Antiviral Research 94 (2012) 98–102

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short Communication

Induction of type I interferon by high-molecular poly-γ-glutamate protects B6.A2G-*Mx1* mice against influenza A virus

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ARTICLE INFO

Article history:
Received 29 October 2011
Revised 17 February 2012
Accepted 21 February 2012
Available online 27 February 2012

Keywords:
High-molecular-weight poly-γ-glutamate
Myxovirus resistance protein
Interferon type I
B6.A2G-*Mx1* mouse
Influenza A virus

ABSTRACT

In addition to development of vaccines and synthetic antiviral drugs, recent studies have advocated the use of natural substances that inhibit or prevent viral infections. High-molecular-weight poly-γ-glutamate (HM-γ-PGA) produced by *Bacillus subtilis chungkookjang* was evaluated for anti-influenza virus activity. HM-γ-PGA induced type I interferons (IFNs), which in turn stimulated expression of Myxovirus resistant 1 protein and IFN-related proteins *in vitro*. In the B6.A2G-*Mx1* mouse model, which mimics the innate immune system of humans, treatment with HM-γ-PGA enhanced the antiviral state of mice and protected them against highly pathogenic influenza A virus. Naturally synthesized HM-γ-PGA has potent anti-influenza activity and may be a useful means for control of influenza virus.

Innate and adaptive immunity provides defense against influenza A virus by producing type I interferons (IFNs), neutralizing antibodies, and activating NK cells and cytotoxic T lymphocytes (Davenport et al., 2007; Vanlarer et al., 2008). Among innate immune responses, the exact functions of Myxovirus resistant proteins have not been proven. The Myxovirus resistant 1 (Mx1) gene produces a crucial cellular protein that inhibits virus replication at an early stage of infection. Basal expression of the *Mx1* gene is normally below detectable level but is rapidly induced upon viral infection through the action of virus-induced type I (α/β) or type III (λ) IFNs (Haller et al., 2007). Antiviral-active Mx proteins are found in most vertebrate species, including birds, fish and humans (Dittmann et al., 2008). However, inbred strains of laboratory mice, most of which are extremely susceptible to influenza virus infection, harbor large deletions or nonsense mutations in the Mx genes (Staeheli et al., 1988). To determine the role HM-γ-PGA plays in the antiviral response to influenza virus, we used the congenic B6.A2G-*Mx1* mouse strain, in which *Mx1* gene expression is restored.

Aside from the development of antiviral drugs and vaccines, many natural substances are increasingly being tested for potent antiviral activity. One of these substances is high-molecular-weight (⩾3000 kDa) poly-γ-glutamate (HM-γ-PGA). Recently, various beneficial functions of the HM-γ-PGA, a copolymer of D- and L-glutamic acid derived from *Bacillus subtilis* subsp. *chungkookjang* have been reported. HM-γ-PGA was reported to be a safe and edible substance (Lee et al., 2009; Prodhomme et al., 2003) that is widely applicable as a nontoxic, biodegradable, and inexpensive polymer (Buescher and Margaritis, 2007). Moreover, several studies have shown HM-γ-PGA is functionally superior to low molecular weight γ-PGA (10–1000 kDa) when used as an adjuvant, for immune stimulation, and for its antitumor activity (Lee et al., 2009; Yoshikawa et al., 2008). Here, treatment with HM-γ-PGA was evaluated as an inducer of IFNs which specifically stimulate

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doi:10.1016/j.antiviral.2012.02.010

Mx1 defense mechanism to protect against lethal influenza virus infection in the congenic B6.A2G-*Mx1* mouse strain.

Endotoxin free HM-γ-PGA was prepared as described previously (Kim et al., 2007) and provided by BioLeaders Corporation (Daejeon, Korea) in 150 mM sterile NaCl solution. To investigate the HM-γ-PGA antiviral effects *in vitro*, RAW264.7 cells, a murine macrophage cell line (ATCC TIB-71), were treated with 1 mg/ml HM-γ-PGA for 12 h before infection, and infected with 1 multiplicity of infection (MOI) of a A/Puerto Rico/8/34 (PR8, H1N1) reporter virus that encodes green fluorescence protein (GFP) and express GFP in infected cells. At 12 h post-infection (hpi), GFP expression in the HM-γ-PGA treated RAW264.7 cells was substantially lower than that of untreated cells (Fig. 1A). The infectivity of HM -γ-PGA-treated cells was reduced more than 70% at 12 hpi, and less than 5% cell death was observed by Trypan Blue staining in its sole treatment at 24 hpi (Fig. 1B and C). These results indicated that HM-γ-PGA had an antiviral effect with negligible cytotoxicity *in vitro*.

