Catalyser-21TM, a mineral water derived from leaf soil, inhibits tumor cell invasion and angiogenesis

Jun Ye · Yuping Li · Takeki Hamasaki · Noboru Nakamichi · Takeshi Kawahara · Kazuhiro Osada · Kiichiro Teruya · Yuko Kato · Kazuko Toh · Masumi Abe · Yoshinori Katakura · Katsumi Noguchi · Sanetaka Shirahata

Received: 8 March 2007 / Accepted: 30 March 2007 / Published online: 20 July 2007
© Springer Science+Business Media B.V. 2007

Abstract Catalyser-21TM is a mineral water derived from natural leaf soil containing various organic and inorganic substances. Previous reports suggested a possibility that Catalyser-21TM has antioxidative potential and could inhibit angiogenesis and cancer cell invasiveness. Angiogenesis is a prerequisite for cancer cells to spread to surrounding tissues. Vascular endothelial growth factor (VEGF) is a major angiogenic factor in the formation of blood capillaries by cancer cells to supply nutrients and oxygen for their sustained growth. Matrix metalloproteinase-2 (MMP-2) is another key enzyme for cancer cell metastasis. To assess the anti-angiogenic activity of

Catalyser-21TM, we first examined cell viability using a human cervical cancer cell line, HeLa, and a fibrosarcoma cell line, HT1080. The results showed that Catalyser-21TM decreased the viability of both cell types in a dose-dependent manner. Flow cytometric analysis proved that Catalyser-21TM scavenges intracellular H₂O₂ in both cell types. RT-PCR demonstrated that both VEGF and MMP-2 gene transcription was suppressed after Catalyser-21TM treatment. Both Matrigel and tubule formation experiments showed an effect of Catalyser-21TM. These results suggest that Catalyser-21TM has potential as an anti-tumor agent.

J. Ye · K. Teruya · Y. Katakura · S. Shirahata
Graduate School of Systems Life Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

J. Ye
Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Science, Xiamen University, Fujian, China

Y. Li
School of Life Sciences, Nanchang University of Science and Technology, Nanchang 330006 Jiangxi Province, China

Y. Li · T. Hamasaki · K. Osada · K. Teruya · K. Toh · M. Abe · Y. Katakura · S. Shirahata (✉)
Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
e-mail: sirahata@grt.kyushu-u.ac.jp

N. Nakamichi
Functional Water Cell Analysis Center Co. Ltd, Fukuoka 812-0000, Japan

T. Kawahara
Integrated Department of Sciences of Functional Foods, Graduate School of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan

Y. Kato
Department of Industrial Chemistry, Faculty of Engineering, Tohwa University, 1-1-1 Chikushigaoka, Minami-ku, Fukuoka 815-8510, Japan

K. Noguchi
Noguchi General Institute Co. Ltd, 4-5-8 Kokyu, Miyazaki 880-0913, Japan

Keywords Angiogenesis · Anti-tumor agent · Catalyser-21TM · Hydrogen peroxide · Invasion · Matrix metalloproteinase-2 (MMP-2) · Oxidative stress · Vascular endothelial growth factor (VEGF)

Introduction

Approximately 30 million years ago, geographical instability led to movements of the Earth's mantle and created isolated seawater lakes enclosing various living organisms such as fish, shellfish, sea weeds, mosses, microorganisms, and minerals. Further mantle movements, land slides and volcanic activity could have overlaid these isolated lakes with soil and/or ash. For many years thereafter, fermentation and decomposition reactions involving these organic materials, mineral substances and microorganisms in the lake bottom produced a unique stratum. At present, this stratum is situated approximately 40 m beneath the Earth's surface. Material excavated from this depth has been further fermented with soil bacteria in a bioreactor followed by three consecutive filtration steps to produce a mineral water designated Catalyser-21TM. Due to the origin of the stratum from which it is derived, numerous substances including minerals, amino acids, vitamins, lipo-amino acids and carbohydrates are expected to be contained in Catalyser-21TM.

Catalyser-21TM was reported to induce the antitumor cytokine interleukin (IL)-12 p40 subunit in murine macrophage cell lines and BALB/C mouse-derived peritoneal cavity macrophages (Kawahara et al. 2001). Another study of Catalyser-21TM demonstrated IL-12 p40 subunit-inducing substances in the fraction containing nine carbohydrates (Kato et al. 2005). These results suggest that constituents of Catalyser-21TM might act as anticancer agents.

