provide an overview of the ghrelin and its receptor role in tumor progression.

Key words: ghrelin, growth hormone secretagogue receptor, cancer, canine mammary cancer

Introduction

Ghrelin was isolated from the human and rat stomach by Kojima et al. in 1999 as an endogenous ligand for growth hormone secretagogue receptor type 1a (GHS-R1a). The GHS-R activation considerably augments growth hormone (GH) secretion, which is the main effect of ghrelin activity. Ghrelin is highly conserved among various species, particularly ten amino acids at the N terminus. It shows significant homology to motilin (Kojima et al. 1999, Kojima et al 2001).

Ghrelin is mainly produced by the submucosal layer of the stomach by endocrine X/A-like cells. It is secreted directly into the blood, as these cells are situated close to vascular capillaries (Date et al. 2000). However, the presence of ghrelin has been also determined in other areas of the gastrointestinal tract, as well as in the central nervous system (Cowley 2003). Furthermore, ghrelin has been also found in the lung, heart, lymphatic tissue, endocrine pancreas, adrenal cortex, kidney, testis, ovary, placenta, thyroid and parathyroid glands, bone, adipose tissue, prostate and immune cells (Hattori et al. 2001, Gnanapavan et al.

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2002, Fukushima et al. 2005, Raghay et al. 2006, for review see: Leite-Moreira and Soares 2007). Moreover, ghrelin has been identified in a variety of cancer tissues and related cancer cell lines (Korbonits et al. 2001, Cassoni et al. 2004, Gaytan et al. 2004, Jeffery et al. 2005, Raghay et al. 2006, Ekeblad et al. 2007, Wasco et al. 2008, Alnema et al. 2010).

Ghrelin is not only one peptide

Ghrelin is derived from polypeptide precursor composed of 117 amino acids (AA) called pre-proghrelin, which is encoded by *GHRL* gene. At first, a signal peptide of 23 aminoacids is cleaved from pre-prohormone to yield proghrelin (94 AA). Then, the proghrelin undergoes proteolytic process which results in the mature ghrelin (28 AA) and the C-terminal propeptide C-ghrelin (66 remaining AA) production. C-ghrelin may be further cleaved to discharge obestatin (23AA) and/or other peptides (Pemberton et al. 2008, Seim et al. 2010).

Mature ghrelin is subjected to a post-translational octanoic acid esterification of the third amino acid, Serine. However, the other types of esterification have been also observed (Hosoda et al. 2006). This unique modification is required for activation of GHS-R type 1a and further pituitary GH releasing (Bednarek et al. 2000).

Approximately 10-20% of the circulating ghrelin is n-octanoylated and might be transported across the blood-brain barrier to stimulate GH-secretion. Contrary, the remaining 80-90% of the circulating ghrelin is non-octanoylated. This isoform is called des-acyl ghrelin (alias des ghrelin, unacylated ghrelin, or UAC) and may be unacylated form of ghrelin or desoctanoylated form of acyl-ghrelin (Hosoda et al. 2006). The proteolysis of acylated ghrelin has not been reported in human or rat sera so far, but occurs in tissue homogenates (De Vriese et al. 2004).

In spite of des-acyl ghrelin is a major form of the circulating hormone, this form of ghrelin is unable to activate GHS-R1a in the pituitary and thus, does not influence the GH-release and food intake (Hosoda et al. 2006, Neary et al. 2006). Therefore, des-acyl ghrelin was thought to be inactive form of the hormone. However, the recent studies have identified biological activity of this peptide which is independent of GHS-R1a (Broglio et al. 2004, Delhanty et al. 2006). Des-acyl ghrelin promotes adipogenesis, similarly to acyl ghrelin (Thompson et al. 2004), and inversely affects glucose output by primary hepatocytes (Gauna et al. 2005). Thus, it is hypothesized that there is an alternative ghrelin receptor (Thompson et al. 2004).

The alternative splicing of the *GHRL* may lead to various peptides production (des-Gln^{14}ghrelin occurring in rat, exon 3 or exon 4 delated pre-proghrelin variant and trincated C-ghrelin) (for review see: Hosoda 2000). These peptides have been found in human prostate and breast cancer cell lines (Jeffery et al. 2005). Thus, their potential role in tumorigenesis is suspected.

