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Original research article

Interferon alpha and rapamycin inhibit the growth of carcinoid and medullary thyroid cancer *in vitro*

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ABSTRACT

Neuroendocrine tumors (NETs) are highly vascularized neoplasms characterized by rising incidence. Moreover, the neuroendocrine cells were shown to express vascular endothelial growth factor (VEGF) and VEGF receptors. Therefore, angiomodulators could be potentially a new group of drugs enhancing still unsatisfactory effectiveness of NET therapy.

The aim of this study was to assess the direct influence of angiomodulators: VEGF and five endogenous and exogenous antiangiogenic compounds (endostatin, interferon alpha [IFNα], rapamycin, JV1-36, semaxinib [SU5416]) on the growth of two NET cell lines: lung carcinoid H727 cell line and medullary thyroid cancer TT cell line *in vitro*.

IFNα and rapamycin induced the inhibitory effect on H727 and TT cell viability and proliferation, increasing apoptosis and arresting the cell cycle. Also semaxinib (10^{-5} M) inhibited proliferation of both cell lines. VEGF and endostatin did not influence the growth of H727 and TT cells.

The inhibitory effect of IFNα, rapamycin and semaxinib on carcinoid and medullary thyroid cancer growth was revealed in our in vitro study, although some other antiangiogenic agents did not directly influence H727 and TT cell growth. Thus, IFNα and mTOR inhibitors as multidirectionally acting drugs with antiangiogenic effect could be potentially efficient in treatment of neuroendocrine tumors and are worth further studies.

Introduction

Neuroendocrine tumors (NETs) are rare malignancies but their incidence has risen in the last years. NETs comprise a heterogeneous group of neoplasms with a wide spectrum of clinical behavior, which require specially tailored therapies. Although somatostatin analogs are routinely used to control hormone-mediated symptoms (carcinoid syndrome), the antitumor efficacy of traditional cytotoxic drugs is limited. These have led to the search for new targeted drugs based on the biological features of NETs, such as extraordinary vascularization with high expression of several proangiogenic molecules [1,2]. Moreover, as the neuroendocrine cells were shown to secret VEGF [3–5] and express VEGFR [6,7], the paracrine and autocrine action of VEGF is possible. The role of angiogenesis in neoplastic transformation was also suggested in medullary thyroid carcinoma (MTC) [8–12], which as a malignant tumor arising from the neuroendocrine calcitonin-producing parafollicular C cells is in broad understanding included to NETs. These facts suggest that antiangiogenic compounds could be potentially a group of drugs enhancing still unsatisfactory efficacy of NET therapy. They could act not only on endothelial cells, but also directly on neuroendocrine cells, which were shown to overexpress VEGF

Abbreviations: bFGF, basic fibroblast growth factor; BrdU, 5-bromo-2′-deoxyuridine; FDA, Food and Drug Administration; FITC, fluorescein isothiocyanate; GHRH, growth hormone releasing hormone; IFNα, interferon alpha; IGFs, insulin-like growth factors; LSD, least significant difference; MTC, medullary thyroid carcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NET, neuroendocrine tumors; OD, optical density; PI, propidium iodide; PI3K/AKT/mTOR-pathway, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin-pathway; PNETs, pancreatic neuroendocrine tumors; SU5416, semaxinib; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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and VEGFR (vascular endothelial growth factor receptor) [5,8,11].

The aim of this paper was to study the direct influence of proangiogenic (VEGF) and antiangiogenic (rapamycin, interferon alpha, endostatin, JV1-36, SU5416) factors on the growth of two human neuroendocrine tumors cell lines: lung carcinoid H727 and medullary thyroid cancer TT line in vitro. We also decided to assess the influence of these substances on calcitonin secretion in TT cells. Calcitonin used in clinical practice as hormonal marker of MTC, was shown to be produced in TT cells [13].

Rapamycin, a naturally occurring macrolide antibiotic, was the first known inhibitor of PI3K/AKT/mTOR-pathway (phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin-pathway), which takes part in regulation of cell growth, metabolism, angiogenesis and is frequently hyperactive in neoplasms [14]. Rapamycin was demonstrated to have immunosuppressive, antiangiogenic and antitumor activity and was proven to be effective in a number of neoplasms [14,15], including neuroendocrine tumors [16]. In 2011, the FDA (Food and Drug Administration) approved an mTOR inhibitor – everolimus – for the treatment of progressive pancreatic neuroendocrine tumors (PNETs) (NCI; http://www.cancer.gov/cancertopics/druginfo/fda-everolimus).

JV1-36 belongs to GHRH (growth hormone releasing hormone) antagonists, which were shown to exert antiproliferative effects in different tumors. The mechanism of GHRH antagonist action is complex and apart from inhibition of IGFs (insulin-like growth factors) production, the suppression of VEGF and bFGF (basic fibroblast growth factor) expression in neoplasms is suggested [17].

