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ANTI-INFLAMMATORY AND ANTI-MUTAGENIC EFFECT OF YHK PHYTOCOMPOUND IN HEPATOCYTES: IN VIEW OF AN AGE-MANAGEMENT LIVER PROTECTING APPROACH

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Complete List of Authors:	Catanzaro, Roberto; University of Catania, Dept of Internal Medicine Celep, Gulcin; Gazi University, Family and Consumer Sciences Department, Nutrition and Food Technology Division Illuzzi, Nicola; ReGenera Research group for Aging Intervention, Milazzo, Michele; University of Catania, Dept of Internal Medicine Rastmanesh, Reza; Shahid Beheshti University of Medical Sciences, National Nutrition and Food Technology Research Institute Yaduvanshi, SK; Jiwaji University, School of Studies in Biochemistry He, Fang; Sichuan University, Department of Nutrition and Food Hygiene, West China School of Public Health Trushin, Maxim; Kazan University, Deparment of Genetics, Institute of Sapienza, Chiara; University of Catania, Dept of Internal Medicine Srivastava, N; Jiwaji University, School of Studies in Biochemistry Marotta, Francesco; Regenera Research Group for Aging Intervention,
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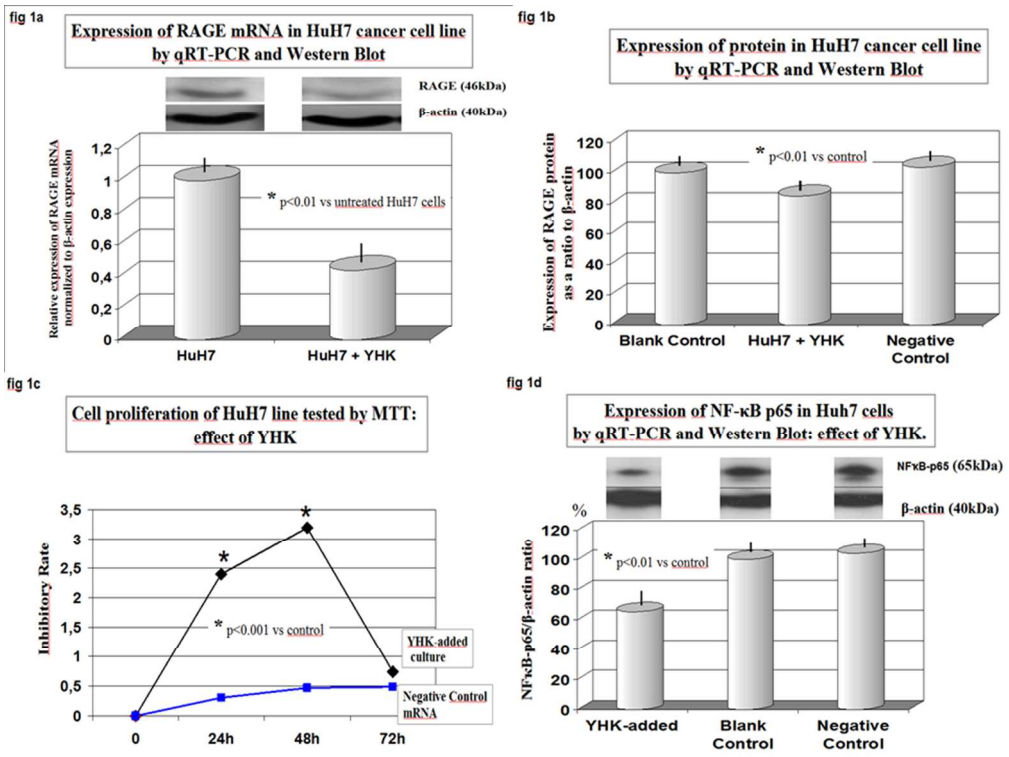

Expression of RAGEmRNA, NFkB and protein in HuH7 cell line and their proliferation rate
254x190mm (96 x 96 DPI)

Introduction

Hepatocellular carcinoma (HCC) is one of the most widely examined inflammation-related oncogenic processes because over 90% of HCCs develop in the setting of chronic liver disease with and inflammatory pattern. HCC is detected in more than half a million people each year and represents the third most common cause of tumor mortality worldwide (1). Although the short-term prognosis of patients with HCC has substantially improved due to better modalities for early diagnosis and treatment, long-term prognosis remains disappointing with a low overall survival at 10 years. By employing the quantitative analysis of RAGE mRNA expression, it has been recently clarified that HCC patients show an overexpression of RAGE and this also when compared to surrounding para-neoplastic liver tissue (2). AGEs are the products of non-enzymatic, irreversible glycation of proteins and the causative factors of several pathological processes such as inflammation and cancers. Moreover, the AGEs/RAGE system is known to activate nuclear factor (NF)-κB (3) and these events trigger the production of proinflammatory cytokines (4).