The complicated ghrelin biosynthesis shows that this peptide is the "one piece of a puzzle" which contains many other peptides obtained from alternative splicing of the same gene or from extensive post-translational modifications.

Structure of the ghrelin receptor

Ghrelin acts via the specific receptor (GHS-R), which belongs to G protein coupled receptor family. GHS-R has two splice variants: functional type 1a, which contains seven transmembrane domains and truncated type 1b, composed of only the first five transmembrane domains. It arises from an alternative splicing. GHS-R1a is a specific receptor for ghrelin, whereas the function of the 1b type of receptor is still unclear (Howard et al. 1996, Gnanapavan et al. 2002).

GHS-R1a expression has been demonstrated in wide range of tissues, including: central nervous system (mainly: hypothalamus, thalamus, hippocampus, cortex, regions of appetite control, food intake and energy homeostasis), thyroid and parathyroid glands, pancreas, spleen, myocardium, cardiovascular system, adrenal glands, kidney, ovaries, testis and prostate (Gnanapavan et al. 2002, Gaytan et al. 2004, Raghay et al. 2006, for review see: Leite-Moreira and Soares 2007).

GHS-R1b is also widely distributed in various tissues but interestingly it is also over expressed in many tumors (Barzon et al. 2005, Jeffery et al. 2005, Takahashi et al. 2006). Although this form of receptor was regarded as non-functional it may play a role in turmorigenesis. It may modulate the function of ghrelin-GHS-R axis presumably by increasing internalisation of GHS-R1a. Moreover it can act as a dominant-negative mutant of GHS-R1a and transform its signaling (Leung et al. 2007).

Multiple studies have suggested the occurrence of other ghrelin receptors, such as: receptor for des-acyl ghrelin or common receptor for ghrelin and des-acyl ghrelin (Muccioli et al. 2004, Thompson et al. 2004, Gauna et al. 2005, Delhanty et al. 2006). This problem is still unclear especially in the face of fact that some researchers suggest that des-acyl ghrelin might also act through GHS-R, which was ruled out so far (Gauna et al. 2007).

Wide range of possible ghrelin effects

The main function of ghrelin is the stimulation of pituitary hormones secretion (mainly GH), which was reported in both *in vivo* and *in vitro* studies. This activity is even stronger than the effect of growth hormone releasing hormone (GHRH). However, high concentrations of ghrelin demonstrated lack of specificity by elevating adrenocorticotropic hormone (ACTH), cortisol, and prolactin (PRL) levels as well as GH. Another important role of ghrelin is an energy homeostasis control and stimulation of appetite and food intake (Hosoda et al. 2006). During the starvation, ghrelin is thought to activate hypothalamic neurons to promote the resumption of feeding. Thus, ghrelin is one of the factors responsible for the long-term regulation of body weight. Moreover, it also modulates gastrointestinal functions. Ghrelin stimulates gut motility, accelerates gastric emptying, increases gastric acid secretion and causes a gastroprotective effect against stress-, ethanol-, cysteamine-induced ulcers (for review see: Peeters 2007). Recent studies have revealed that ghrelin also modulates cardiovascular functions. Indeed, intravenous administration of ghrelin decreases mean arterial pressure in humans, without any changes in heart rate (Nagaya et al. 2001). Ghrelin may improve cardiac contractility and left ventricular function (increasing cardiac output) in chronic heart failure. Moreover, it inhibits apoptosis of cardiomiocytes and endothelial cells *in vitro* (Isgaard and Johansson 2005). Ghrelin also inhibits apoptosis of osteoblasts, which are responsible for the deposition of new bone matrix. Even 70% of them undergo apoptosis during bone remodeling process, thus ghrelin may play a role in human osteoporosis pathogenesis (Kim et al. 2005). Ghrelin has also an anti-inflamatory effect. It reduces production of proinflammatory cytokines in human endothelial cells, T cells and monocytes. Ghrelin might oppose inflammation of the cardiovascular system (for review see: De Vriese and Delporte 2007).