To establish the mechanism of HM-γ-PGA antiviral activity, we compared the amount of secreted IFN-β from RAW264.7 cells (3×10^5/well of a 24-well tissue cell culture plate) treated with 1 mg/ml HM-γ-PGA to that of cells treated with 10 ng/ml *Escherichia coli* lipopolysaccharide (Sigma–Aldrich, USA). Secreted IFN-β was measured with the Verikine™ murine IFN-β ELISA Kit (PBL Interferon Source, USA). At 10 h post-treatment (hpt), HM-γ-PGA induced similar levels of secreted IFN-β as did lipopolysaccharide treatment, a known potent interferon agonist (Fig. 1D). We tested whether HM-γ-PGA upregulated Mx1 protein expression by immunoblotting using a monoclonal anti-Mx1/2/3 (C-1) antibody (Santa Cruz Biotechnology, USA). Cells were treated with 1 mg/ml HM-γ-PGA, 500 IU/ml IFN-β, or infected with 1 MOI of H5N2 virus (mouse-adapted A/Aquatic bird/Korea/W81/05) for the indicated times in the Fig. 1E. As shown in the figure, there was basal expression of Mx1 protein, but expression increased after stimulation with HM-γ-PGA or IFN-β. These data from Fig. 1D and E suggested that secreted IFNs stimulated by HM-γ-PGA induced Mx1 protein expression. This potent antiviral activity suggested that HM-γ-PGA could inhibit influenza virus infection *in vitro* by establishing a cellular antiviral state.

Based on the *in vitro* studies, we next evaluated anti-influenza virus activity of HM-γ-PGA *in vivo* by challenging HM-γ-PGA-treated B6.A2G-*Mx1* mice with the mouse-adapted A/Aquatic bird/Korea/W81/05 H5N2 virus. According to the previous research, B6.A2G-*Mx1* mice are highly resistant to HPAI viruses, and therefore, over 100,000 times higher mouse lethal dose of the A/Vietnam/1203/04 (H5N1) virus for mouse carrying non-functional Mx1 was required to induce 50% mortality in $Mx1^{+/+}$ mice (Salomon et al., 2007). Instead of the A/Vietnam/1203/04 (H5N1) virus, we have been carried out using A/EM/Korea/W149/06 (H5N1) virus which is highly virulent to normal inbred mouse at low challenge dose to evaluate antiviral effect of HM-γ-PGA. However, B6.A2G-*Mx1* mice were not completely killed by this virus. For the challenge test using B6.A2G-*Mx1* mice, mouse-adapted A/Aquatic bird/Korea/W81/05 virus, more virulent than A/EM/Korea/W149/06 virus to the congenic mice, was used. This virus was obtained from a fecal specimen of wild bird habitat and identified as H5N2 subtype (Baek et al., 2010; Song et al., 2009). After the serial mouse lung to lung passages, the virus was used for this challenge test when the virus obtained the highly pathogenic property in B6.A2G-*Mx1* mice.