The phenomenon of binding of RAGE and ligand is crucial for the triggering of signal transduction events which brings about the upregulation of RAGE and pro-inflammatory genes that are associated with the pathogenesis of chronic diseases such as diabetes, non-alcoholic steatohepatitis and dementia (5-7). Moreover, ligand- RAGE binding can activate signaling pathways, which interfere with cancer cell biology thus worsening a number of deleterious tumour characteristics such as its invasiveness and metastatic progression (8). Since HCC is characteristically an inflammation-associated malignancy, the aim of the present study was to test the in vitro effect of YHK, a nutraceutical with prior data suggesting its anti-inflammatory/antioxidant hepatocyte protecting role and antimutagenic effect (9-11), in regulating RAGE in the proliferation of HCC cell line HuH7, as well checking also its potential modulation in the expression of the transcriptional factor NF-κB p65.

Materials and Methods.

HuH7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) in an incubator with a 5% CO_2 atmosphere.

Polymerase chain reaction. Cells were plated at 1×10^5/well in 24-well plates and cultured for 24 h. Then, YHK (0.5 μg/mL) was added to the medium and culture was maintained for further 24 h. Total RNA (1 μg) was reverse transcribed to cDNA, using random hexamer primers, per the manufacturer's recommendations. Final reaction concentrations were as follows: 1× TaqMan buffer, 5.5 mmol/L $MgCl_2$, 500 μmol/L each dNTP, 2.5 μmol/L random hexamer, 400 kU/L (0.4 U/μL) RNase inhibitor, and 1.25 kU/L (1.250 U/μL) Multiscribe reverse transcriptase. Reverse transcription was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. Twenty-five percent of the synthesized cDNA served as substrate for PCR amplification. Quantitative RT-PCR was performed in 96-well plates using Tg-specific primers and probe with the ABI PRISM 7700 Sequence Detection System. This system identifies and quantifies amplified Tg product at 7-s intervals during PCR amplification. Tg-specific primers that spanned a 1.5-kb intron were designed to amplify an 87-bp product from bp 262 to bp 348 in the cDNA sequence as follows: The sequences of the primers were as follows: β-actin: sense-primer 5′-GGACTTCGAGCAAGAGATGG-3′, anti-sense 5′-AGCACTGTGTTGGCGTACAG-3′; RAGE: sense-primer 5′-CACACTGCAGTCGGAGCTAA-3′, anti-sense 5′-GCTACTGCTCCACCTTCTGG-3′. Each sample was assayed in triplicate. Final reaction conditions were as follows: 1× TaqMan buffer; 0.05 g/L gelatin; 0.1 mL/L Tween 20; 80 mL/L glycerol; 5.5 mmol/L $MgCl_2$; 200 μmol/L dATP, dCTP, and dGTP; 400 μmol/L dUTP; 200 μmol/L each primer; 100 μmol/L TaqMan oligoprobe; 10 kU/L AmpErase UNG; and 25 kU/L AmpliTaq Gold. The cycling conditions included an initial phase of 2 min at 50 °C, followed by 10 min at 95 °C for AmpErase, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. In addition to quantitative analysis of PCR product amplification using the 7700 Sequence Detection System, all RT-PCR products were analyzed by electrophoresis in 3% agarose gels followed by ethidium bromide staining to

ensure amplification of the appropriately sized product. Samples omitting reverse transcriptase and template were included for each sample to identify contamination. The level of expression was calculated using the formula: Relative expression (*t*-value) = (Copy number of target molecule/Copy number of β-actin) × 1000.

Intra- and interassay variation. The threshold cycle was determined in triplicate for each calibrator in six independent analytical runs. The measured threshold cycle of the triplicate calculations of each calibrator was used to assay the intraassay CV. An intraassay CV was determined for each of the calibrators in each of the six analytical runs. Finally, the mean threshold cycle measured for each calibrator in six independent analytical runs on separate days was used to assess the interassay CV.