A fundamental process of tumor development and metastatic spread is angiogenesis—the formation of new blood vessels. Tumor angiogenesis begins with the secretion by tumor cells of angiogenic factors, especially vascular endothelial growth factor (VEGF)-A (Hicklin and Ellis 2005), which plays a pivotal role in the regulation of normal and abnormal angiogenesis (Ferrara 1993; Takahashi and Shibuya 2005). VEGF-A is overexpressed at both mRNA and protein levels in a high percentage of malignant animal and human tumors and in many immortalized and transformed cell lines (Berse et al. 1992; Brown

et al. 1993a, b; Sato et al. 1994). The VEGF-A gene transcript undergoes alternative splicing to yield nine isoforms of 121, 145, 148, 162, 165b, 165, 183, 189, and 206 amino acids. VEGF₁₆₅ appears to predominate quantitatively and functionally in most angiogenic states (Takahashi and Shibuya 2005). VEGF₁₂₁ and VEGF₁₆₅ are secreted as soluble compounds, whereas VEGF₁₈₉ and VEGF₂₀₆ remain associated with the cell surface or are primarily deposited in the extracellular matrix (Ferrara 1993). The matrix metalloproteinases (MMPs) also play a key role in tumor metastasis. MMP-2 is one of the enzymes used by cancer cells to degrade type IV collagen, a major component of the basement membrane (Liotta 1986; Chakrabarti 2005). Increased expression of MMP-2 and VEGF, which is strongly related to intracellular oxidative status, is now known to be a characteristic of most malignant tumors (Berse et al. 1992; Brown et al. 1993a, b; Eyries et al. 2004; Iwata et al. 1996; Monte et al. 1997; North et al. 2005; Sato et al. 1994; Tokuraku et al. 1995; Ushio-Fukai 2006). Furthermore, cancer cells produce large amounts of H₂O₂, thereby creating an intracellular oxidative state (Szatrowski et al. 1991).

Recently, a nutrient mixture containing lysine, proline, arginine, ascorbic acid, and green tea extract was shown to be effective in the inhibition of cancer development and spread via retardation of MMP expression (Roomi et al. 2006a, b, c). This suggests that other combinations of amino acids and appropriate substances could have an anticancer effect. Catalyser-21TM is thought to be a candidate anticancer agent due to its constituents such as carbohydrates, amino acids, and other substances. However, other than for carbohydrates, detailed studies correlating Catalyser-21TM constituents with antitumor activity have not been performed. As a first step in the characterization of Catalyser-21TM, we investigated whether it has H₂O₂ scavenging potential and whether it suppresses VEGF and MMP-2 expression in cancer cells.

Materials and methods

Preparation of Catalyser-21TM stock solution and reagents

Crude Catalyser-21TM solution was obtained from Noguchi General Laboratory (Miyazaki, Japan). The

solution (pH 2.8–2.9) was neutralized to pH 7.0 with NaOH. The particulates were removed by centrifugation at 2000×g for 15 min. The supernatant was then sterilized by filtration through a 0.2 µm pore filter (Millipore, MA, USA) and stored as 100% Catalyser-21TM stock solution at 4°C. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Sigma Chemical Co. (St Louis, MO, USA). WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was purchased from Kishida (Osaka, Japan). Matrigel was purchased from Funakoshi (Tokyo, Japan). A human VEGF immunoassay kit (QuantiGlo[®]) was purchased from R&D Systems (Minneapolis, MN, USA). An angiogenesis tubule staining kit (for staining von Willebrand factor) was obtained from TCS Cellworks (Buckingham, UK).

Cell culture and treatment

HeLa cells (a human cervical cancer cell line), HT1080 cells (a human fibrosarcoma cell line), and TIG-1 cells (normal human cells) were obtained from Health Science Research Resources Bank and cultured in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Biowest, France). Human umbilical vein endothelial cells (HUVEC) were obtained from Kurabo (Osaka, Japan) and were cultured in EBM-2 medium (Cambrex, MD, USA). Catalyser-21TM was mixed with 10× MEM that had been diluted with MilliQ water.

Cell viability assay

HeLa cells and HT1080 cells, each at 5×10^5 cells/well, were cultured in a 24-well microtiter plate with MEM containing 10% FBS in a 5% CO₂ atmosphere for 24 h at 37°C. At the end of the incubation period, the culture medium was replaced with fresh MEM containing 10% FBS, and various concentrations (3–50% v/v) of Catalyser-21TM were added; the incubation was then continued for another 24 h. The medium was then removed and 500 µl of WST-1 reagent was added to each well, followed by incubation for 2 h at 37°C. The absorbance of the treated and untreated samples was measured using a microtiter plate reader (Tecan Spectra, Wako, Japan) at 450 nm.

Measurement of H₂O₂ generated by cancer cells

Endogenous H₂O₂ production in HeLa cells was measured using DCFH-DA (Molecular Probes Inc., Eugene, OR, USA) as a substrate. DCFH-DA is hydrolyzed by cellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by the action of intracellular H₂O₂. HeLa and HT1080 cells were pretreated with MEM containing Catalyser-21TM for 30 min, then incubated with 5 µM DCFH-DA for 30 min at 37°C. The cells were washed with phosphate-buffered saline to remove the DCFH-DA. H₂O₂ levels were measured using flow cytometry (EPICS XL System II; Beckman Coulter, USA) by determining the intensity of the fluorescence relative to that of control cells.

