EXPRESSION OF INSULINE-LIKE GROWTH FACTOR-1 RECEPTOR mRNA IN COLORECTAL CARCINOMA PATIENTS

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The IGF-1R signalling pathway can positively regulate cell-cycle progression and thus play a critical role in cancer development. Although recent studies provide sufficient evidence supporting the functional importance of IGF-1R in cancer, the prognostic significance of IGF-1R expression levels to colorectal cancer (CRC) remains obscure. Expression of IGF-1R mRNA was examined in paired samples of CRC and adjacent normal mucosa, as well as in CRC patients’ venous blood. We also investigated the effect of two monocytes stimulus - C3bgp and LPS on induced mRNA of IGF-1R from normal and colorectal human monocytes. The role of a member of MAPK signal transduction pathways - JNK in IGF-1R expression was assessed. The expression of IGF-1R mRNA was measured by relative RT-PCR. The results demonstrated that expression of IGF-1R mRNA in venous blood from CRC was down-regulated compared to venous blood from healthy donors (1.426 vs. 0.645; p=0.024). Mean IGF-1R mRNA level was found to be approximately 5.8 fold higher in tumour tissue compared to adjacent normal mucosa (p=0.02). Strong IGF-1R mRNA expression was found for early stages of CRC. The results showed that both stimuli used, strongly up-regulated mRNA expression for IGF-1R in CRC monocytes, then in monocytes from healthy donors. The highest level of IGF-1R expression was detected after C3bgp stimulation, regardless of JNK inhibitor presence. The significance for increasing expression of IGF-1R was observed for early CRC when patients were divided according to the tumour stage. We can conclude that down-regulation of mRNA expression of IGF-1R in peripheral blood cells and stimulated monocytes from patients with advanced stages of colorectal cancer are a hallmark of tumour progression and can be used as a prognostic factor.

Keywords: colorectal cancer, IGF-1R, qRT-PCR

Introduction

Colorectal cancer (CRC) is the second most common cancer as it is responsible for 20% of all cancer deaths in the developed countries. In Bulgaria, the incidence of colorectal cancer is in the top third within the European Union and the second most common cancer-related death in men and women nationwide (Ministry of Health, Bulgaria; WHO). Although the primary therapy of CRC is surgical, the elucidation of different novel prognostic markers that could also serve as therapeutic targets is necessary to better understand this cancer entity and to improve outcome. The very early events that rescue cells from cell cycle arrest are mediated through signals transmitted by a group of peptides, collectively known as growth factors (5, 9). These molecules can be classified into two subgroups, namely the “competence” factors, such as the platelet-derived growth factor that enable cells to enter into the G1 phase, and the “progression” factors, such as the insulin-like growth factor (IGF) that are required for progression from G1 into the S phase and, ultimately, cell division (7).

The IGF system is comprised of ligands (IGF-1 and IGF-2), receptors (IGF-1R and IGF-2R), and a family of six binding proteins. The IGF1R is activated by binding of either IGF1 or IGF2. Binding of ligand induces a conformational change and autophosphorylation of key residues in the β subunits of the receptor, and docking proteins then interact with the phosphorylated residues of the activated receptor. Activation of the receptor and transduction of the intracellular signalling kinase cascades culminates in cell proliferation and anti-apoptotic effects (2, 16).

The IGF-1R and its associated signalling pathway is an important growth regulatory pathway...
that plays a crucial role in cancer development (3, 11, 12). Increased levels of IGF ligands and over-expression of IGF-1R have been observed in prostate, breast and colorectal cancer development and have been shown to affect proliferation, differentiation, migration and apoptosis of cancer cells (1, 10, 15). In tumours, such as colorectal, which may drive their own growth and survival through over-expression of autocrine IGF-II, the role of IGF-1R is especially critical (6, 8). Although recent studies provide sufficient evidence supporting the functional importance of IGF-1R in cancer, the prognostic significance of IGF-1R expression levels to colorectal cancers (CRC) still remains elusive.

The IGF-1R gene is constitutively expressed in most cells. The promoter of IGF-1R is CG-rich and lacks TATA and CCAAT elements (13), but has elements found in housekeeping genes, containing regulatory elements characteristic for highly regulated genes (20). Its expression is altered in certain diseases, including cancer (2, 13). The vast expression of IGF-1R in neoplastic cells and tissues combined with its crucial roles in cancer cell growth is making this tyrosine receptor an attractive target to combat malignant diseases.