Nevertheless, ghrelin plays various roles in other physiological processes including glucose and lipid metabolism, regulation of reproductive functions as well as embryo development and implantation, or modulation of pulmonary functions (for review see: Leite-Moreira and Soares 2007).

The role of ghrelin in cancer

The ghrelin expression has been described in several endocrine and non-endocrine tumors and related cell lines in humans, such as: thyroid follicular cancer and parathyroid adenomas (Volante et al. 2003, Raghay et al. 2006), pituitary adenomas and other neuroendocrine tumors (Kim et al. 2001, Korbonits et al. 2001, Wasko et al. 2008), oral squamous cell carcinoma (Alnema et al. 2010), gastric carcinoids and colon cancer (Papotti et al. 2001, Waseem et al. 2008), pancreatic-related endocrine tumors (Duxbury et al. 2003, Ekeblad et al. 2007), renal carcinoma (Dagli et al. 2009), bronchial carcinoid (Arnaldi et al. 2003), testicular and ovarian tumors (Gaytan et al. 2004, 2005), adrenocortical tumors (Barzon et al. 2005, Delhanty et al. 2007), prostate cancer (Cassoni et al. 2004, Jeffery et al. 2005), and breast cancer (Cassoni et al. 2001, Jeffery et al. 2005). Moreover, in these tissues the n-octanoylated ghrelin and des-acyl ghrelin concentrations might reach even higher levels than those in the blood, through local production.

The majority of these ghrelin-producing neoplasms and related cell lines express also GHS-R1a and/or GHS-R1b or other specific binding sites, which may differ from classic receptor but can recognize octanoylated ghrelin, des-acyl ghrelin as well as synthetic peptidyl and non-peptidyl GH secretagogues.

The coexpression of ghrelin and its receptor in various tumors and cancer cell lines may indicate their autocrine/paracrine role in the tumor development. Thus, the role of ghrelin in cancer cells has been investigated in multiple experiments *in vitro*, however the results are confusing. Some investigators have shown ghrelin as antiproliferative factor, but the others have found it as a tumor development promoting factor which stimulates cancer cell proliferation. Probably the effect of ghrelin depends on its concentration and cell type (Table1).

Cassoni and co-workers were the first who described a negative impact of ghrelin on cell proliferation in multiple study on thyroid, breast, lung and prostate cancer cells (Cassoni et al. 2000, Cassoni et al. 2001, Ghe et al. 2002). The authors found that ghrelin as well as synthetic peptidyl growth hormone secretagogues (hexarelin and their analogues) inhibited cell growth. Moreover, this effect was supposed to be reached through the activation of specific binding sides other than GHS-R1a, because the examined cancer cell lines did not demonstrate the expression of GHS-R1a. Similar effect was obtained by Volante et al. (2003) who have confirmed antiproliferative effect of ghrelin in thyroid cancer cell line. Interestingly, the ghrelin binding activity was greater in well differentiated papillary and follicular carcinomas than in poorly differentiated carcinomas, anaplastic carcinomas or non-tumoral thyroid parenchyma or follicular adenomas. The ghrelin effect was depended not only on the grade of the tumor cell differentiation but also on its concentration and exposure time. Cassoni et al. (2004) have shown various

Table 1. The effect of ghrelin on proliferation of various cancer cell lines.

Stimulation of proliferation	Inhibition of proliferation
– human hepatocellular carcinoma cell line (HepG2 cells) – Murata et al. 2002 – human oestrogen-dependent and independent breast cancer cell lines -Jeffery et al. 2005 – human prostate cancer cell lines – Jeffery et al. 2005 – rat pituitary cell line – Nanzer et al. 2004 – human pancreatic adenocarcinoma cell lines – Duxbury et al. 2003 – human adrenocortical carcinoma cell line – Delhanty et al. 2007 – human colon cancer cell lines – Waseem et al. 2008	– human thyroid cancer cell line – Cassoni et al. 2000 – human nonendocrine lung carcinoma cell line – Ghe et al. 2001 – human oestrogen-dependent and independent breast cancer cell lines – Cassoni et al. 2001 – human prostate cancer cell lines – Cassoni et al. 2004 – human thyroid cancer cell line – Volante et al. 2003 – human adenocortical carcinoma cell line – Barzon et al. 2005

dose-dependent effect of ghrelin treatment on prostate cancer cells.

