

Anti-Inflammatory and Anti-Mutagenic Effect of the YHK Phytocompound in Hepatocytes: In View of an Age-Management Liver-Protecting Approach

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Abstract

The receptor for advanced glycation end products (RAGE) regulates cellular proliferation in hepatocellular carcinoma (HCC). The aim of this study was to test the *in vitro* effect of Yo Jyo Hen Shi Ko (YHK), a nutraceutical with prior data suggesting its hepatocyte-protecting role, in regulating RAGE in the proliferation of the HCC cell line HuH7 as well checking also its potential modulation in the expression of the transcriptional factor nuclear factor- κ B (NF- κ B) p65. Our study showed that YHK significantly reduced cellular growth in the HuH7 cell line ($p < 0.05$). Moreover, this phytocompound partly reduced gene expression of NF- κ B p65 (by 35%, $p < 0.05$). These data suggest that YHK has a potential role as a modulator of RAGE and RAGE ligands for potential healthy liver intervention in HCC prevention strategies.

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 an 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 improved substantially due to better modalities for early diagnosis and treatment, long-term prognosis remains disappointing, with a low overall survival of 10 years. By employing quantitative analysis of receptor for advanced glycation end products (RAGE) mRNA expression, it has been recently clarified that HCC patients show an over-expression of RAGE and also when compared to surrounding para-neoplastic liver tissue.² Advanced glycation end products (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- κ B (NF- κ B),³ and these events trigger the production of pro-inflammatory cytokines.⁴

The binding of RAGE and ligand is crucial for the triggering of signal transduction events that bring about the up-regulation 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 that interfere with cancer cell biology, thus worsening a number of deleterious tumor characteristics, such as invasiveness and metastatic progression.⁸ Because HCC is characteristically an inflammation-associated malignancy, the aim of the present study was to test the *in vitro* effect of Yo Jyo Hen Shi Ko (YHK), a nutraceutical, with prior data suggesting its anti-inflammatory/anti-oxidant hepatocyte-protecting role and anti-mutagenic 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- κ B p65.

Materials and Methods

HuH7 cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) in an incubator with a 5% CO₂ atmosphere.

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Polymerase chain reaction

Cells were plated at 1×10^5 /well in 24-well plates and cultured for 24 hr. Then, YHK (0.5 $\mu\text{g}/\text{mL}$) was added to the medium, and the culture was maintained for a further 24 hr. Total RNA (1 μg) was reverse transcribed to cDNA, using random hexamer primers, per the manufacturer's recommendations. Final reaction concentrations were as follows: $1 \times$ TaqMan buffer, 5.5 mmol/L MgCl_2 , 500 $\mu\text{mol}/\text{L}$ each deoxyribonucleotide (dNTP), 2.5 $\mu\text{mol}/\text{L}$ 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 specific primers and probes with the ABI PRISM 7700 Sequence Detection System. This system identifies and quantifies amplified Tg product at 7-sec 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: $1 \times$ TaqMan buffer, 0.05 gram/L gelatin, 0.1 mL/L Tween 20, 80 mL/L glycerol, 5.5 mmol/L MgCl_2 , 200 $\mu\text{mol}/\text{L}$ dATP, dCTP, and dGTP, 400 $\mu\text{mol}/\text{L}$ dUTP, 200 $\mu\text{mol}/\text{L}$ each primer, 100 $\mu\text{mol}/\text{L}$ 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 sec 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) \times 1000.

Intra-assay and inter-assay 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 intra-assay coefficient of variation (CV). An intra-assay 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 phosphate-buffered saline (PBS), lysed with

200 μL of 0.5% (wt/vol) sodium dodecyl sulfate (SDS), and centrifuged at 10,000 rpm. The supernatants were adjusted by dilution so as to contain the same amounts of protein, as ascertained by a BCA Protein Assay Kit (Pierce, Rockford, IL). Samples (20 μg of protein) were run on 12.5% (wt/vol) SDS-polyacrylamide gel electrophoresis (PAGE) with 10% gel and electroblotted onto polyvinylidene fluoride (PVDF) membranes. The blots were halted for 1 hr with 5% (wt/vol) non-fat milk powder and 0.1% (vol/vol) 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 2 hr at room temperature. The immune complex was visualized using the Enhanced Chemiluminescence Western blot detection system (Pierce, Rockford, IL). The amount of β -actin as an internal control was also examined using a specific antibody (Cytoskeleton Inc., Denver, CO).