The aim of our study was to examine the expression of IGF-1R mRNA in paired samples of CRC and adjacent normal mucosa, as well as in CRC patients’ venous blood compared to healthy controls’ venous blood. We also investigated the effect of two monocytes stimuli - C3bgp and LPS on induced mRNA of IGF-1R from normal and colorectal human monocytes. We assessed the role of the member of MAPK signal transduction pathways - c-Jun N-terminal kinase (JNK) in IGF-1R expression with a selective anthrapyrazolone inhibitor.

Materials and Methods

Reagents

Lipopolysaccharide (LPS) from Escherichia coli serotype 026:B6, Histopaque-1077, specific inhibitor of JNK SP600125 and all culture reagents, including fetal bovine serum (FBS-heat inactivated, sterile-filtered, cell culture tested) were obtained from Sigma, St. Louis, MO. Polystyrene materials were manufactured by Corning Inc., Corning, NY, and Nunc, Roskilde, Denmark. C3 binding glycoprotein (C3bgp) was isolated as described previously (22).

Blood, tumour and non-tumour tissue specimens

A group of 20 Bulgarian patients with CRC, who underwent surgical resection of the tumor, were included in the study. Cases with new diagnosis of CRC attending the University hospital and St. Ivan Rilsky Hospital in Stara Zagora, Bulgaria between October 2008 and November 2010 were selected. The histopathological examination confirmed the diagnosis of cancer. The mean age of total group of CRC patients was 65±7.3 years. Tumor grading and staging was performed according to the tumor–node–metastasis (TNM) classification. Paired samples of colorectal cancer free mucosa and adenocarcinomas derived from patients with the consent of the local ethics board. Criteria’s for eligible patients included no history of prior surgery for colon or rectal tumours; no known hereditary cancer, ulcerative colitis or Crohn’s disease. Tumour samples were taken from vital areas of histopathologically confirmed carcinomas. Mucosa samples were derived from unaffected mucosa 5 cm distal to the resection margin by sharp dissection. The tissues were harvested immediately after resection and used for RNA isolation.

Patient’s bloods were taken one day before surgery (preoperative). Blood samples were taken from 18 healthy donors for controls, matched for sex and age with the cancer patients. The peripheral venous blood (3 ml) was collected from patients and healthy donors in sterile tubes with EDTA and 1 ml was used for RNA isolation immediately. All patients and healthy volunteers gave an informed consent for this research.

Monocytes purification and stimulation

Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077 density gradient centrifugation. The interface containing PBMC was harvested and washed twice with cold RPMI-1640 medium. Purification of monocytes was done by plastic adherence. Briefly, PBMC were seeded in 24-well cell-culture plates. After 2 hours nonadherent cells were discarded, wells were gently washed and adherent monocytes were used for stimulation.

Monocytes cultures (1x10⁵ cells/ml) were carried out in RPMI-1640 supplemented with: 10% FBS, 100 U/ml penicillin, 100 µg/ml gentamicin and 0.3 mg/ml L-glutamine. The cells were stimulated with 30 µg/ml C3bgp or 1 µg/ml LPS. Monocytes cultures were incubated at 37°C for 3h.
For inhibition of c-jun N-terminal kinase we used the selective anthrapyrazolone inhibitor SP600125. It competitively inhibits JNK 1, 2 and 3 with >20 – fold selectivity versus wide range of kinases according to Bennet et al. (4). 20µM SP600125 were added 1 hour before stimulation to monocytes cultures. SP600125 was dissolved in 100% dimethylsulphoxide (DMSO) and the final concentration of DMSO in cultures was 0.1%. To avoid the influence of DMSO on the cytokine synthesis, nonstimulated cell cultures (controls) with 0.1% DMSO were seeded.

RNA extraction
Tumour and normal mucosa (30 mg) samples were homogenized, washed and re-suspended in Lysis Solution RA1 supplied with a column-based illustra RNAspin mini RNA isolation kit (GE Healthcare, UK). The following steps for total RNA isolation were performed according to the manufacturer’s instructions.

Total RNA from peripheral blood (1 ml) and monocytes cultures was isolated using immuPREP blood RNA isolation kit AJ Roboscreen (Leipzig; Germany) with additional step of treatment with DNase I (Fermentas) to remove traces of genomic DNA. The total RNA was quantified by spectrophotometrical analysis.