Matrigel invasion assay

A 24-well microtiter plate was coated with 700 µl of serum-free MEM containing 10 µg of fibronectin; 8 µm chambers (Kurabo, Osaka, Japan) were then coated with 20 µl of Matrigel on the upper parts and 10 µl of collagen on the lower parts. The chambers were then placed in the 24-well microtiter plate. HT1080 cells were pretreated with Catalyser-21TM for 24 h, then inoculated at 1×10^5 cells/well onto the chamber and incubated for 12 h at 37°C. The cells that invaded the lower collagen surface were fixed and stained with Diff-Quik (Sysmex, IL, USA). The invading cells were counted in three random fields under a light microscope.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using a GenEluteTM Mammalian Total RNA kit (Sigma, St Louis, MO, USA) following the protocol provided by the supplier. The primer sequences for cDNA synthesis and PCR amplification were: GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse); MMP-2, 5'-TTCTATGCTGCCCAAGGAGAGCTGCAAC-3' (forward) and 5'-CAGCTCAGCAGCCTAGCCAGTCGGATTGA-3' (reverse). The common primers for VEGF amplification were 5'-GGGCCTCCGAAACCATGAC-3' (forward) and 5'-CTGGTTCCCGAAACCTGAG-3' (reverse), which simultaneously generate

DNA fragments of 634 bp for VEGF₁₆₅ and 502 bp for VEGF₁₂₁ (Tischer et al. 1991; Takahashi and Shibuya 2005). The PCR conditions were 94°C for 3 min, 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 30 cycles for GAPDH; 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min for 30 cycles for MMP-2 and VEGF. The RT-PCR products were resolved by 1% agarose gel electrophoresis and photographs were documented by a digital camera (Print Graph AE-6915DX, ATTO, Tokyo, Japan). For densitometric analysis, recorded images were analyzed by the NIH image analyzer program (Image 1.62f) using a personal computer. The amount of template DNA was varied per assay, and a linear range of the band intensity of RT-PCR product versus template DNA was determined to measure gene expression level semi-quantitatively.

Measurement of VEGF secretion in culture medium

HeLa cells (5×10^4 cells/well) were seeded in a 24-well microtiter plate with MEM containing 10% FBS and incubated overnight. The medium was replaced with serum-free MEM containing Catalyser-21TM and incubated for another 24 h. The conditioned medium was collected to measure VEGF, according to the manufacturer's instructions of the human VEGF immunoassay kit. The monoclonal antibody in the kit recognizes not only VEGF₁₆₅ but also both VEGF₁₂₁ and naturally occurring human VEGF.

Preparation of conditioned medium and tubule formation assay using co-culture system

HeLa cells (1×10^6 cells) were seeded in a 100 mm dish with MEM containing 10% FBS and incubated for 24 h. The spent medium was then replaced with serum-free MEM or MEM containing Catalyser-21TM and the cells were incubated for an additional 72 h to generate conditioned medium. The conditioned medium was collected and filtered through a 0.2 µm filter. Aliquots were stored in a refrigerator at -80°C. HUVEC were mixed with TIG-1 cells in the proportion 1:40, seeded in a 24-well microtiter plate in EBM-2 medium and incubated overnight. The medium was then removed and a mixture of HeLa cell conditioned medium and EBM-2 medium in the proportion 2:1 was added to the co-cultured cells. The conditioned medium was changed every

2 days. On day 11, tubule formation was detected by the tubule staining kit. Tubule formation in the co-culture system was visualized under a phase-contrast microscope, and photomicrographs were taken with a digital camera (Olympus, Japan). The images were analyzed using software for the quantification of angiogenesis (AngioSys 1.0; TCS CellWorks, UK). Twelve random fields per well were taken for assessment of tubule formation.

Results

Effect of Catalyser-21TM on proliferation of HeLa cells

To examine the effect of Catalyser-21TM on HeLa cell growth, cells were treated with different concentrations (3–50%) of Catalyser-21TM for 2 days and cell proliferation was evaluated by a WST-1 assay. As shown in Fig. 1, the viability of HeLa cells decreased in a dose-dependent manner. HT1080 cells subjected to the same viability test also showed a dose-dependent reduction of viable cell numbers (data not shown). These results indicate that Catalyser-21TM contains an agent or agents that could

Fig. 1 Effect of Catalyser-21TM on HeLa cell proliferation. HeLa cells were cultured in a 24-well microtiter plate with MEM containing 10% FBS for 24 h. The culture medium was then removed and replaced with MEM containing 10% FBS and various concentrations (3–50%) of Catalyser-21TM, and the cells were incubated for another 24 h. A WST-1 assay was performed, and the data are expressed as the mean $\pm$ SD of three independent experiments

regulate cancer cell growth. To minimize the toxic effect, if any, of Catalyser-21TM in the study, a 3% concentration was used throughout the experiments.