Contrary to the reports mentioned, the pro-proliferative role of ghrelin has been also documented. In 2002, Murata and co-workers described the positive impact of ghrelin on cell proliferation in hepatoma cell line through insulin-like signaling. The group of Jeffery (Jeffery et al. 2002, Jeffery et al. 2005, Yeh et al. 2005) have found that much lower doses of ghrelin, which encompasses normal circulating ghrelin levels, may stimulate breast and prostate cancer cells proliferation. All the examined cancer cell lines demonstrated pre-proghrelin and GHS-R1a expression and moreover, prostate cancer cell line secreted mature ghrelin into the medium. The potential tumor-promoting role of ghrelin was supported by Delhanty and co-workers (2003) who revealed that ghrelin increases proliferation rate in adrenocortical cancer cell lines. It seems an interesting fact that cancer cells showed very low, if any, expression of GHS-R1a, however were still capable to respond to ghrelin treatment, which was proved by blocking the effects of ghrelin on proliferation using the GHS-R1a antagonist [d-Lys3]GHRP6.

To resolve doubts in the role of ghrelin in cell proliferation, Delhanty and co-workers (2007) examined the cell cycle. This study demonstrated that although ghrelin did not change the proportion of cells in the G0/G1, S or G2/M phases, it caused a decrease in the number of apoptotic cells in sub-G1 phase. Thus, they hypothesized, that ghrelin may be the antiapoptotic factor. They confirmed these results and showed that both acylated and des-acylated ghrelin increased cell growth through reduction of apoptotic rate (Delhanty et al. 2007). The antiapoptotic activity of ghrelin has been shown also in colonic cancer cells (He et al. 2011) as well as in many other types of cells like adipocytes (Kim et al. 2004), osteoblastic cells (Kim et al. 2005), or human endothelial cells (Xiang et al. 2011).

The role of ghrelin in cancerogenesis – new possibilities

The role of ghrelin in cell proliferation and apoptosis has previously been described, however several studies implicate that ghrelin may influence the cancer cells motility or ability to metastasis. Duxbury and co-workers (2007) investigated the role of ghrelin in metastatic potential of poorly differentiated human pancreatic cancer cells. They found that ghrelin increased not only the proliferation rate of cancer cells but also cellular invasiveness (even up-to 60%). Another study showed the colorectal cancer cells proliferation and promotion of invasion by ghrelin in an autocrine and paracrine manner. This effect was almost completely abolished when the cells were pre-treated with either GHS-R antagonist (D-[Lys3]GHRP6) or a neutralizing ghrelin – specific antibody (Waseem et al. 2008).

Furthermore, Dixit et al. (2006) analyzed the impact of ghrelin on brain cancers. They found that ghrelin treatment stimulates astrocytoma cell migration and invasiveness. They also revealed, that ghrelin acting via functional GHS-R1a increases intracellular calcium mobilization and leads to membrane ruffling which results in higher motility and invasion of astrocytoma cells (Dixit et al. 2006).

The role of ghrelin in veterinary oncology

So far there are no published studies about the role of ghrelin in cancers in animals. Recent reports have confirmed that ghrelin administration has impact on pituitary growth hormone secretion in many species, including rats, goats, cats, cattle and pigs (Hayashida et al. 2001, Hashizume et al. 2003, Ida et al. 2007). Ghrelin induces also GH secretion in dogs (Yokoyama

