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Role of type I- and type II-interferon in expression of melanoma differentiation-associated gene-5 in HSC-3 oral squamous carcinoma cells

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ABSTRACT

Melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid-inducible gene-I (RIG-I) are members of DExH family of proteins, and known to play important roles in antiviral responses to induce type I interferons (IFNs). MDA-5 has been thought to sense RNA virus with long (>1 kb) double-stranded RNA. However, MDA-5 is also induced by type II IFN that is involved in acquired immunity, suggesting that role of MDA-5 remains to be elucidated. In addition, no study regarding MDA-5 in oral region has been performed. Here we investigated the role of MDA-5 in HCS-3 squamous carcinoma cells derived from oral epithelial cells. Treatment of HCS-3 cells with IFN-α2b or IFN-γ significantly induced MDA-5 as well as RIG-I. IFN-α2b exerted anti-proliferative effect in HSC-3 cells while no such effect was observed in the cells treated with IFN-γ. MDA-5 is known to be associated with tumor cell growth in melanoma. However, overexpression of MDA-5 did not alter the proliferation in HSC-3 cells, indicating that MDA-5 is unrelated to the cell growth in this type of cells. We conclude that MDA-5 is induced by both type I- and type II-IFNs in HSC-3 cells, and this suggests MDA-5 may play a role in immune responses in oral cavity.

Interferons (IFNs) are cytokines that play a key role in antiviral activities, enhancement of innate and acquired immunity, and apoptosis (22). IFNs are divided into at least three distinct types: type I, II, and III (18). Type I IFNs are composed of various genes including IFN-α, -β, -ε, -κ, and -ω (18). In human, type I IFN is characterized as follows: type I IFN genes are clustered in one locus on chromosome 9 (1), are intronless (7), and bind to unique type I IFN receptor (2). Type I IFN is known to exert antiviral activities (23). Upon viral infection, viral components such as viral nucleic acids and viral proteins are sensed by pattern recognition receptors (PRRs). Subsequently, the recognition triggers activation of the antiviral signaling, leading to the production of type I IFN (12).

IFN-γ is the unique type II IFN. Although all types of cells can induce at least one of the type I IFN upon viral infection, IFN-γ is produced by T cell in response to microbial infection (21). IFN-γ exerts its biological activities by binding to a specific cell surface receptor, type II IFN receptor. In addition to its anti-viral and anti-tumor properties, IFN-γ plays an essential role in definition of Th1/Th2 balance. IFN-γ stimulates Th0 cells to differentiate into Th1 cells and to suppress Th2 differentiation (14).

In 2004, retinoic acid-inducible gene-I (RIG-I) was shown to be a cytoplasmic RNA virus sensor (25). RIG-I is an RNA helicase, consisting of N-terminal caspase domain (CARD), a central DExD/H box RNA helicase domain, and a C-terminal regula-

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tory domain (16). Melanoma differentiation-associated gene-5 (MDA-5) also has N-terminal tandem CARDs and the DExH box domain (10). Molecular analysis revealed that RIG-I and MDA-5 share structural and functional similarities (26). RIG-I and MDA-5 are both RNA virus sensors with distinct role: RIG-I recognizes relatively short double-stranded (ds) RNA and 5' triphosphate-single-stranded (ss) RNA while MDA-5 senses long dsRNA (11). Experimental evidence indicates that RNA viruses sensed by MDA-5 are limited (*e.g. picornaviridae*) whereas many other RNA viruses including Influenza virus and Hepatitis C virus are sensed by RIG-I (16). Thus, most of the research working on RNA virus sensor has been focused on the function of RIG-I; however, the role of MDA-5 is incompletely understood. MDA-5 was originally reported as a growth suppressor of melanoma (10). We found a high level of MDA-5 expression in glomeruli of patients with severe lupus nephritis or IgA nephropathy (9), and also in gastric mucosa infected with *Helicobacter pylori* (24). Of note, no significant RIG-I expression that was expected in the patient with severe lupus nephritis was observed. Collectively, MDA-5 may play multiple roles in addition to viral sensing. Following the viral RNA recognition, MDA-5 mediates its signal to a downstream adaptor molecule, mitochondrial antiviral signaling protein (MAVS), resulting in induction of type I IFN (26). As the induced IFN enhances MDA-5 expression (10), there must be a positive feedback loop in antiviral innate immune response.