Intracellular H₂O₂ scavenging activity of Catalyser-21TM in HeLa cells

As cancer cells are reported to produce large amounts of H₂O₂, we investigated whether Catalyser-21TM can act as an H₂O₂ scavenger. H₂O₂ production was measured by flow cytometry. A shift of the signal curve obtained with Catalyser-21TM-treated HeLa cells toward the left compared with that of untreated control HeLa cells would indicate scavenging of H₂O₂. Pretreatment of HeLa cells for 30 min with MEM containing 3% Catalyser-21TM resulted in a reduction of intracellular H₂O₂, as the signal curve was shifted to the left (Fig. 2). We performed the same assay using HT1080 cells and obtained similar results (data not shown). These findings indicate that Catalyser-21TM can scavenge endogenous H₂O₂ in two cancer cell types and imply that this effect could be applicable to other types of cancer.

Fig. 2 Detection of H₂O₂ scavenging activity of Catalyser-21TM. Cultured HeLa cells were pretreated for 30 min with MEM containing 10% FBS with 3% Catalyser-21TM, then incubated with 5 μ M DCFH-DA for 30 min at 37°C. The fluorescence intensity of DCF was measured with a flow cytometer. The fluorescence intensity relative to that of control cells is presented as curves. Curve (a) is the fluorescence intensity obtained from control HeLa cells. Curve (b) is the fluorescence intensity obtained from HeLa cells treated with 3% Catalyser-21TM. H₂O₂ scavenging activity was judged positive, as the Catalyser-21TM-treatment curve (b) was shifted to the left compared with the control curve (a)

Catalyser-21TM down-regulates VEGF gene expression in HeLa cells

Solid tumor cells stimulate angiogenesis by releasing many angiogenic factors, including VEGF. We first examined transcription of the VEGF gene with and without Catalyser-21TM treatment. RT-PCR was used to detect VEGF transcripts as a measure of angiogenesis and GAPDH transcripts as an internal standard. As shown in Fig. 3A, transcription of VEGF₁₂₁ was reduced by 35% compared with the control level after 24 h of incubation in medium containing Catalyser-21TM, while VEGF₁₆₅ transcription showed little change. This is noteworthy because VEGF isoforms are generated as a result of alternative splicing of the primary VEGF mRNA transcript, and our findings imply that Catalyser-21TM impedes one or more of the steps involved in this differential splicing.

We then measured secreted VEGF protein to determine whether the reduction in mRNA was correlated with a reduction in the secretion of VEGF in HeLa cells. Catalyser-21TM reduced the secretion of VEGF protein approximately threefold compared with the amount observed with untreated control HeLa cells (Fig. 3B). Thus, the release of VEGF protein into the culture medium appears to be regulated by mRNA levels.

Catalyser-21TM down-regulates vascular tubule formation

VEGF remodels the vasculature by inducing proliferation and differentiation of endothelial cells, leading to increased permeability that in turn induces abnormal angiogenesis (Yancopoulos et al. 2000). Conversely, angiogenesis could be expected to be suppressed by reduction of VEGF secretion. Using a HUVEC/TIG-1 co-culture system, we investigated whether reduced release of VEGF from HeLa cells treated with Catalyser-21TM affected vascular tubule formation. Four criteria (total area, number of junctions, number of tubules, and total tubule length) were measured and compared for untreated HeLa cells (Cont), conditioned medium produced by HeLa cells (HeLa CM), and conditioned medium produced by Catalyser-21TM-treated HeLa cells (Catalyser-HeLa CM) (Fig. 4). Representative photographs are shown in Fig. 4A, and the images were quantified as

Fig. 3 Suppression of VEGF transcription (**A**) and protein secretion (**B**) in HeLa cells by Catalyser-21™. (**A**) HeLa cells were cultured for 24 h in MEM containing 10% FBS. The culture medium was then removed and serum-free MEM containing 3% Catalyser-21™ was added, followed by an additional 24 h incubation. VEGF and GAPDH transcripts were detected by RT-PCR using total RNA isolated from the treated and untreated HeLa cells and appropriately designed primers. Lane a, DNA fragments were amplified from total RNA of untreated HeLa cells. Lane b, DNA fragments were amplified from total RNA of 3% Catalyser-21™-treated HeLa cells. Amplified products were resolved by agarose gel electrophoresis and photographs were documented by a digital camera. Recorded images were analyzed by the NIH Image analyzer program (Image 1.62f) using a personal computer. Values below the panel were normalized by arbitrarily setting the density of the VEGF₁₆₅ and VEGF₁₂₁ bands of untreated HeLa cells to 1.0. GAPDH transcripts were used as an internal control for cellular activity. (**B**) HeLa cells were treated as above and each supernatant was collected to measure VEGF secretion. The procedure for VEGF protein measurement is described in the Materials and methods. Differences were analyzed by Student's *t* test (values are the mean $\pm$ SD; *n* = 3) and the asterisk represents a significant difference compared with the control (**p* < 0.05)