Cell proliferation assay by MTT

Cell viability was monitored after incubation for 24, 48, and 72 hr by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Briefly, cells were seeded in 96-well tissue culture plates at a concentration of 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 hr. At the end of culture, the medium was replaced with medium containing MTT (50 $\mu\text{g}/\text{mL}$) and further maintained for 2 hr at 37°C. Afterward, the blue formazan crystals were dissolved in 1 mL of isopropanol and the absorbance at 570 and 630 nm was measured with an enzyme-linked immunosorbent assay (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 0.5 $\mu\text{g}/\text{mL}$ YHK for 24 hr, then harvested by trypsinization and washed twice with PBS. The annexin V binding assay was performed using an Annexin V-FITC Apoptosis Kit. At least 1×10^6 cells were incubated with fluorescein isothiocyanate (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 the Student t -test and one-way analysis of variance (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 inhibition of cell growth of

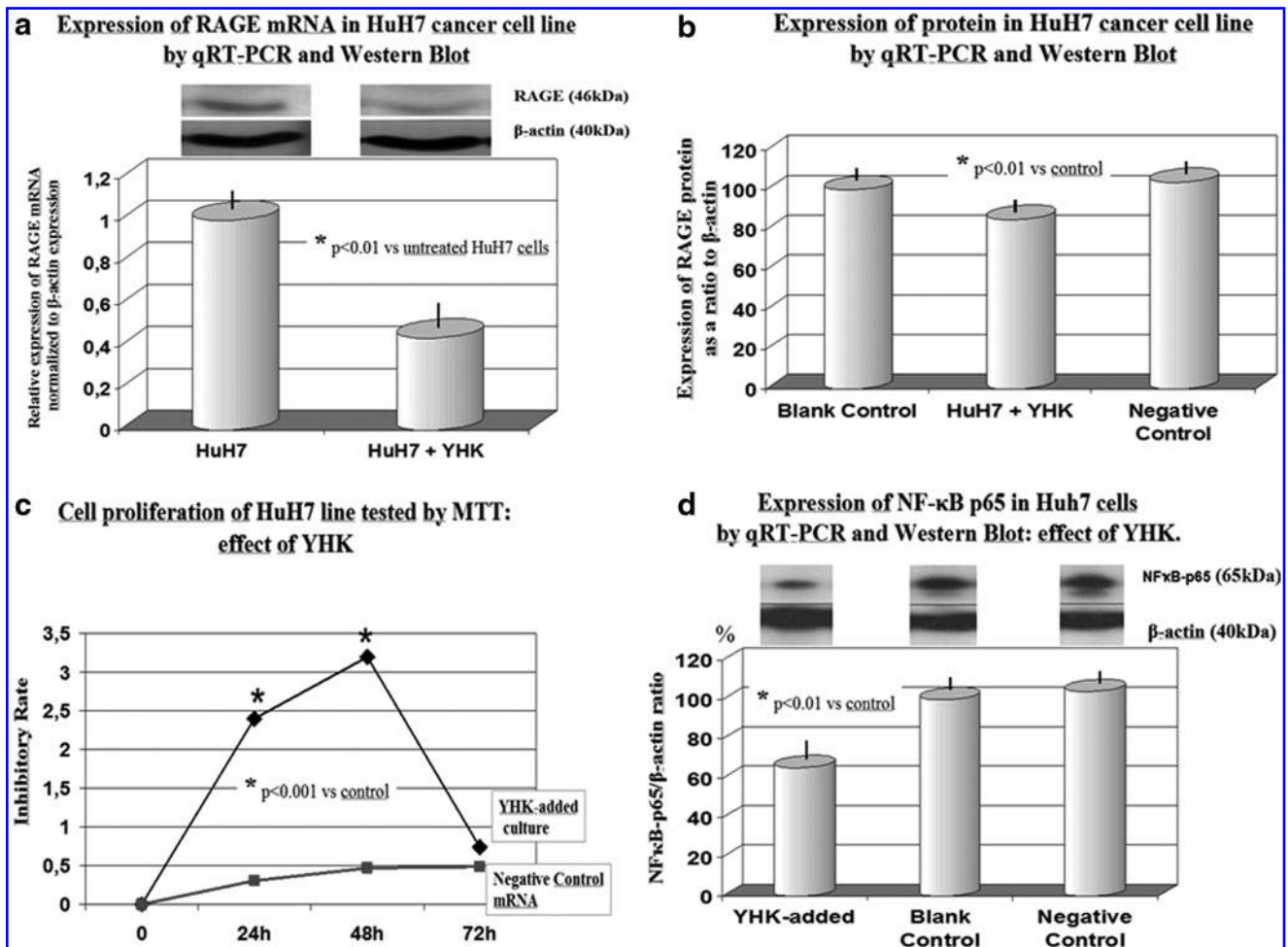


FIG. 1. Expression of receptor for advanced glycation end products (RAGE) mRNA, nuclear factor- κ B (NF- κ B), and protein in the HuH7 cell line and their proliferation rate.

the HCC cell line HuH7, peaking at 48 hr (Fig. 1C; $p < 0.01$). Such actions were associated to a partly, but significantly, reduced gene expression of NF- κ B p65 (by 35% expressed as relative expression of NF- κ B 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 envisages a host-based disease of persistent oxidative stress and inflammation that starts locally and then is amplified systemically in the host to an overall catabolic cascade. In this context, RAGEs have attracted a great deal of attention because they are oncogenic and RAGEs appear to be activated along with the pathogenetic mechanisms linked to a number of chronic degenerative-inflammatory diseases and cancers. Indeed, the binding of AGEs to their receptor 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, and so is amenable for potential therapeutic intervention in HCC prevention strategies within broader health plans. It is conceivable that some YHK components endowed by potent anti-oxidant properties^{9–11} might have further contributed to such an effect, although more detailed mechanisms have to be ascertained as yet. As a matter of fact, signal transduction begins with RAGE through NF- κ B, leading to enhanced expression of cyclin D1, which in its turn hastens 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- κ B,¹⁸ which in our study was down-regulated by YHK. The NF- κ 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,¹⁹ acting on several factors along the signaling process. Thus, further, more specific, anti-inflammatory pathways, such high-mobility box 1 protein, that may be affected by YHK represent our future research goals. 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.

