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ORIGINAL PAPER

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

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Abstract Catalyser-21^{TM} is a mineral water derived from natural leaf soil containing various organic and inorganic substances. Previous reports suggested a possibility that Catalyser-21^{TM} 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-21^{TM}, we first examined cell viability using a human cervical cancer cell line, HeLa, and a fibrosarcoma cell line, HT1080. The results showed that Catalyser-21^{TM} decreased the viability of both cell types in a dose-dependent manner. Flow cytometric analysis proved that Catalyser-21^{TM} scavenges intracellular H_2O_2 in both cell types. RT-PCR demonstrated that both VEGF and MMP-2 gene transcription was suppressed after Catalyser-21^{TM} treatment. Both Matrigel and tubule formation experiments showed an effect of Catalyser-21^{TM}. These results suggest that Catalyser-21^{TM} has potential as an anti-tumor agent.

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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-21^{TM}. 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-21^{TM}.

Catalyser-21^{TM} 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-21^{TM} demonstrated IL-12 p40 subunit-inducing substances in the fraction containing nine carbohydrates (Kato et al. 2005). These results suggest that constituents of Catalyser-21^{TM} 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. $VEGF_{165}$ appears to predominate quantitatively and functionally in most angiogenic states (Takahashi and Shibuya 2005). $VEGF_{121}$ and $VEGF_{165}$ are secreted as soluble compounds, whereas $VEGF_{189}$ and $VEGF_{206}$ 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 H_2O_2, 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-21^{TM} 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-21^{TM} constituents with antitumor activity have not been performed. As a first step in the characterization of Catalyser-21^{TM}, we investigated whether it has H_2O_2 scavenging potential and whether it suppresses VEGF and MMP-2 expression in cancer cells.

Materials and methods

Preparation of Catalyser-21^{TM} stock solution and reagents

Crude Catalyser-21^{TM} 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-21™ 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 (Mineapolice, 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-21™ 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% CO_2 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-21™ 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 H_2O_2 generated by cancer cells

Endogenous H_2O_2 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 dichlorofluorescin (DCF) by the action of intracellular H_2O_2. HeLa and HT1080 cells were pretreated with MEM containing Catalyser-21™ 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. H_2O_2 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-21™ 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 GenElute™ 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′-TTCTATGGCTGCCCCAAGGAGAGCTGCAAC-3′ (forward) and 5′-CAGCTCAGCAGCCTAGCCAGTCGGATTTGA-3′ (reverse). The common primers for VEGF amplification were 5′-GGGCCTCCGAAACCATGAAC-3′ (forward) and 5′-CTGGTTCCCGAAACCCTGAG-3′ (reverse), which simultaneously generate

DNA fragments of 634 bp for $VEGF_{165}$ and 502 bp for $VEGF_{121}$ (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,7 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-21™ 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 $VEGF_{165}$ but also both $VEGF_{121}$ 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-21™ 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 EMB-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-21™ on proliferation of HeLa cells

To examine the effect of Catalyser-21™ on HeLa cell growth, cells were treated with different concentrations (3–50%) of Catalyser-21™ 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-21™ contains an agent or agents that could

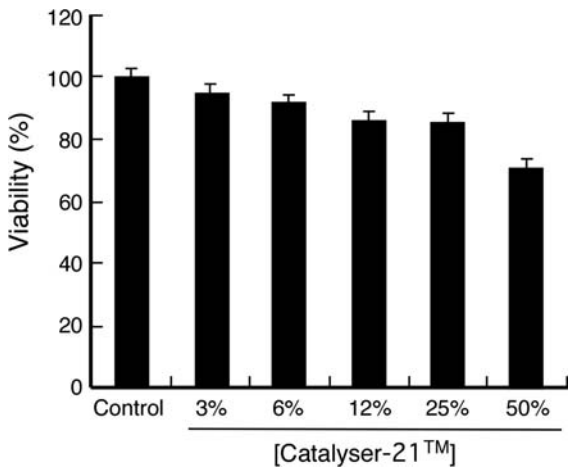

Fig. 1 Effect of Catalyser-21™ on HeLa cell proliferation. HeLa cells were cultured in a 24-well microtiter plate with MEM containing 10% FBS for 24 h. The culture medium was then removed and replaced with MEM containing 10% FBS and various concentrations (3–50%) of Catalyser-21™, and the cells were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

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

Intracellular H_2O_2 scavenging activity of Catalyser-21^{TM} in HeLa cells

As cancer cells are reported to produce large amounts of H_2O_2, we investigated whether Catalyser-21^{TM} can act as an H_2O_2 scavenger. H_2O_2 production was measured by flow cytometry. A shift of the signal curve obtained with Catalyser-21^{TM}-treated HeLa cells toward the left compared with that of untreated control HeLa cells would indicate scavenging of H_2O_2. Pretreatment of HeLa cells for 30 min with MEM containing 3% Catalyser-21^{TM} resulted in a reduction of intracellular H_2O_2, 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-21^{TM} can scavenge endogenous H_2O_2 in two cancer cell types and imply that this effect could be applicable to other types of cancer.