Reverse transcription
Synthesis of cDNA was performed manually according to manufacturer’s instructions with High-Capacity cDNA Archive kit (Applied Biosystems, USA) that uses random primers and MultiScribe TM MuLV reverse transcriptase enzyme. Incubation conditions for reverse transcription were 10 min at 25°C followed by 2 hours at 37°C. Reverse transcription was performed on a GeneAmp PCR System 9700 (Applied Biosystems, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)
Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The validated PCR primers and TaqMan MGB probes (6FAM-labeled) used were IGF-1R (assay ID Hs00153126_m1) and 18S ribosomal RNA (Hs99999903_m1) as endogenous control.

An aliquot of 5 µl of the RT reaction was amplified in duplicate at a final volume of 20 µl using a TaqMan Universal PCR Master Mix and Gene Expression Assay mix, containing specific forward and reverse primers and labelled probes for target genes and endogenous control (Applied Biosystems, USA). The thermocycling conditions were: initial 10 min incubation at 95°C followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. PCR data were collected with Sequence Detection System (SDS) software, version 1.3.1. (Applied Biosystems).

Statistical and data presentation
Relative quantitative (RQ) evaluation of mRNAs was performed by the comparative ΔΔCt method. The mean ΔCt obtained in normal mucosa from a healthy donor and in peripheral blood from healthy donor were used as calibrators for the IGF1R after normalization to endogenous control - 18S rRNA. As calibrators for the target gene in monocytes was used the mean ΔCt obtained in the non-stimulated cell cultures after normalization to endogenous control.

Non-parametric tests were used because of the non-normal distributions of data for mRNA expression levels. The RQ values of the tumour and corresponding mucosa, preoperative and healthy donors’ blood as well as not stimulated and stimulated monocytes were compared by t-test. Spearman test was used for correlation analysis. All statistical analysis was made on the statistical platform of StatSoft6. Differences were considered significant when the p value was less than 0.05.

Results are presented as n-fold mean difference relative to calibrator (RQ=2 -ΔΔCt) calculated by 7500 system SDS software, version 1.3.1 (Applied Biosystems).

Results and Discussion
IGF-1R is expressed in normal colonic epithelial cells and colorectal cell lines. However, there are conflicting data regarding the relative levels of IGF-IR mRNA in human colorectal carcinoma compared with normal mucosa. Zenilman et al. reported no significant differences in IGF-1R mRNA expression in the colon during neoplasia (21). Weber et al. analysed the expression of IGF-IR in 40 paired samples of normal and carcinomatous colonic tissue by competitive RT-PCR demonstrating that the IGF-IR mRNA ratio in paired tumour and adjacent normal mucosa was higher than 2.0 in 80% of samples and the overall
mean IGF-IR mRNA level was five-fold higher in tumour versus adjacent normal mucosa (19). In attempt to clarify this discrepancy and to evaluate whether IGF-IR is overexpressed in colonic carcinomas we compared the expression of IGF-IR mRNA in twenty paired samples of normal and carcinomatous colonic tissue. At Fig. 1 are presented results of qRT-PCR for RQ level of IGF-IR mRNA expression in tumour versus adjacent normal mucosa. The mean IGF-IR mRNA level was found to be approximately 5.8 fold higher in tumor tissue compared to adjacent normal mucosa (RQ=22 vs. RQ=3.8; p=0.02). Our results are in the same direction as results from Weber et al. Moreover, we investigated the timing of these changes in expression during the evolution of CRC. The patient’s group was divided in stages of disease by TNM classification and grouped to early and advanced stage as following: I, II stages – early and III, IV stages – advanced. We demonstrated a stage-dependent expression of IGF-1R mRNA. Strong and statistically significant upregulated IGF-1R mRNA expression was found only for the group of early stages of CRC. Similar results have been reported by Allison et al., by means of Northern blotting and immunohistochemistry, which showed a high-level IGF-1R expression in colorectal neoplasia, initiated by an abnormality of stem cell programmed differentiation in the aberrant crypt focus and low-level of the IGF-1R expressed in invasive foci (1). The prognostic value of IGF-1R expression has been evaluated in 161 patients with curatively resected Dukes’ C CRC, who had not received neoadjuvant or adjuvant therapy and had at least 5 years follow-up in the study of Nakamura et al. (14). Their results indicated an association between low IGF-1R membrane expression and increased risk of metastasis in Dukes’ C CRC (13). Moreover, Schnarr et al., reported a down regulation of IGF-1R in advanced human breast cancer (18).