Oral cavity is one of the initial sites where pathogens enter from the external environment; it is speculated that the innate immune system is highly developed in oral mucosa. To date, however, there is no report about the presence or expression of MDA-5 in oral mucosa. Here, we report the expression of MDA-5 in response to IFN in oral epithelial cell line.

MATERIAL AND METHODS

Reagents. Culture dishes and fetal calf serum (FBS) were from Asahi Techno Glass (Tokyo, Japan). Recombinant human IFN-α2b was obtained from ProSpecTany TechnoGene (East Brunswick, NJ, USA). Recombinant human IFN-γ was purchased from Roche Diagnostics (Mannheim, Germany). Antibiotic-antimycotic, FBS, oligo$(dT)_{12–18}$, Superscript II, and Trizol reagent were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant ribonuclease inhibitor Rnasin was from Promega (Madison, WI, USA). The specific primers were synthesized by Hokkaido System Science (Sapporo, Japan). Immobilon polyvinylidene fluoride (PVDF) membrane was from Millipore Japan (Tokyo, Japan). An anti-MDA-5 antibody was purchased from Immuno-Biological Laboratories (Maebashi, Japan). Monoclonal mouse anti-RIG-I antibody was from Enzo Life Sciences (Miami, FL, USA). Rabbit anti-actin antibody was from Sigma-Aldrich (St. Louis, MO, USA). Bovine anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (HRP) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. HSC-3 cells, a human oral squamous carcinoma cell line (National Institute of Health Science, Tokyo, Japan), were cultured in DMEM containing 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamicin (80 μg/mL) in an atmosphere of 95% air and 5% CO_2 at 37°C. HSC-3 cells were stimulated with a series of concentrations of IFN-α2b (0.008–5 ng/mL) and IFN-γ (0.04–25 ng/mL) for up to 48 h.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the cells using Trizol reagent. Single-stranded cDNA for a PCR template was synthesized from 1 μg total RNA using a primer oligo $(dT)_{12–18}$ and the Superscript II reverse transcriptase under the conditions indicated by the manufacturer. A CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) was used for the quantitative analyses of MDA-5, RIG-I, and 18S ribosomal RNA (rRNA). The sequences of the primers were as follows: MDA-5-F (5'-GTTGAAAAGGCTGGCTGAAAAC-3'), MDA-5-R (5'-TCGATAACTCCTGAACCACTG-3'), RIG-I-F (5'-GTGCAAAGCCTTGGCATGT-3'), RIG-I-R (5'-TGGCTTGGGATGTGGTCTACTC-3'), 18S rRNA-F (5'-ACTCAACACGGGAAACCTCA-3'), and 18S rRNA-R (5'-AACCAGACAAATCGCTCCAC-3'). The amplification reactions were performed with SsoFast EvaGreen Supermix (BioRad) according to the manufacturer's specifications. The amplification conditions were as follows: heat for 30 s at 98°C, followed by heating consecutively at 98°C and 58°C for 5 s each for 40 cycles. After amplification was completed, a melting curve was generated by slowly heating from 65°C to 95°C at 0.5°C increments with 5 s per step, with continuous monitoring of the fluorescence. The melting curves and quantitative analysis of the data were performed using a CFX manager (BioRad).

Western blot analysis. SDS-PAGE and western blot analysis were performed as previously reported (15). Briefly, after two washes with phosphate-buffered saline, pH 7.4 (PBS), the cells were lysed in hypotonic lysis buffer (10 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40) containing 0.2% protease inhibitors. The lysates were cleared by centrifugation at 12,000 rpm for 5 min at 4°C. Five micrograms of the cell lysate was subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. The proteins were then transferred to PVDF membranes, which were then blocked for 1 h at room temperature in TBST buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20) containing 1% nonfat dry milk (blocking buffer). The membranes were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-MDA-5 (1 : 250 dilution), mouse anti-RIG-I (1 : 10,000 dilution), or rabbit-β-actin (1 : 10,000 dilution). After five washes with TBST, the membranes were further incubated for 1 h at room temperature with HRP-conjugated secondary antibodies in blocking buffer. The washes were repeated using TBST, and then immunoreactive bands were visualized using the Luminata Forte Western HRP Substrate (Millipore).