Immunoblot analysis. After cells had been collected, they were washed twice with cold PBS, lysed with 200 μL of 0.5% (w/v) SDS, and centrifuged at 10 000 r/min. The supernatants were adjusted by dilution so as to contain same amounts of protein, as ascertained by BCA Protein Assay Kit (Pierce, Rockford, IL). Samples (20 μg protein) were run on 12.5% (w/v) SDS-PAGE with 10% gel and electroblotted onto PVDF membranes. The blots were halted for 1 h with 5% (w/v) non-fat milk powder and 0.1% (v/v) Tween 20 in Tris-NaCl, then exposed to the primary antibody at a 1000-fold dilution overnight at 4°C. After extensive washing, the blots were incubated with the secondary horseradish-peroxidase-conjugated antibody (1:2000) for 2h at room temperature. The immune complex was visualized using the Enhanced Chemiluminescence Western blot detection system (PIERCE, Rockford,IL, USA). The amount of β-actin as an internal control was also examined using a specific antibody (Cytoskelton Inc., Denver, CO, USA).

Cell proliferation assay by MTT. Cell viability was monitored after incubation for 24, 48, and 72 hours by MTT assay. Briefly, cells were seeded in 96-well tissue culture plates at a concentration 4 × 10^3 cells per well. When cells reached approximately 70% confluence, the medium was changed

to DMEM for serum starvation and maintained for 24 hours. At the end of culture, the medium was replaced with medium containing MTT (50 μg/mL) and further maintained for 2 hours at 37°C. Afterwards, the blue formazan crystals were dissolved in 1 mL isopropanol and the absorbance at 570 and 630 nm was measured with an ELISA reader. The actual counts were calculated by subtracting the absorbance at 570 nm with background subtraction at 650 nm, using a spectrophotometric plate reader (Benchmark Plus Microplate Spectrophotometer, Bio-Rad Laboratories, Inc., Hercules, CA). Each assay was performed in triplicate and the average absorbance was calculated.

Apoptosis assay. Cells were cultured under control condition, or with YHK 0.5 μg/mL for 24 h, then harvested by trypsinization and washed twice with PBS. The annexin V binding assay was performed using an Annexin V-FITC Apoptosis At least 1×10^6 cells were incubated with FITC-conjugated annexin V at room temperature for 15 min, and the cells were then analyzed on a FACscan (Becton-Dickinson).

Statistical Analysis

Statistical analysis for all the biological tests and real time PCR was carried out using Student's *t* test and one-way ANOVA and *p* values less than 0.05 were regarded as significant.

Results

Our data showed that YHK significantly reduced RAGE gene expression and protein (fig 1a-b, $p<0.05$). Moreover it also exerted a significant cell growth inhibition of HCC cell line HuH7 peaking at 48h (fig 1c, $p<0.01$). Such actions were associated to a partly but significantly reduced gene expression of NF-κB p65 (by 35% expressed as relative expression of NF-κB p65 mRNA normalized to β-actin expression, fig 1d, $p<0.05$) and an increase of 16% of apoptosis (data not shown).

Discussion

There is a growing evidence that activated oncogenes and chronic inflammation have local and systemic metabolic effects, which establish metabolic symbiosis between epithelial cancer cells and cancer-associated fibroblasts (12). Accordingly, an updated approach to cancer pathophysiology is envisaging a host-based disease of persistent oxidative stress and inflammation that starts locally and then amplified systemically in the host up to an overall catabolic cascade. In this context RAGEs have attracted a great deal of attention since they are oncogenic and RAGE appears to be activated along with the pathogenetic mechanisms linked to a number of chronic degenerative-inflammatory diseases and cancers. Indeed, the binding of advanced glycation end products (AGEs) to their receptor (RAGE) increases oxidative stress and inflammation and may be involved in liver injury and subsequent carcinogenesis (13-17). The present data suggest that YHK has a potential role as a modulator of RAGE and RAGE ligands so to be amenable for potential therapeutic intervention in HCC prevention strategies within broader health plans. It is conceivable that some YHK components endowed by potent antioxidant property (9-11) might have further contributed to such effect although more detailed mechanisms have to be ascertained as yet. As a matter of fact signal transduction begins with RAGE through NF-κB leading to enhanced expression of cyclin D1 which in its turn fastens the progression to S phase and increased proliferation of cancer cells. Moreover, the increase in RAGE expression is likely to follow a positive feedback from the RAGE promoter through RAGE activation of NF-κB (18) which is our study was down-regulated by YHK. The NFκ B complex consists of a family of dimeric transcription factors and its multi-step signaling pathway plays a crucial role in the control of cell survival, tumor invasion and inflammatory stress response (Baker et al., 2011; Ben-Neriah and Karin, 2011) acting on several along the signalling process and thus further, more specific, anti-inflammatory pathways such high mobility box 1 protein which may be affected by YHK represent our future research goal. At the same time, studies on YHK are ongoing

to enhance the anti-inflammatory mechanisms array and its bioavailability and for which clinical applicability remains to be confirmed.

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