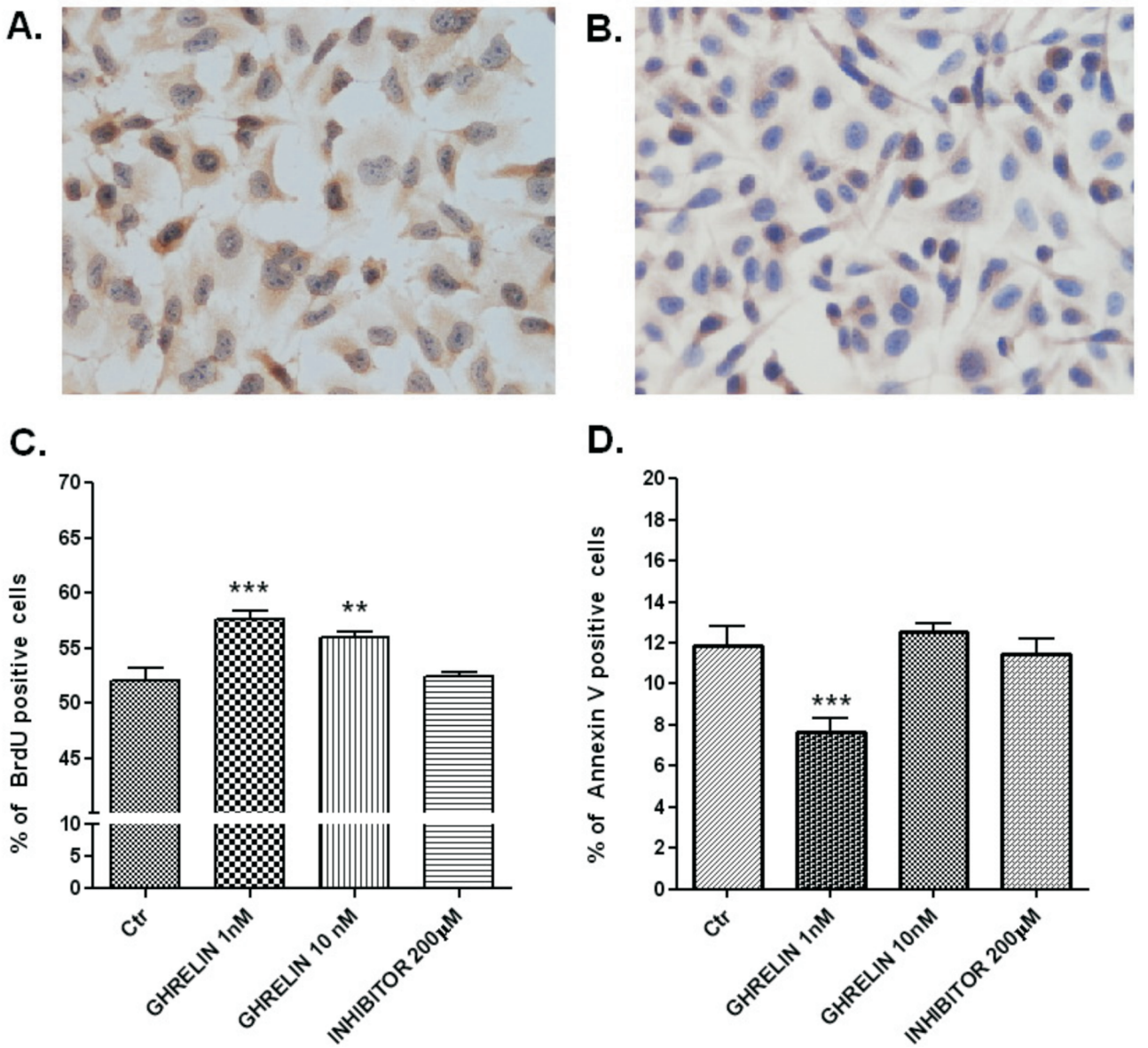

Fig. 1. Representative picture of **A**. ghrelin and **B**. growth hormone secretagogue receptor (ghrelin receptor, GHS-R) expression in canine mammary adenocarcinoma cell line determined by immunohistochemistry using EnVision System (DAB) (Dako, Denmark). The majority of the cancer cells show a strong cytoplasmic staining reaction. Pictures were obtained with Olympus BX60 (x20). **C.** The effect of ghrelin and GHS-R antagonist (D-Lys^3-GHRP6) on proliferation of canine mammary cancer cells determined using BrdU incorporation test (Becton Dickinson, USA). Proliferation, represented as a percent of BrdU positive cells obtained with FACS Aria II (Becton Dickinson, USA) is significantly increased after the ghrelin treatment at dose of 1 and 10nM ($P<0.01$, and $P<0.001$ were marked as ** and ***, respectively). **D.** The effect of ghrelin and GHS-R antagonist (D-Lys^3-GHRP6) on apoptosis of canine mammary cancer cells determined by Annexin-V FITC Apoptosis Kit (Becton Dickinson, USA). Rate of apoptosis, represented as a percent of Annexin-V positive cells established with FACS Aria II (Becton Dickinson, USA) is significantly decreased after the ghrelin treatment at dose of 1nM ($P<0.001$). The statistical analysis was conducted using Prism 5.0 (GraphPad Software, USA). The one-way ANOVA and Tukey post-hoc tests were applied.

et al. 2005). The distribution of ghrelin-immunoreactive cells in stomach is similar in all of the species mentioned (Date et al. 2000, Hayashida et al. 2001, Yokoyama et al. 2005). In healthy dogs, ghrelin regulates feeding behavior and energy metabolism. Plasma ghrelin levels increase before the feeding time, and decrease after eating. These changes are associated with the insulin and glucose concentration (Yokoyama et al. 2005, Bhatti et al. 2006). Some studies in dogs suggest a role of ghrelin in the development of adiposity because its abnormal circulating levels have been observed in obese dog compared with normal or lean

animals (Jeusette et al. 2005, Yokoyama et al. 2005). However, contrary to humans in dogs ghrelin does not stimulate the motor activity of the digestive tract (Ohno et al. 2006).

Our studies of gene expression in canine mammary cancer cell lines revealed that cell lines with the highest proliferative potential showed up-regulation of growth hormone receptor (*ghr*) and growth hormone secretagogue receptor (*ghsr)*. We proposed, that ghrelin through its specific receptor stimulates the GH production by cancer cells, which acts via GHR on cell proliferation (Król et al. 2010 a).