Plasmid construction. To clone the full-length human MDA-5 gene, we amplified cDNA isolated from HeLa cells treated with IFN-γ for 24 h. For amplification, we used *Phusion* DNA polymerase (Thermo Scientific, Pittsburgh, PA, USA) and specific primers harboring a NotI site (shown in lowercase font) or a SalI site (shown in lowercase font), the sequences of which were as follows: MDA-NotI-F (5'-CTTgcggccgcGATGTCGAATGGGTATT CCACA-3') and MDA-SalI-R (5'-TAGAgtcgacCTA ATCCTCATCACTAAAT-3'). The products were purified, digested with NotI and SalI, and then cloned into a p3xFLAG-CMV7.1 expression vector (p3x FLAG-MDA).

Transfection. The day prior to transfection, HSC-3 cells were seeded at a density of 0.5×10^5 cells. The next day, the cells were transfected with p3xFLAG-MDA or control p3xFLAG-expression vectors using polyethylenimine (PEI)-Max (Polysciences, Warrington, PA, USA).

Microscopic analysis. Following stimulation with IFNs or transfection with MDA-5, the cells were monitored for up to 24 h under a phase-contrast microscopy (Ti-E; Nikon, Tokyo, Japan).

Statistics. Values are expressed as means S.D. (n = 3), and statistical significance was analyzed by Student's *t*-test. The probability (*P*) values were based on two-tailed tests, and $P < 0.05$ was considered to be significant.

RESULTS

Expression of MDA-5 in response to type I and type II IFNs in HSC-3 cells

Since MDA-5 is reported to be induced by IFNs (10), we first confirmed the expression of MDA-5 in response to IFNs in HSC-3 oral epithelial cell line. IFN-α2b is known to have the highest affinity to the type I IFN receptor in type I IFN family (19); indeed, IFN-α2b is used as a therapeutic agent against certain types of malignant tumor such as leukemia (13) and melanoma (6). Therefore, we chose IFN-α2b as the type I IFN in this study. The expression of MDA-5 mRNA was upregulated by stimulation with either IFN-α2b or IFN-γ in a concentration-dependent manner. The expression of MDA-5 was induced by the treatment with 0.04 ng/mL IFN-α2b (Fig. 1A). The maximal effect of IFN-α2b was observed at 1 ng/mL. A similar pattern of RIG-I mRNA expression was observed in HSC-3 cells treated with IFN-α2b (Fig. 1B). Protein levels of MDA-5 and RIG-I in response to IFN-α2b were in agreement with those of the mRNA expressions (Fig. 1C). Fig. 2 shows concentration-dependent effect of IFN-γ on MDA-5 and RIG-I expressions in HSC-3 cells. mRNA levels of both MDA-5 and RIG-I were started to increase by 0.2 ng/mL IFN-γ treatment in HSC-3 cells (Fig. 2 A, B). The protein levels of both MDA-5 and RIG-I were observed from the treatment with 0.04 ng/mL IFN-γ (Fig. 2C).

Kinetics of MDA-5 in response to IFNs in HSC-3 cells

MDA-5 mRNA was rapidly induced and reached the maximal level 2 h after treatment of the cells with IFN-α2b (Fig. 3A). RIG-I mRNA was also rapidly induced and reached the maximal level 4 h after the stimulation (Fig. 3A). The time course of MDA-5 protein production lagged slightly behind that of the mRNA expression (Fig. 3B). RIG-I production was observed 4 h after the treatment with IFN-α2b, and the increased protein levels of RIG-I was found to prolong up to 48 h after the stimulation (Fig. 3B). IFN-γ also induced mRNA expressions of MDA-5 and RIG-I in time-dependent manner, but with delayed kinetics compared to IFN-α2b (Fig. 4A). Thereafter, mRNA expression of MDA-5 and RIG-I began to increase again after 24 h, and it was a ten-