SU5416 (semaxinib), a 3-substituted indolinone compound, was the first VEGFR tyrosine kinase inhibitor tested clinically as antiangiogenic treatment in cancer [18]. This strategy was continued in trials with another multitargeted, orally available tyrosine kinase inhibitor – sunitinib – (structurally related to semaxinib), which was approved by the FDA for treatment of several neoplasms, including PNETs (NCI; http://www.cancer.gov/cancertopics/druginfo/fda-sunitinib-malate).

Endostatin is a proteolytic fragment of collagen XVII. It is an endogenous inhibitor of angiogenesis acting by blocking the binding of VEGF to VEGFR [19] and down-regulating VEGF expression in tissues [20]. Endostatin was also shown to exert an antineoplastic action [21,22]. Although endostatin suppressed the growth of pancreatic neuroendocrine tumor in transgenic RT2 (RIP-Tag2) mice [23], the clinical study did not confirm the efficacy of endostatin in PNET treatment [24].

Interferon alpha (IFNα) is a pleiotropic cytokine with proven antitumor activity triggered in the direct (through prolongation of the cell cycle time and enhancement of apoptosis in malignant cells) and indirect (interaction with other cytokines, immunomodulatory and antiangiogenic influence) mechanism [25]. IFNα as an angiogenesis inhibitor was first reported in 1980 [26] and then was confirmed by other authors [19,25]. The clinical use of IFNα includes treatment of neuroendocrine tumors, renal cell carcinoma, melanoma, and some other neoplasms [25].

Materials and methods

Cell line and culture condition

The human lung carcinoid cell line H727 and the human medullary thyroid cancer cell line TT obtained from the American Type Culture Collection (ATCC) were used in the experiments. The cells were routinely grown as a monolayer in a humidified incubator at 37 °C with 5% CO_2 in RPMI medium (ATCC) for H727 line or DMEM medium for TT line, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin solution (Sigma) and 10% fetal bovine serum (Biochrom). The cells were passaged every 7 days with 0.05% trypsin/0.02% EDTA (Trypsin–EDTA, Sigma) and the medium was changed every 3–4 days.

Substances

The following substances were used in the experiments: human IFNα (Sigma), rapamycin (Sigma), JV1-36 (Bachem), SU5416 (Sigma), human endostatin (Sigma), human VEGF (Sigma).

IFNα, JV1-36, endostatin and VEGF were diluted in serum free culture medium and added to the appropriate wells at the final concentrations as indicated in figures. The control group in these experiments received the medium.

Rapamycin was dissolved in 96% ethanol and serum free culture medium (5% of 96% ethanol in stock solution) and added to the appropriate wells at the final concentrations as indicated in figures. The appropriate volume of serum free culture medium and 96% ethanol at the final concentration identical with the solvent concentration in 10^{-5} M group was added to the control wells.

SU5416 was dissolved in DMSO and serum free culture medium (10% of DMSO in stock solution) and added to the appropriate wells at the final concentrations as indicated in figures. The appropriate volume of serum free culture medium and DMSO at the final concentration identical with the solvent concentration in every examined group was added to the control wells.

The action of all the agents in a wide range of concentrations was determined using an MTT colorimetric assay. Following this, based on the results of the MTT study, some concentrations of the substances were further assessed using the BrdU incorporation method, cell cycle analysis and apoptosis examination. However, the MTT method was not used to estimate the influence of SU5416 on the H727 and TT cell growth because of the potential interference of this yellow-dyeing compound with manufacture's procedures.

Cell viability/cytotoxicity study/Mosmann method

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) colorimetric assay was used as an indicator of cell viability/cytotoxicity. The cells were seeded at a density of 20×10^3 cells/well (H727) or 40×10^3 cells/well (TT) into 96-well microplates in 180 μl/well of complete culture medium. After preincubation (24 h) the cells were cultured for further 72 h in the presence of various concentrations of the examined substances (20 μl: IFNα, rapamycin, JV1-36, endostatin, VEGF). Cell viability was assessed using the EZ4U assay kit (The 4th Generation Non Radioactive Cell Proliferation & Cytotoxity Assay, Biomedica Gesellschaft mbH, Biomedica Gruppe Austria.) according to the manufacturer's instructions. The optical density (OD) of each sample was measured by a microplate reader at 450 nm.

Cell proliferation study

Colorimetric immunoassay based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis was used for the quantification of H727 and TT cell proliferation. The cells were seeded at a density of 20×10^3 cells/well (H727) or 40×10^3 cells/well (TT) into 96-well microplates in 90 μl/well of complete culture medium. After preincubation (24 h), the cells were cultured for further 72 h in the presence of various concentrations of the examined substances (10 μl: IFNα, rapamycin, JV1-36, SU5416, endostatin, VEGF). Cell proliferation was assessed using the BrdU assay (Cell proliferation ELISA, BrdU, Roche) following the producer's instructions. The optical density (OD) of each sample was measured by a microplate reader at 450 nm.