In summary we suppose that low levels of the IGF-1R expression in advanced CRC are related to cancer cells dedifferentiation and loss of cell adhesion. Previous studies reveal that at early stages the tumour cells are exhibiting growth factor depending progression of tumorgenesis (2, 3, 14). During later stages, such cells may become growth factor-independent for continued progression. For example, early-stage melanoma cells have recently been shown to be exquisitely sensitive to IGF-I. At these early stages, IGF-I activates the MAP kinase pathway and the PI3 kinase pathway, which triggers both proliferation and promotes cell survival. At later stages of development, in malignant melanoma cells, IGF-I has been unable to further activate these systems (17).

We also investigated the expression of IGF-1R in patients’ blood cells one day before surgery (preoperative). The patients’ data were compared to the data from the blood cells taken from normal healthy volunteers. The results showed that the expression of IGF-1R mRNA in the venous blood from CRC was down-regulated compared to the venous blood from healthy donors with statistically significant differences (RQ=1.426 vs. RQ=0.645; p=0.024; (Fig. 1). We found a mathematical...
correlation expressed by a linear equation in levels of IGF-1R mRNA in peripheral blood between the whole group of patients and advanced stages of colorectal cancer (Spearman test R=0.80, t=3.26; p=0.017). The observed down-regulation of IGF-1R expression in venous blood cells and up-regulation of IGF-1R expression in tumour tissue from early CRC patients demonstrated the differences in local and systemic regulation of the IGF-1R mRNA expression during development of the adenocarcinoma. The specific pattern of expression could be due to the involvement and crosstalk of different intracellular pathways in activated peripheral blood cells and transformed epithelial cells. To investigate specificity of the cellular response to stimulation we isolated monocytes from healthy donors and CRC patients and cultured with two monocytes stimuli (LPS or C3bgp) in the presence or absence of JNK inhibitor (SB600125). Results are presented at Fig. 2. In the group of healthy donors significantly increased expression of IGF-1R was observed in the monocytes stimulated with LPS in the presence of JNK inhibitor compared to LPS stimulated monocytes without SB600125 (RQ=0.42 vs. RQ=1.05; p=0.026). According to the results both used stimuli strongly up-regulated mRNA expression for IGF-1R in CRC monocytes, then in monocytes from healthy donors. In the same time the inhibition of JNK signalling pathway through specific JNK inhibitor did not influence regulation of IGF-1R expression in peripheral monocytes from CRC patients. The highest level of IGF-1R expression was detected after C3bgp stimulation, regardless of JNK inhibitor presence (RQ=0.57 vs. RQ=1.81; p=0.04; RQ=0.65 vs. RQ=1.24; p=0.04 in presence of SB600125). When the patients were divided according to the tumour stage (Fig. 3) it is obvious that increased expression of IGF-1R in the stimulated monocytes was observed for the early CRC compared to both the healthy controls and advanced stages of CRC. These results show that the regulation of the IGF-1R expression in stimulated peripheral monocytes exerts a similar pattern as the regulation of the IGF-1R expression in colorectal tissue. A down regulation was detected in both stimulated monocytes and tumour tissue samples from patients with advanced stages then early stages of CRC. An explanation of these results could be the involvement of common regulatory mechanisms concerning IGF-1R expression, emphasizing the pathophysiologic relevance of the progression of colorectal carcinomas. Data included in this paper suggest that functional changes of the IGF-1R expression participate in the maintenance of colorectal cancer progression rather than in its initial development.

Fig. 2. Expression of EGF1R mRNA in in vitro stimulated monocytes from patients with colorectal carcinoma
Fig. 3. Comparison of IGF1R mRNA expression from in vitro stimulated monocytes from healthy donors and patients with colorectal carcinoma

**Conclusions**

According to our results we can conclude that the down-regulation of IGF-1R mRNA expression in peripheral blood cells and stimulated monocytes from patients with advanced stages of colorectal cancer is a hallmark of tumour progression and can be used as a prognostic factor in diagnostics. The up-regulation in tumour tissue and stimulated CRC monocytes from early stages reveal the role of IGF1R in tumour initiation.

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**REFERENCES**