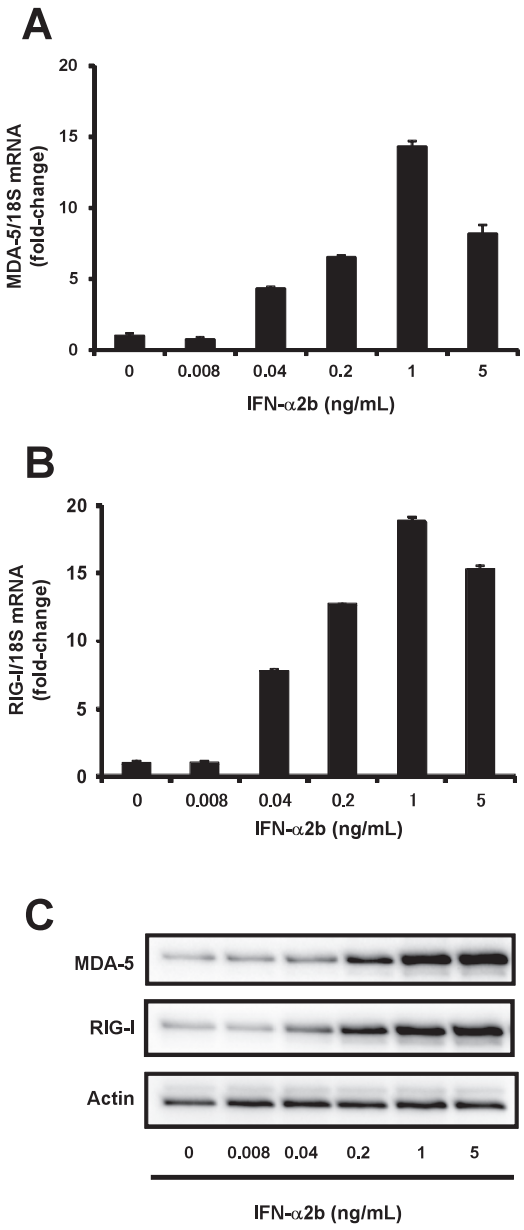

Fig. 1 IFN-α2b induces the expression of MDA-5 and RIG-I in HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with various concentrations of IFN-α2b for 4 h (A, B) or 8 h (C). Total RNA was extracted from the cells and the mRNA expressions of MDA-5 (A) and RIG-I (B) were examined by real-time RT-PCR analysis. (C) MDA-5 and RIG-I proteins in cell lysates were detected by western blot analysis.