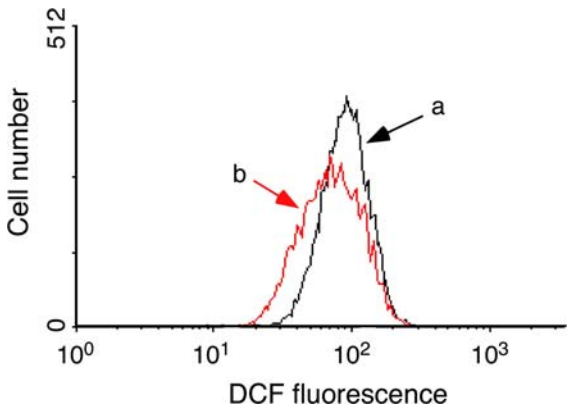

Fig. 2 Detection of H_2O_2 scavenging activity of Catalyser-21^{TM}. Cultured HeLa cells were pretreated for 30 min with MEM containing 10% FBS with 3% Catalyser-21^{TM}, then incubated with 5 μM DCFH-DA for 30 min at 37°C. The fluorescence intensity of DCF was measured with a flow cytometer. The fluorescence 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-21^{TM}. H_2O_2 scavenging activity was judged positive, as the Catalyser-21^{TM}-treatment curve (b) was shifted to the left compared with the control curve (a)

Catalyser-21^{TM} 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-21^{TM} 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 $VEGF_{121}$ was reduced by 35% compared with the control level after 24 h of incubation in medium containing Catalyser-21^{TM}, while $VEGF_{165}$ 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-21^{TM} 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-21^{TM} 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-21^{TM} 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 (Yancopolos 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-21^{TM} 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-21^{TM}-treated HeLa cells (Catalyser-HeLa CM) (Fig. 4). Representative photographs are shown in Fig. 4A, and the images were quantified as

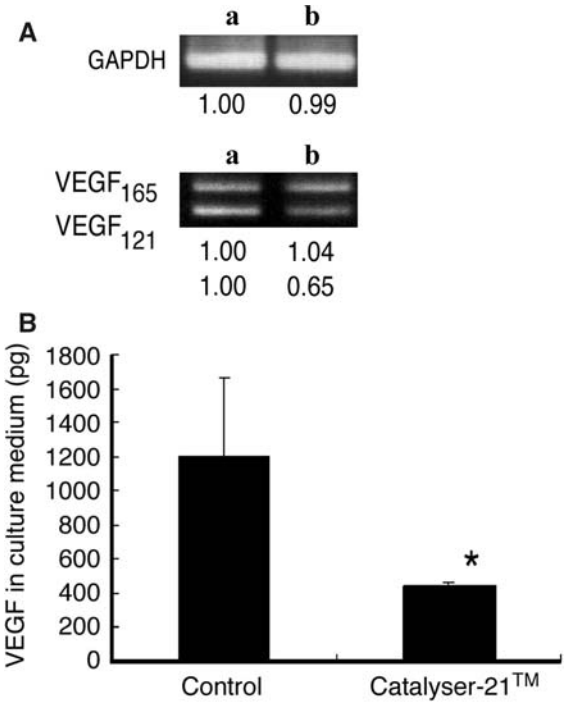

Fig. 3 Suppression of VEGF transcription (**A**) and protein secretion (**B**) in HeLa cells by Catalyser-21^{TM}**.** (**A**) HeLa cells were cultured for 24 h in MEM containing 10% FBS. The culture medium was then removed and serum-free MEM containing 3% Catalyser-21^{TM} was added, followed by an 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^{TM}-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 $VEGF_{165}$ and $VEGF_{121}$ 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 ± 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 ^{TM} itself did not affect the tube formation (data not shown). These results strongly suggest that Catalyser-21^{TM} contains a factor or factors that down-regulate VEGF transcription, leading to reduced tubule formation (i.e. suppression of angiogenesis).

Catalyser-21^{TM} 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^{TM} 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^{TM} 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 Catalyzer-21^{TM} ($^*p < 0.05$; $n = 3$). These data together suggest that Catalayser-21^{TM} 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^{TM}, a natural product, has an anticancer effect. Using HeLa and HT1080 cells, our results indicate that Catalyser-21^{TM} 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