shown in Fig. 4B. All four criteria of tubule formation were increased with in the HUVEC/TIG-1 co-culture system. With Catalyser-HeLa CM, all criteria were significantly reduced except the number of tubules formed (*p* < 0.05; *n* = 3). Catalyser-21™

itself did not affect the tube formation (data not shown). These results strongly suggest that Catalyser-21™ contains a factor or factors that down-regulate VEGF transcription, leading to reduced tubule formation (i.e. suppression of angiogenesis).

Catalyser-21™ suppresses MMP-2 transcription and invasiveness of HT1080 cells

MMP-2 is a key enzyme involved in cancer metastasis. As a preliminary experiment, we determined whether Catalyser-21™ has an antimetastatic effect against HT1080 cells. These cells are from a highly metastatic and aggressive human fibrosarcoma of connective tissue origin (Papagelopoulos et al. 2002). We first examined MMP-2 transcription using RT-PCR, and compared MMP-2 mRNA levels and metastasis using a Matrigel invasion assay. As shown in Fig. 5A, Catalyser-21™ reduced MMP-2 expression by about 19% compared with GAPDH, suggesting reduced breakdown of the extracellular matrix. We therefore extended our investigation to determine whether down-regulated MMP-2 expression could be related to reduced invasiveness. The results, shown in Fig. 5B, indicate that HT1080 cell invasion was significantly decreased when the cells were treated with Catalyser-21™ (**p* < 0.05; *n* = 3). These data together suggest that Catalyser-21™ suppressed the invasiveness of HT1080 cells as a result of down-regulation of MMP-2 gene transcription and a consequent reduction of MMP-2 protein secretion.

Discussion

This study attempted to determine whether Catalyser-21™, a natural product, has an anticancer effect. Using HeLa and HT1080 cells, our results indicate that Catalyser-21™ does indeed have anticancer effects via reduction of VEGF and of MMP-2 gene transcription and protein secretion. In recent years, natural antioxidants in foodstuffs have attracted much attention because of their health-promoting and disease-preventing effects, including cancers (Jung et al. 2001; Oak et al. 2003, 2005; Tang et al. 2006). A mixture of green tea extract, vitamin E, and several amino acids was shown to reduce the development of cancer and metastasis by suppressing MMP

Fig. 4 Effect of Catalyser-21TM on HeLa cell conditioned medium-induced vascular tubule formation. A HUVEC/TIG-1 co-culture in a 24-well plate was challenged with mixtures of EGM-2 medium and non-conditioned MEM (Cont), HeLa cell conditioned MEM (HeLa CM) and Catalyser-21TM-treated HeLa cell conditioned MEM (Catalyser-HeLa CM) in the proportion 1:2, respectively. Each medium was changed every 2 days. On day 11, tubule formation was detected by a tubule staining kit and visualized under a phase-contrast microscope.

Digital images were taken, and representative photographs are shown (A). The images were analyzed using angiogenesis quantification software (B). Twelve random fields per well were used for assessment of tubule formation. Data are expressed as percentages of the total area, number of junctions, number of tubules, and total tube length in untreated control cells (mean $\pm$ SE; $n = 3$). The asterisk represents a significant difference compared with the control (* $p < 0.05$) and p values of < 0.05 are considered statistically significant

expression (Roomi et al. 2006a, b, c). Based on these reports and the natural history of Catalyser-21TM, several substances yet to be identified in this agent are likely to be responsible for the anticancer effects observed in the present study.

The development, progression, and metastasis of cancer involve multiple factors, including VEGF and MMPs. When a tumor grows to a certain size, its further enlargement is limited unless a supply of nutrients, oxygen, and other factors can be secured by the development of a new vascular system, a process called angiogenesis. VEGF serves as a major angiogenic factor by initiating the formation of capillaries through the recruitment and proliferation of endothelial cells (Lelkes et al. 1998). It has been known for

several years that, in contrast to normal cells, cancer cells produce more reactive oxygen species (ROS) and can withstand a high level of oxidative stress that triggers ROS-sensitive gene expression (Gupta et al. 1999). ROS, particularly H₂O₂, are reported to up-regulate VEGF and MMP-2 gene expression (Belkhir et al. 1997; Yoon et al. 2002; Cho et al. 2001; Chua et al. 1998; Kuroki et al. 1996) and thus play an important role in angiogenesis (Yoshikawa 2002; Monte et al. 1997; Stone and Collins 2002; Qian et al. 2003; Ushio-Fuaki 2006; Chua et al. 1998; Inoue et al. 2001). Although nine isoforms of VEGF-A are known to be generated by alternative splicing, most cells appear to produce predominantly VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ (Takahashi and