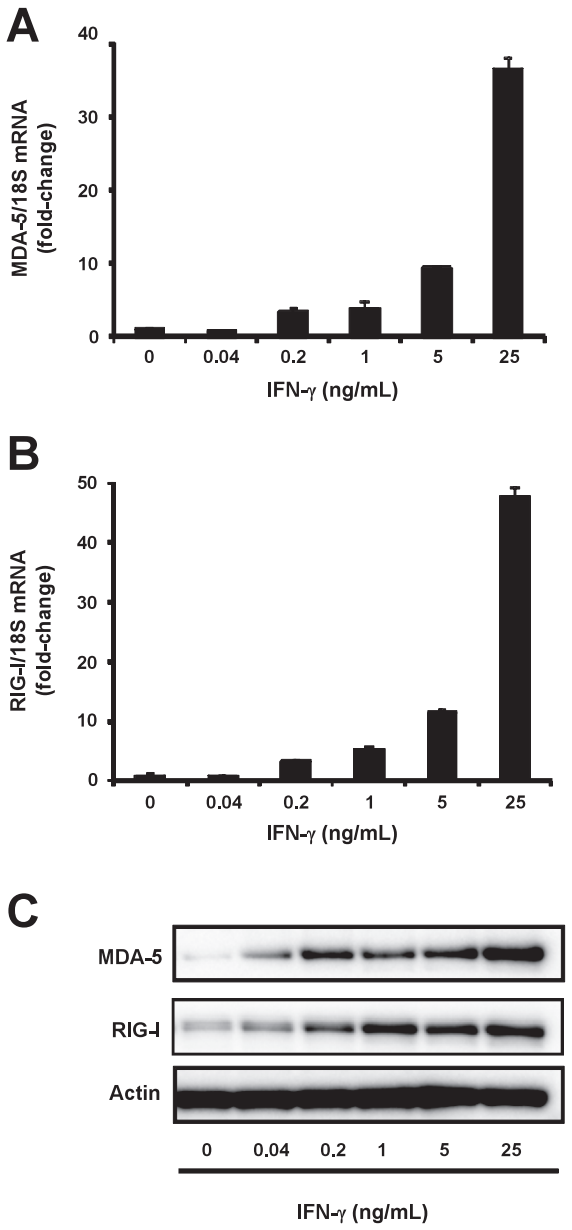

Fig. 2 Concentration-dependent stimulation by IFN-γ of the MDA-5 and RIG-I expression in HSC-3 cells. HSC-3 cells were treated with IFN-γ (0-25 ng/mL) for 8 h (A, B) or 24 h (C). Total RNA was extracted from the cells and the mRNA expressions of MDA-5 (A) and RIG-I (B) were examined by real-time RT-PCR analysis. (C) The cell lysate was subjected to SDS-PAGE and visualized by immunostaining for MDA-5, RIG-I, and Actin.

dency to increase after 48 h (Fig. 4A). Prolongation of both mRNA expressions was observed at least up to 72 h after the stimulation (data not shown). The levels of both proteins were enhanced at 8 h after the treatment of IFNs (Fig. 4B).

Effect of MDA-5 on cell proliferation in HSC-3 cells

IFNs are known to regulate cell proliferation and to induce apoptosis; therefore they are used as anti-tumor agents (17). We initially examined the effect of IFNs on the cell growth on HSC-3 cells. As shown in Fig. 5, IFN-α2b exerted marked suppressive ac-

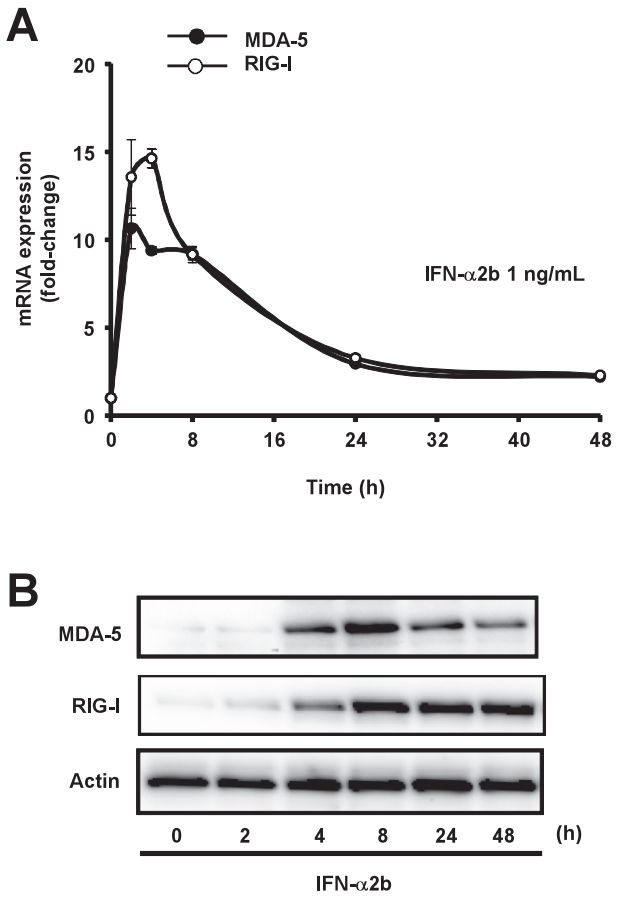

Fig. 3 Time-course of the MDA-5 expression in HSC-3 cells in response to IFN-*α*2b. HSC-3 cells were treated with 1 ng/mL IFN-*α*2b for up to 48 h. **A**. Total RNA extracted from the cells was subjected to quantitative RT-PCR for MDA-5 and RIG-I. **B**. MDA-5 and RIG-I proteins in cell lysates were examined by western blot.