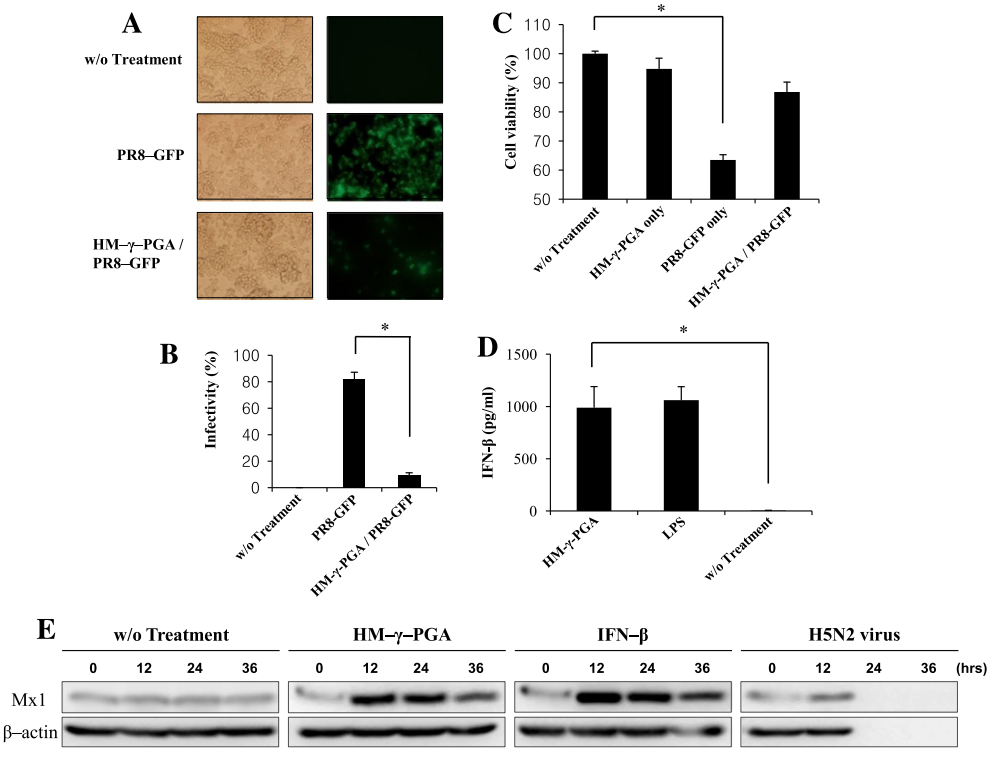

Fig. 1. Antiviral function of HM-γ-PGA in murine macrophage cell line. (A) GFP expression levels of media treated, 1 mg/ml HM-γ-PGA treated 12 h before infection or GFP-expressing A/PR/8/34 (PR8) virus treated cells were determined at 12 hpi (200× magnification). (B) Infectivity (%). Number of GFP-expressed cells was determined by Image J program (http://rsb.info.nih.gov/ij) based on Fig. 1A, and then the infectivity was obtained at 12 hpi by dividing the determined number of GFP-positive cells by total cell number. (C) Cell viability was determined by Trypan Blue exclusion at 24 hpi. The results are presented as a percentage of the control (cells without treatment). Error bars on Fig. 1A and B indicate the range of values obtained from triplicate counting in three experiments (*$P < 0.05$, those compared groups by student *t*-test are significantly different). (D) Ability of HM-γ-PGA to induce secretion of IFN-β in RAW264.7 cells. The data shows representative mean ± SD of 4 independent assays (*$P < 0.05$, significantly different). (E) Expression level of Mx1 protein over time in each treatment group was confirmed by immunoblot. H5N2 virus infection caused substantial cell death at 24 and 36 hpi, so immunoblot was not able to be performed on these samples.

To demonstrate the prophylactic effectiveness of HM-γ-PGA, eleven heads (5 head for survival test and 6 heads for virus titration in lung at 3 and 5 days post-infection (dpi)) of 7-week-old B6.A2G-*Mx1* mice and normal C57BL/6 mice were treated with 30 μl of 1 mg/ml HM-γ-PGA intranasally 12 h prior to virus challenge, 20 μl mouse IFN-β (100 U/g mouse, Sigma–Aldrich) 8 h prior to virus challenge, or 30 μl saline (150 mM) as a negative control. Timing of the HM-γ-PGA and IFN-β was such that the peak response occurred at the time of virus challenge. Afterward, mice were challenged intranasally with 30 μl containing 1.23×10^4 plaque forming units (PFU) of the H5N2 virus which is equivalent of 2 times of 50% mouse lethal dose to B6.A2G-*Mx1* mouse, and body weight, survival, and virus lung titer were monitored for 9 dpi.

After virus challenge, the HM-γ-PGA or IFN-β treated B6.A2G-*Mx1* mice groups showed relatively negligible symptoms (data not shown), but the other groups showed prominent signs of infection, such as weight loss, ruffled fur, hunched back, and labored breathing from 2 dpi. As shown in Fig. 2A, the HM-γ-PGA or IFN-β treated groups had weight loss of less than 10% out to 9 dpi, but the other groups had weight loss of more than 20% by 3 dpi. Moreover, 100% of the HM-γ-PGA and IFN-β treated animals survived, but the other groups showed 100% mortality at 3 dpi (Fig. 2B). Finally, we measured the infectious virus at 3 and 5 dpi in lung tissue by plaque assay. Infectious virus was virtually eliminated from HM-γ-PGA or IFN-β treated lung tissue, but high levels of infectious virus remained in lung tissues of the other group animals (Fig. 2C). To correlate these results with pathogenicity, histological sections of lung tissue taken at 3 dpi were observed. Lung tissues were sectioned and stained hematoxylin and eosin. Tissue sections from HM-γ-PGA and IFN-β treated B6.A2G-*Mx1* mice displayed moderate signs of virus infection and the tissue structure remained intact. In contrast, tissues from the other groups showed severe lung tissue damage with disrupted epithelial cell and alveoli, and perivascular spaces seemed to be infiltrated with immune cells such as macrophages and neutrophils (Fig. 2D). Together, these results show that HM-γ-PGA treatment in the context of a functional Mx1 gene was protective against highly pathogenic influenza virus infection.