Moreover, we found the *ghsr* up-regulation in metastatic cancer cell lines (isolated from canine mammary cancer metastases to the lungs) what may indicate its role in metastasis (Król et al. 2010 b). Recently, we have confirmed the expression of ghrelin and GHS-R in adenocarcinoma cell lines isolated from canine mammary gland at mRNA level by real-time qPCR and immunohistochemically at the protein level (Fig. 1A,B). We also examined the role of ghrelin in proliferation and apoptosis of cancer cells. The wide FACS analyses (FACS Aria II) revealed that the ghrelin treatment (1 and 10nM) stimulated proliferation of canine mammary cancer cells evaluated by BrdU incorporation test (Fig. 1C). The ghrelin receptor antagonist ([D-Lys^3]-GHRP6) after one hour pre-incubation completely abolished the stimulatory effect of ghrelin on cell proliferation evaluated by MTT assay (data not shown). Moreover, ghrelin treatment (1nM) significantly decreased the number of apoptotic cells in the cancer cell line examined (Annexin V and propidium iodide dual staining) (Fig. 1D). These preliminary results have encouraged us to further studies in this field.

Conclusions

The possible role of ghrelin in the cancer development is equivocal. Presumably the crucial factor is ghrelin concentration. Higher doses of ghrelin inhibit cell growth whereas lower doses stimulate cell proliferation. Ghrelin activity depends also on a type of cancer cell (poorly or well-differentiated cancer). The next important issue is an expression of GHS-R1a or other binding sites in cells investigated. Some authors suggest that proliferative and invasive behavior of malignant cancer cells is mediated by ghrelin through the "non-functional" GHS-R1b or other unknown receptor subtype, which is responsible for the non-endocrine activities of ghrelin. Despite the effect of ghrelin on cancer cell lines has proven controversial, the studies conducted by Jeffery and co-workers (2002, 2003, 2005) are considered the most convincing and providing the basis for investigation on molecular mechanisms of action of ghrelin.

Nevertheless, further studies are required to assess turmorigenic potential of grelin axis and fully understand its role in cancerogenesis.

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