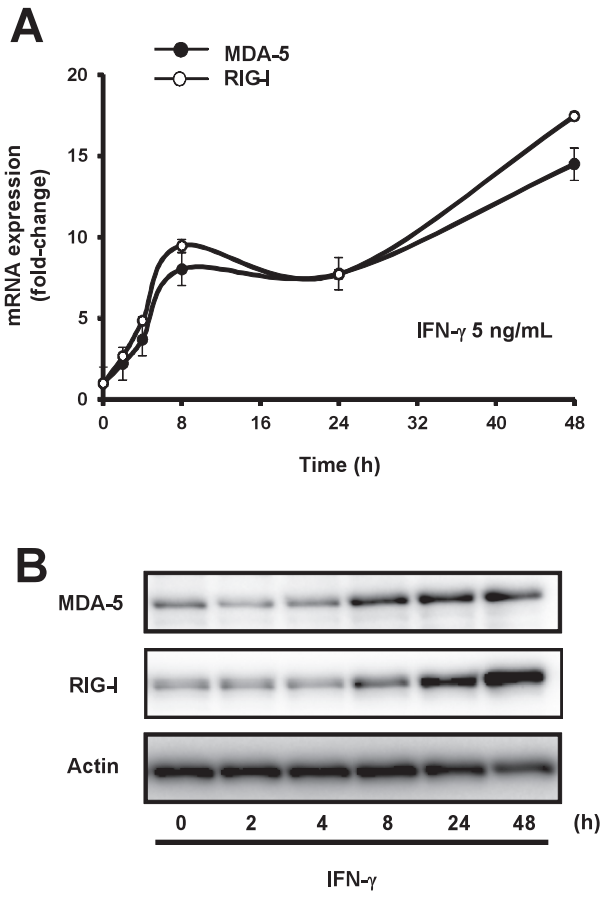

Fig. 4 Time-course mRNA expression of MDA-5 in HSC-3 cells stimulated with IFN-*γ*. HCS-3 cells were treated with 5 ng/mL IFN-*γ* for up to 48 h. **A**. mRNA expression of MDA-5 and RIG-I were analyzed by real-time RT-PCR. **B**. The protein levels of MDA-5 and RIG-I were analyzed by immunoblotting.

tivity in the growth of HCS-3 cells. In contrast, no such effect was observed in the cells treated with IFN-γ. Our results from the present study showed that both IFN-α2b and IFN-γ induce MDA-5; therefore, we next investigated the effect of MDA-5 on the cell proliferation of HSC-3 cells. When the cells were transfected with control expression vector for 24 h, the cells grew almost normally with slight cytotoxity probably due to the transfection (Fig. 6A). Transfection of cells with MDA-5 expression construct did not alter the proliferation of HSC-3 cells (Fig. 6B). We note that overexpression of MDA-5 was confirmed by western blot (data not shown).

DISCUSSION

An earlier study has clearly shown that the expression of MDA-5 is IFN-dependent (10). Type I- and type II-IFN bind their specific cell surface receptors to activate individual intracellular signal transduction pathway. Type II IFN induces homodimerization of signal transducer and activator of transcription (STAT)1 to induce certain IFN-stimulated genes (ISGs), while type I IFN stimulates formation of complexes composed of STAT1, STAT2, and ISG factor 3 (ISGF3) (20). Therefore, these two types of IFNs have been known to have distinct functions in immune responses. In the present study, we first asked whether or not oral epithelial cell line can induce MDA-5 in response to IFNs. We found that both IFNs can induce the expression of MDA-5 as well as RIG-I. We further explored if either type I- or type II-IFN induces the expression of MDA-5 with similar kinetics. Our results showed that type II IFN induces the MDA-5 expression with delayed kinetics as compared with type I IFN. This result