To confirm that the antiviral function of HM-γ-PGA in the cellular level of the HM-γ-PGA treated mice, we tested the stimulation of Mx1 protein expression in lung tissue homogenates of treated mice. Normal mice (C57BL/6) were intranasally treated with 30 μl HM-γ-PGA (1 mg/ml) and 4 groups of B6.A2G-*Mx1* mice were intranasally treated with 30 μl HM-γ-PGA (1 mg/ml), 20 μl IFN-β or IFN-α (100 U/g mouse, Sigma–Aldrich), or 30 μl H5N2 virus (1.23×10^4 PFU). Lungs were harvested and homogenized at 0, 6, 12, 24, and 36 hpt and Mx1 levels detected by immunoblotting using anti-Mx1/2/3 antibody. Loading was normalized by immunoblotting for β-actin using a specific monoclonal antibody (Sigma–Aldrich). HM-γ-PGA treated mouse lungs showed continuous stimulation of Mx1 protein expression out to 36 hpt, but treatment with type I IFNs or infection with H5N2 influenza virus showed initial stimulation of Mx1 expression followed by a decrease in expression by 36 hpt (Fig. 3A).

These results suggested that HM-γ-PGA induced a potent antiviral state both *in vitro* and *in vivo*. Consequently, HM-γ-PGA treatment could inhibit virus replications and enhance clearance of viruses from lung cells and mouse survival against the virus infection through stimulation of Mx1 protein expression mediated by type I IFNs. Likewise, mRNA expression levels of Mx1, IFNs, tumor necrosis factor (TNF)-α, and interferon-stimulated genes (ISGs) were measured from harvested lungs or bone marrow-derived