Fig. 5 Suppression of MMP-2 expression (**A**) and invasiveness (**B**) of HT1080 cells by Catalyser-21TM. (**A**) HT1080 cells were cultured for 24 h with MEM containing 10% FBS. The culture medium was replaced by fresh serum-free MEM containing Catalyser-21TM and the cells were incubated for another 24 h. Total RNA was purified from Catalyser-21TM-treated and untreated HT1080 cells and used for RT-PCR templates. Amplified products were resolved by agarose gel electrophoresis and analyzed as described in the legend of Fig. 3. Lane a, DNA fragments amplified from total RNA of untreated HT1080 cells. Lane b, DNA fragments amplified from total RNA of 3% Catalyser-21TM-treated HT1080 cells. Values below the panels were normalized by arbitrarily setting the density of the MMP-2 and GAPDH bands of untreated HT1080 cells to 1.0. GAPDH transcripts were used as an internal control for cellular activity. (**B**) HT1080 cells were pretreated with Catalyser-21TM for 24 h and then inoculated at 1×10^5 cells/well of the Matrigel chamber. The chambers were incubated at 37°C for 12 h, after which the cells that had invaded the lower collagen surface were fixed and stained with Diff-Quick (Sysmex, IL, USA). The invading cells were counted in three random fields under a light microscope. Differences were analyzed by Student's *t* test (values are the mean $\pm$ SD; *n* = 3) and the asterisk represents a significant difference compared with the control (**p* < 0.05)

Shibuya 2005). They are known to differ in their solubility; VEGF₁₈₉ is completely cell matrix bound, VEGF₁₂₁ is released from the cell, and VEGF₁₆₅ has intermediate properties. A rabbit corneal angiogenesis assay revealed that the VEGF₁₂₁ isoform is more angiogenic and tumorigenic than VEGF₁₆₅ and VEGF₁₈₉ (Zhang et al. 2000). A possible explanation is their solubility; VEGF₁₆₅ contains 15 basic amino acid residues encoded by exon 7 (Ferrara and Henzel 1989; Keck et al. 1989; Leung et al. 1989), giving rise to a moderate affinity for heparin, while VEGF₁₂₁

does not contain such residues and thus is freely diffusible from the cell (Zhang et al. 2000). Considering all of these data, it is reasonable to hypothesize that a reduction in the intracellular oxidative state could down-regulate VEGF and MMP-2 gene expression, and consequently suppress angiogenesis and cancer metastasis. The present results are intriguing because Catalyser-21TM scavenged H₂O₂ in HeLa and HT1080 cells, and specifically reduced VEGF₁₂₁ in HeLa cells. Catalyser-21TM also significantly reduced vascular tubule formation. These results indicate that Catalyser-21TM contains anti-angiogenic factors.

In concert with VEGF-A, MMP-2 and MMP-9 play key roles in cancer cell invasion, metastasis and angiogenesis through digesting the extracellular matrix (Chakarabarti and Patel 2005). MMPs degrade the type IV collagen of the basement membrane, which is the front-line barrier against cancer invasion. Although the relationship between MMP levels and tumor progression is not necessarily strong in all neoplasias (Talvensaaari-Mattila et al. 1998; Scorilas et al. 2001), there are reports indicating that they are closely linked (Brooks 1998; Itoh 1998). Thus, regulation of MMP-2 and MMP-9 activity remains a way of controlling cancer progression. A brown algal extract was shown to inhibit MMP activity and was proposed as a candidate antimetastatic agent as well as for use in other chronic disorders (Ye et al. 2005; Kim 2006). It has been reported that a mixture of four herb extracts inhibits the invasive potential of hepatocellular carcinoma by inhibiting MMP activity (Ha et al. 2004), and an approach to the development of an anticancer agent by inhibiting MMP activity has been demonstrated (Roomi et al. 2006a, b, c). Our preliminary study aimed to determine whether Catalyser-21TM has a suppressive effect on cancer cells via MMP-2 transcription, and on invasive ability using a Matrigel invasion assay. Catalyser-21TM had an inhibitory effect on HT1080 cell invasiveness via inhibition of MMP-2 expression.

It could be concluded that Catalyser-21TM, through intracellular H₂O₂ elimination, decreased MMP-2 and VEGF expression, consequently inhibiting cancer cell invasion and angiogenesis. Our characterization of Catalyser-21TM gave promising results, and further studies are required to elucidate which of the ingredients is responsible for its effects.