Author Disclosure Statement

No competing financial interests exist.

References

1. Fares N, Péron JM. Epidemiology, natural history, and risk factors of hepatocellular carcinoma. *Rev Prat* 2013;63:216–222.
2. Hiwatashi K, Ueno S, Abeyama K, Kubo F. A novel function of the receptor for advanced glycation end-products (RAGE) in association with tumorigenesis and tumor differentiation of HCC. *Ann Surg Oncol* 2008;15:923–933.
3. Okamoto T, Yamagishi S, Inagaki Y, Amano S, Koga K, Abe R, Takeuchi M, Ohno S, Yoshimura A, Makita Z. Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin. *FASEB J* 2002;16:1928–1930.
4. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999;285:248–251.
5. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest* 2001;108:949–955.
6. Sato T, Shimogaito N, Wu X, Kikuchi S, Yamagishi S, Takeuchi M. Toxic advanced glycation end products (TAGE) theory in Alzheimer's disease. *Am J Alzheimers Dis Other Demen* 2006;21:197–208.
7. Hyogo H, Yamagishi S, Iwamoto K, Arihiro K, Takeuchi M, Sato T, Ochi H, Nonaka M, Nabeshima Y, Inoue M, Ishitobi T, Chayama K, Tazuma S. Elevated levels of serum advanced glycation end products in patients with non-alcoholic steatohepatitis. *J Gastroenterol Hepatol* 2007;22:1112–1119.
8. Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature* 2000;405:354–360.
9. Harada M, Marotta F, Sha SH, Minelli E. YHK, a novel herbal remedy with effective antifibrotic action, in chronic liver disease: A pilot clinical study aiming to a successful integrative medicine development. First JSH Single Topic Conference “Therapy of viral hepatitis and prevention of hepatocellular carcinoma.” November 14–15, 2002, Yamanashi, Japan.
10. Marotta F, Harada M, Goh KL, Lorenzetti A, Marandola P, Minelli E. In vitro study on the mechanisms of action of a novel phytotherapeutic compound against human hepatoma cells. *Ann Hepatol* 2007;6:111–116.
11. Marotta F, Harada M, Goh K, Lorenzetti A, Gelosa F, Minelli E. Phytotherapeutic compound YHK exerts an inhibitory effect on early stage of experimentally-induced neoplastic liver lesions. *Ann Hepatol* 2006;5:268–272.
12. Lisanti M, Martinez-Outschoorn U, Pavlides S, Whitaker-Menezes D, Pestell R, Howell A. Accelerated aging in the tumor microenvironment: Connecting aging, inflammation and cancer metabolism with personalized medicine. *Cell Cycle* 2011;10:2059–2063.
13. Pusterla T, Németh J, Stein I, Wiechert L, Knigin D, Marhenke S, Longnerich T, Kumar V, Arnold B, Vogel A, Bierhaus A, Pikarsky E, Hess J, Angel P. Receptor for advanced glycation endproducts (RAGE) is a key regulator of oval cell activation and inflammation-associated liver carcinogenesis in mice. *Hepatology* 2013;58:363–373.
14. Moy KA, Jiao L, Freedman ND, Weinstein SJ, Sinha R, Virtamo J, Albanes D, Stolzenberg-Solomon RZ. Soluble receptor for advanced glycation end products and risk of liver cancer. *Hepatology* 2013;57:2338–2345.
15. Kohles N, Nagel D, Jünger D, Stieber P, Holdenrieder S. Predictive value of immunogenic cell death biomarkers HMGB1, sRAGE, and DNase in liver cancer patients receiving transarterial chemoembolization therapy. *Tumour Biol* 2012;33:2401–2409.
16. Hirata K, Takada M, Suzuki Y, Kuroda Y. Expression of receptor for advanced glycation end products (RAGE) in human biliary cancer cells. *Hepatogastroenterology* 2003;50:1205–1207.
17. Yan W, Chang Y, Liang X, Cardinal JS, Huang H, Thorne SH, Monga SP, Geller DA, Lotze MT, Tsung A. High-mobility group box 1 activates caspase-1 and promotes hepatocellular carcinoma invasiveness and metastases. *Hepatology* 2012;55:1863–1875.
18. Rojas A, Figueroa H and Morales E. Fueling inflammation at tumor microenvironment: The role of multiligand/RAGE axis. *Carcinogenesis* 2010;31:334–341.
19. Ben-Neriah Y, Karin M. Inflammation meets cancer, with NF- κ B as the matchmaker. *Nat Immunol* 2011;12:715–723.

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