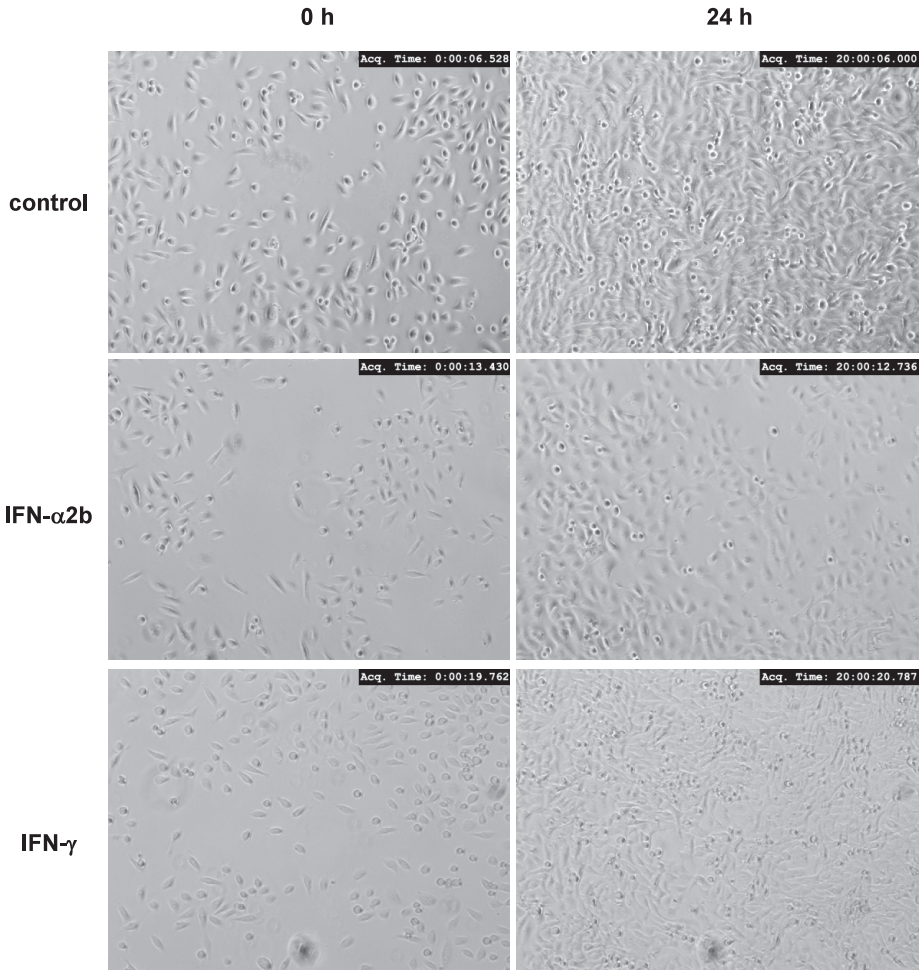

Fig. 5 Effects of IFNs on cell proliferation in HSC-3 cells. HSC-3 cells were grown with IFN-α2b or IFN-γ for 24 h, and then those images were captured under a phase-contrast microscopy. IFN-α2b exerted marked suppressive activity in the growth on HSC-3 cells. In contrast, no such effect was observed in the cells treated with IFN-γ.

agreed with our previous observation that IFN-γ induced the expression of RIG-I in mesangial cells with relatively similar expression pattern (8). These observations allowed us to speculate that type I IFN may be associated with early MDA-5 expression while type II contributes the late MDA-5 expression.

Overexpression of MDA-5 inhibits colony formation of melanoma cells (10). Type I IFN have been used for treatment of melanoma to anticipate terminal differentiation (3). In these contexts, MDA-5 may function as a mediator of IFN-induced growth inhibition and/or apoptosis (10). Several early studies reported that, in contrast to type I IFN, treatment of melanoma with IFN-γ results in biologically more aggressive phenotype including inhibition of melanin synthesis and enhancement of intercellular adhesion molecule-1 (ICAM-1) and very late antigen-2 (VLA-2), both of which are known as progression-associated melanoma markers (4, 5). We also observed that the treatment of IFN-γ had no inhibitory effect on the growth of HSC-3 cells, whereas type I IFN significantly suppressed the tumor cell growth. Our overexpression analysis of MDA-5 clearly indicated no association between MDA-5 and cell proliferation in HSC-3 cells. This result is inconsistent with the report in melanoma cells. From these observations, uncharacterized molecule(s) must participate in the inhibitory effect of type I-IFN on tumor cells growth of oral cancer cells.