References

- Belkhir A, Richards C, Whaley M, McQueen SA, Orr FW (1997) Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal H₂O₂ exposure. *Lab Invest* 77:533–539
- Berse B, Brown LF, Van de Water L, Dvorak HF, Senger DR (1992) Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol Biol Cell* 3:211–220
- Brooks PC, Silletti S, von Schalscha TL, Friedlander M, Chersesh DA (1998) Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. *Cell* 92:391–400
- Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF, Senger DR (1993a) Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. *Am J Pathol* 143:1255–1262
- Brown PD, Bloxidge RE, Stuart NS, Gatter KC, Carmichael J (1993b) Association between expression of activated 72-kilodalton gelatinase and tumour spread in non-small-cell lung carcinoma. *J Natl Cancer Inst* 85:574–578
- Chakarabarti S, Patel KD (2005) Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Exp Lung Res* 31:599–621
- Cho M, Hunt TK, Hussain MZ (2001) Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release. *Am J Physiol Heart Circ Physiol* 280:H2357–H2363
- Chua CC, Hamdy RC, Chua BH (1998) Upregulation of vascular endothelial growth factor by H₂O₂ in rat heart endothelial cells. *Free Radic Biol Med* 25:891–897
- Eyries M, Collins T, Khachigian LM (2004) Modulation of growth factor gene expression in vascular cells by oxidative stress. *Endothelium* 11:133–139
- Ferrara N (1993) Vascular endothelial growth factor. *Trends Cardiovasc Med* 3:244–250
- Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161:851–858
- Gupta A, Rosenberger SF, Bowden GT (1999) Increased ROS levels contribute to elevated transcription factor and MAP kinase activities in malignant progressed mouse keratinocyte cell lines. *Carcinogenesis* 20:2063–2073
- Ha KT, Kim JK, Lee YC, Kim CH (2004) Inhibitory effect of Daesungki-Tang on the invasiveness potential of hepatocellular carcinoma through inhibition of matrix metalloproteinase-2 and -9 activities. *Toxicol Appl Pharmacol* 200:1–6
- Hicklin DJ, Ellis LM (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 23:1011–1027
- Inoue M, Itoh H, Tanaka T, Chun T-H, Doi K, Fukunaga Y, Sawada N, Yamashita J, Masatsugu K, Saito T, Sakaguchi S, Sone M, Yamahara K-i, Yurugi T, Nakao K (2001) Oxidized LDL regulates vascular endothelial growth factor expression in human macrophages and endothelial cells through activation of peroxisome proliferator-activated receptor-γ. *Arterioscler Thromb Vasc Biol* 21:560–566
- Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S (1998) Reduced angiogenesis and tumour progression in gelatinase A-deficient mice. *Cancer Res* 58:1048–1051
- Iwata H, Kobayashi S, Iwase H, Masaoka A, Fujimoto N, Okada Y (1996) Production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human breast carcinomas. *Jpn J Cancer Res* 87:602–611
- Jung YD, Kim MS, Shin BA, Chay KO, Ahn BW, Liu W, Bucana CD, Gallick GE, Ellis LM (2001) EGCG, a major component of green tea, inhibits tumor growth by inhibiting VEGF induction in human colon carcinoma cells. *Br J Cancer* 84:844–850
- Kato Y, Ohta M, Kamei K, Wakita H, Kawahara T, Yamano Y, Hamasaki T, Teruya K, Osada K, Nishikawa R, Noguchi K, Shirahata S (2005) Structure analysis of complex carbohydrate exhibiting IL-12 inducing activity derived from mineral water in natural leaf soil. *Bunseki Kagaku* 54:391–397
- Kawahara T, Teruya K, Katakura Y et al (2001) Anti-tumor activity of Noguchi Catalyser-21 TM, a mineral water containing natural leaf soil. In: Lindner-Olsson et al (eds) *From target to genes. Proceedings of the 17th meeting of European Society for Animal Cell Technology*, June 2001. Kluwer Academic Publishers, the Netherlands, pp 10–14
- Keck PJ, Houser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246:1309–1312
- Kim MM, Ta QV, Mendis E, Rajapakse N, Jung WK, Byun HG, Jeon YJ, Kim SK (2006) Phlorotannins in *Ecklonia cava* extract inhibit matrix metalloproteinase activity. *Life Sci* 79:1436–1443
- Kuroki M, Voest EE, Amano S, Beerepoot LV, Takashima S, Rohan MT, Colby KA, Yeo KT, Adamis AP (1996) Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo. *J Clin Invest* 98:1667–75
- Lelkes PI, Hahn KA, Karmiol S et al. (1998) Hypoxia/reoxygenation enhances tube formation of cultured human micro-vascular endothelial cells: the role of reactive oxygen species. In: Maragoudakis ME (ed). *Angiogenesis: models, modulators, and clinical applications*. Plenum Press, New York, pp 321–336
- Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246:1306–1309
- Liotta LA (1986) Tumor invasion and metastases-role of the extracellular matrix: Rhoads Memorial Award Lecture. *Cancer Res* 46:1–7
- Monte M, Davel LE, de Lustig ES (1997) Hydrogen peroxide is involved in lymphocyte activation: mechanisms to induce angiogenesis. *Eur J Cancer* 33:676–682
- North S, Moenner M, Bikfalvi A (2005) Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. *Cancer Lett* 218:1–14