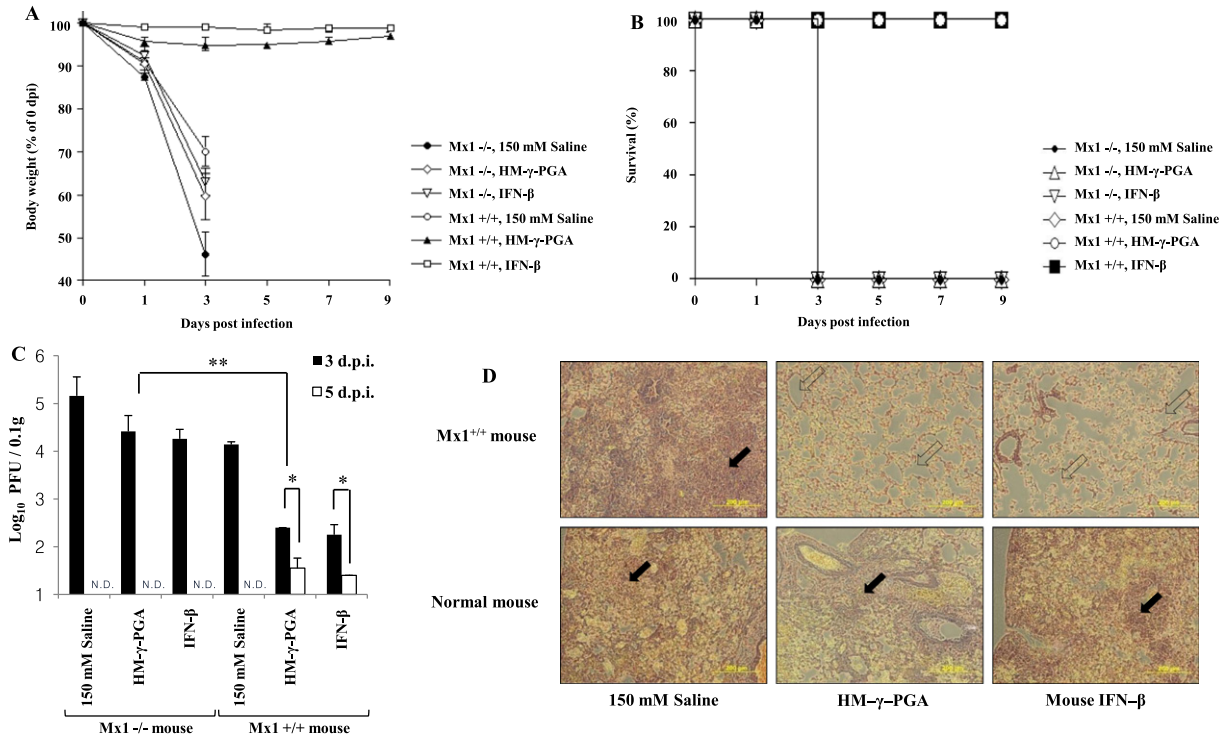

Fig. 2. HM-γ-PGA treatment protects against lethal influenza A virus challenge. Mice were treated intranasally with 30 μl of 1 mg/ml HM-γ-PGA or 20 μl mouse IFN-β (100 U/g mouse), 12 or 8 h prior to virus challenge, respectively. (A) Variation of mouse body weight. Absent data is due to mouse mortality after 3 dpi. (B) Survival (%). HM-γ-PGA and IFN-β inoculated B6.A2G-*Mx1* mice showed 100% survival. The other mice showed 100% mortality. The results of Fig. 2A and B were expressed as mean ± SD of each mouse group and the comparison of 100% survival and 100% mortality on Fig. 2B shows significantly different with $P < 0.01$ by student *t*-test. (C) Virus titers in mice lung. Titers were expressed as mean ± SD and error bars ($^{**}P < 0.01$ and $^{*}P < 0.05$ by student *t*-test, those comparing shows significantly different values). N.D.: no data due to mortality. (D) Histopathologic observation. The filled arrows indicate inflammatory area while the blank arrows indicate intact alveoli and perivascular spaces. Tissues were observed by light microscopy with 200× magnification.