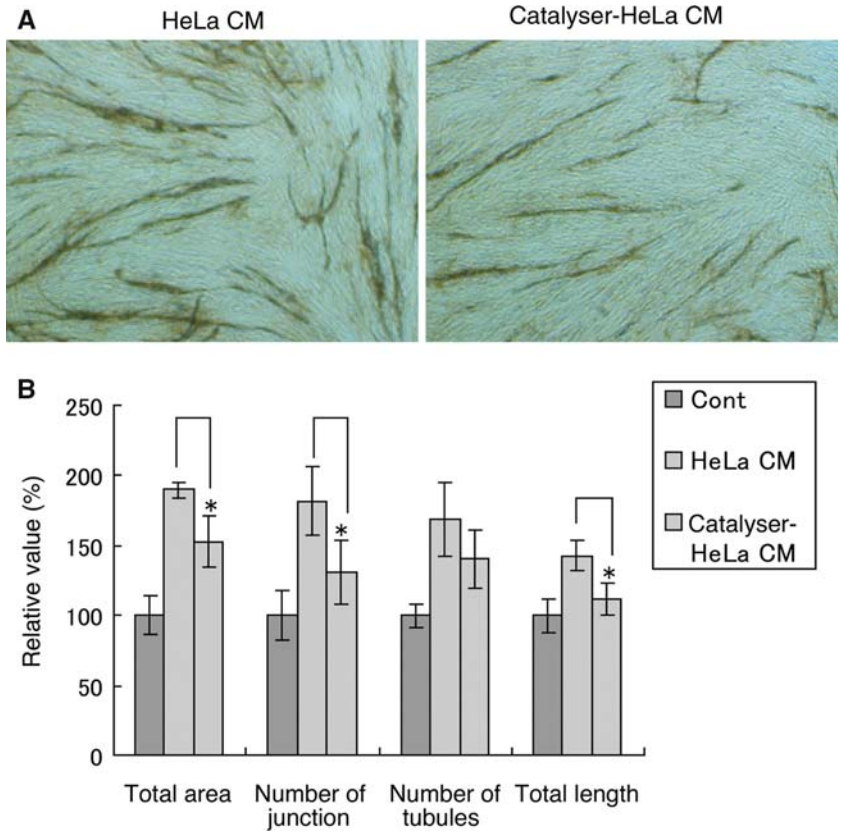

Fig. 4 Effect of Catalyser-21^{TM} on HeLa cell conditioned medium-induced vascular tubule formation. A HUVEC/TIG-1 co-culture in a 24-well plate was challenged with mixtures of EGM-2 medium and non-conditioned MEM (Cont), HeLa cell conditioned MEM (HeLa CM) and Catalyser-21^{TM}-treated HeLa cell conditioned 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 ± 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-21^{TM}, 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 H_2O_2, are reported to up-regulate VEGF and MMP-2 gene expression (Belkhiri 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 $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ (Takahashi and

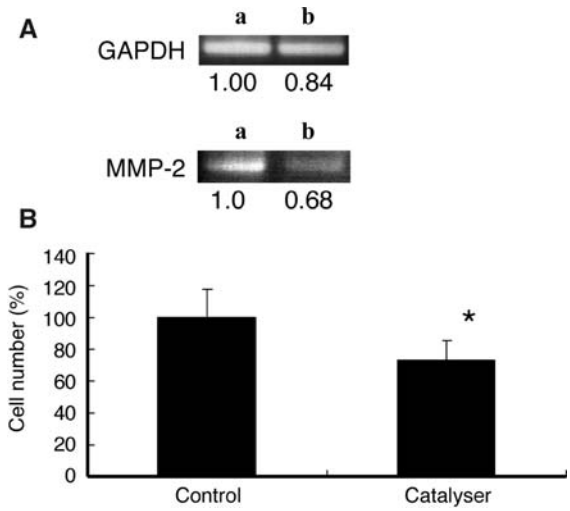

Fig. 5 Suppression of MMP-2 expression (**A**) and invasiveness (**B**) of HT1080 cells by Catalyser-21^{TM}. (**A**) HT1080 cells were cultured for 24 h with MEM containing 10% FBS. The culture medium was replaced by fresh serum-free MEM containing Catalyser-21^{TM} and the cells were incubated for another 24 h. Total RNA was purified from Catalyser-21^{TM}-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-21^{TM}-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-21^{TM} 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 ± 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; $VEGF_{189}$ is completely cell matrix bound, $VEGF_{121}$ is released from the cell, and $VEGF_{165}$ has intermediate properties. A rabbit corneal angiogenesis assay revealed that the $VEGF_{121}$ isoform is more angiogenic and tumorigenic than $VEGF_{165}$ and $VEGF_{189}$ (Zhang et al. 2000). A possible explanation is their solubility; $VEGF_{165}$ 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 $VEGF_{121}$ 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 Catalyzer-21^{TM} scavenged H_2O_2 in HeLa and HT1080 cells, and specifically reduced $VEGF_{121}$ in HeLa cells. Catalyzer-21^{TM} also significantly reduced vascular tubule formation. These results indicate that Catalyser-21^{TM} 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 (Talvensaari-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-21^{TM} has a suppressive effect on cancer cells via MMP-2 transcription, and on invasive ability using a Matrigel invasion assay. Catalyser-21^{TM} had an inhibitory effect on HT1080 cell invasiveness via inhibition of MMP-2 expression.

It could be concluded that Catalyser-21^{TM}, through intracellular H_2O_2 elimination, decreased MMP-2 and VEGF expression, consequently inhibiting cancer cell invasion and angiogenesis. Our characterization of Catalyser-21^{TM} gave promising results, and further studies are required to elucidate which of the ingredients is responsible for its effects.

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