- Oak MH, Bedoui JEL, Schini-Kerth VB (2005) Antiangiogenic properties of natural polyphenols from red wine and green tea. *J Nutr Biochem* 16:1–8
- Oak MH, Chataigneau M, Keravis T, Chataigneau T, Beretz A, Andriantsitohaina R, Stoclet JC, Chang SJ, Schini-Kerth VB (2003) Red wine polyphenolic compounds inhibit vascular endothelial growth factor expression in vascular smooth muscle cells by preventing the activation of the p38 mitogen-activated protein kinase pathway. *Arterioscler Thromb Vasc Biol* 23:1001–1007
- Papageopoulos PJ, Galanis EC, Trantaffyllidis P, Boscainos PJ, Sim FH, Unni KK (2002) Clinicopathologic features, diagnosis, and treatment of fibrosarcoma of bone. *Am J Orthop* 31:253–257
- Qian Y, Luo J, Leonard SS, Harris GK, Millicchia L, Flynn DC, Shi X (2003) Hydrogen peroxide formation and actin filament reorganization by Cdc42 are essential for ethanol-induced in vitro angiogenesis. *J Biol Chem* 278:16189–16197
- Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M (2006a) In vivo and in vitro antitumor effect of ascorbic acid, lysine, proline, arginine, and green tea extract on human fibrosarcoma cells HT-1080. *Med Oncol* 23:105–112
- Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M (2006b) In vivo and in vitro antitumor effect of a unique nutrient mixture on lung cancer cell line A-549. *Exp Lung Res* 32:441–453
- Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M (2006c) Inhibition of matrix metallo- proteinase-2 secretion and invasion by human ovarian cancer cell line SK-OV-3 with lysine, proline, arginine, ascorbic acid, and green tea extract. *J Obstet Gynaecol Res* 32:148–154
- Sato K, Terada K, Sugiyama T, Takahashi S, Saito M, Moriyama M, Kakinuma H, Suzuki Y, Kato M, Kato T (1994) Frequent overexpression of vascular endothelial growth factor gene in human renal cell carcinoma. *Tohoku J Exp Med* 173:355–360
- Scorilas A, Karamelis A, Arnogiannaki N (2001) Overexpression of matrix- metalloproteinase-9 in human breast cancer: a potential favorable indicator in node- negative patients. *Br J Cancer* 84:1488–1496
- Stone JR, Collins T (2002) The role of hydrogen peroxide in endothelial proliferative responses. *Endothelium* 9:231–238
- Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 51:794–798
- Takahashi H, Shibuya M (2005) The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci* 109:227–241
- Talvensaari-Mattila A, Pääkkö P, Höyhty M, Blanco-Sequeiros G, Turpeenniemi-Hujanen T (1998) MMP-2 immunoreactive protein, a marker of aggressiveness in breast carcinoma. *Cancer* 83:1153–1162
- Tang FY, Chiang EP, Shih CJ (2006) Green tea catechin inhibits ephrin-A1-mediated cell migration and angiogenesis of human umbilical vein endothelial cells. *J Nutr Biochem* (in press) doi: 10.1016/j.jnutbio.2006.07.004
- Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA (1991) The human gene for vascular endothelial growth factor. *J Biol Chem* 266:11947–11954
- Tokuraku M, Sato H, Murakami S, Okada Y, Watanabe Y, Seiki M (1995) Activation of the precursor of gelatinase A/72 kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. *Int J Cancer* 64:355–359
- Ushio-Fuaki M (2006) Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc Res* 71:226–235
- Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. *Nature* 407:242–248
- Ye J, Li Y, Teruya K, Katakura Y, Ichikawa A, Eto H, Hosoi M, Hosoi M, Nishimoto S, Shirahata S (2005) Enzyme-digested fucoidan extracts derived from seaweed Mozuku of *Cladosiphon novae-caledoniae* kylin inhibit invasion and angiogenesis of tumor cells. *Cytotechnology* 47:117–126
- Yoon SO, Park SJ, Yoon SY, Yun CH, Chung AS (2002) Sustained production of H₂O₂ activates pro-matrixmetalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-kappa B pathway. *J Biol Chem* 277:30271–30282
- Yoshikawa T (ed) (2002) Vascular dysfunction and free radical. *Free Radic Biol Med* 33:1047–1060
- Zhang HT, Scott PAE, Morbidelli L, Peak S, Moore J, Turely H, Harris AL, Ziche M, Bicknell R (2000) The 121 amino acid isoform of vascular endothelial growth factor is more strongly tumorigenic than other splice variants in vivo. *Br J Cancer* 83:63–68