We conclude that both IFNs induce the expression of MDA-5 in cultured oral cancer cells. It seemed that the induced MDA-5 is unrelated to the tumor cell proliferation. The expressed MDA-5 may be involved specifically in antiviral immune responses in oral epithelial cells.

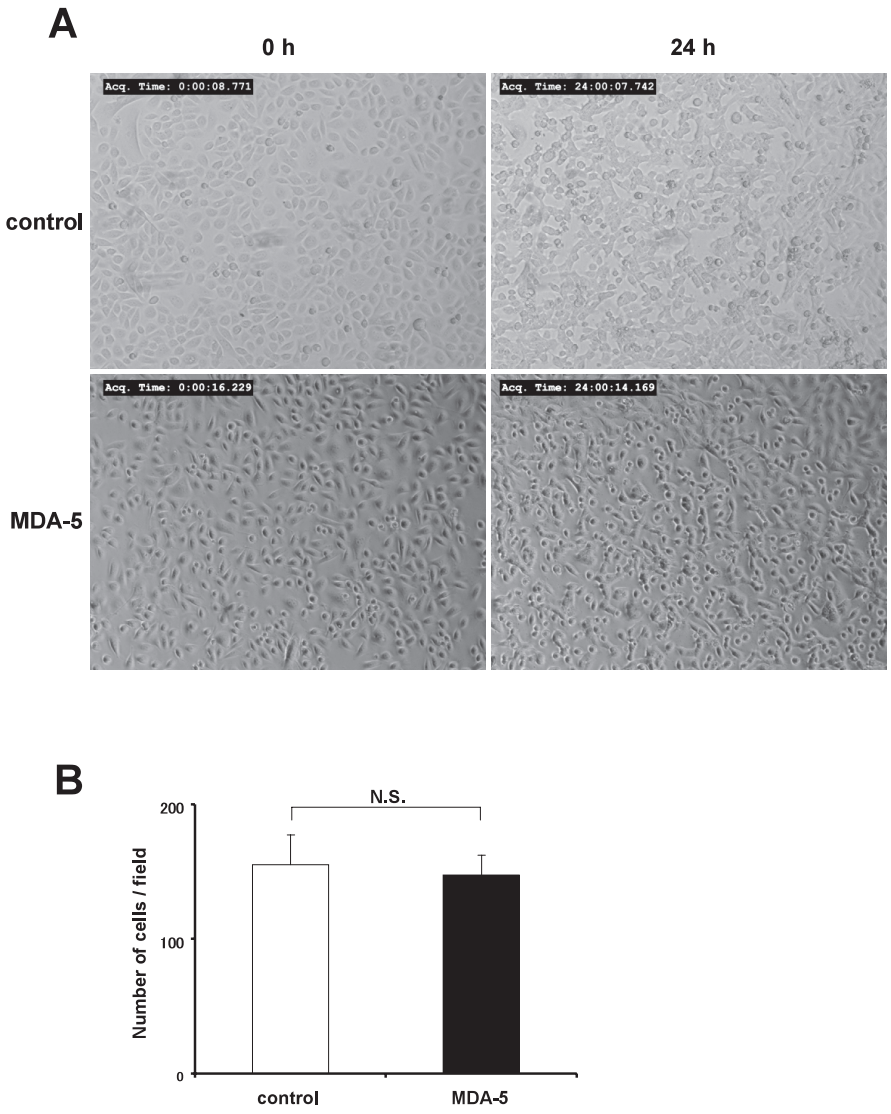

Fig. 6 Effects of MDA-5 overexpression on cell proliferation in HSC-3 cells. HSC-3 cells were transfected with plasmid vector encoding MDA-5 cDNA or control expression vector, then the cells were photographed under the phase-contrast microscopy. **A**. Represented image in each condition. **B**. Four random fields were chosen through the microscope, and numbers of cells were counted. Data are shown as average numbers of cells per field. NS, not significant.

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