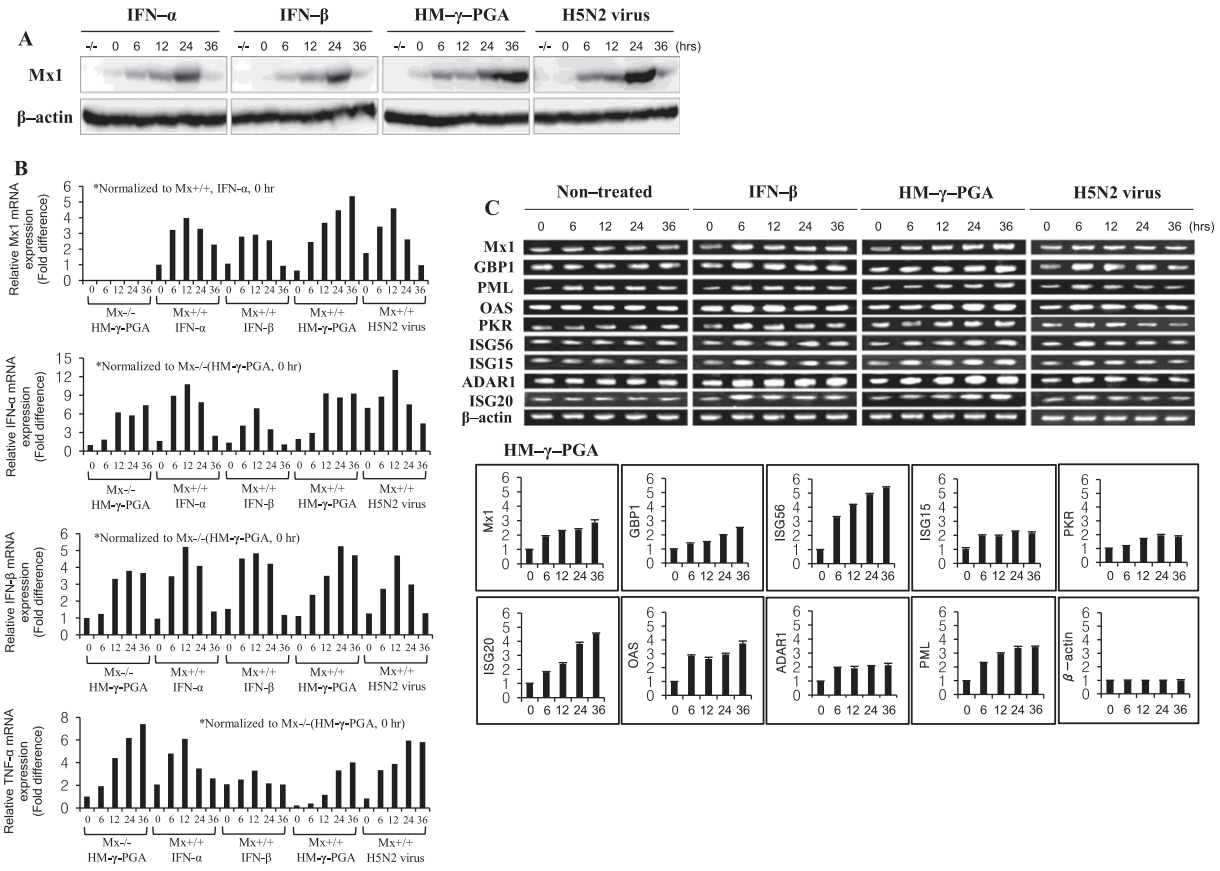

Fig. 3. Stimulation of an antiviral state by HM-γ-PGA treatment. (A) Mice were treated intranasally with 30 μl HM-γ-PGA (1 mg/ml), 20 μl IFN-β (100 U/g mouse), or 30 μl H5N2 virus (1.23×10^4 PFU). The changes in Mx1 protein expression in the lungs of treated mice over time were determined by immunoblot using anti-Mx1/2/3 antibody. (B) Mice were treated intranasally with 30 μl HM-γ-PGA (1 mg/ml), 20 μl IFN-β or α (100 U/g mouse), or 30 μl H5N2 virus (1.23×10^4 PFU). The time-dependent changes in mRNA expression after treatment in both parental (C57BL/6) and transgenic B6.A2G-*Mx1* mouse lungs were confirmed by RT–PCR using the primers which are shown in Table 1. The band intensities of each PCR product on 1% agarose gel were determined by Image J analysis program and each graph has been normalized to each appropriate negative control. (C) BMDMs from B6.A2G-*Mx1* mice were treated with culture media, 1 mg/ml HM-γ-PGA, 500 U of IFN-β, or 1 MOI of H5N2 virus. After treatment, induced mRNA levels of ISGs in BMDM over time were confirmed by RT–PCR using the primers listed on Table 1. Particularly, the band intensity of HM-γ-PGA treated group was determined by Image J analysis program and showed as a bar graph. Error bars indicate the range of values obtained from two independent experiments.

Table 1
Primer sets used to confirm mRNA expression.

Genes	Primers	
	Forward	Reverse
Mx1	5′-ACAAGCACAGGAAACCGTATCAG-3′	5′-AGGCAGTTTGGACCATCTTAGTG-3′
TNF-α	5′-AGCAAACCACCAAGTGGAGGA-3′	5′-GCTGGCACCACTAGTTGGTTGT-3′
IFN-α	5′-CTCTCCTGCCTGAAGGACAGGAAG-3′	5′-GGTGGAGGTCATTGCAGAATGAGT-3′
IFN-β	5′-TCCAAGAAAGGACGAACATTCG-3′	5′-TGCGGACATCTCCCACGTCAA-3′
β-actin	5′-ACCCACACTGTGCCCATCTA-3′	5′-CGGAACCGCTCATTGCC-3′
OAS	5′-GAGGCGGTTGGCTGAAGAGG-3′	5′-GAGGAAGGCTGGCTGTGATTGG-3′
ADAR 1	5′-CCAAAGACACTTCCTCTC-3′	5′-CAGTGTGGTGGTTGTACT-3′
GBP 1	5′-AAAAACTTCGGGGACAGCTT-3′	5′-CTGAGTCACCTCATAAGCCAAA-3′
PML	5′-CCTGCGCTGACTGACATCTACT-3′	5′-TGCAACACAGAGGCTTGGC-3′
PKR	5′-GCCAGATGCACGGAGTAGCC-3′	5′-GAAAACTTGGCCAAATCCACC-3′
ISG56	5′-AGAGAACAGCTACCACCTTT-3′	5′-TGGACCTGCTCTGAGATTCT-3′
ISG20	5′-AGAGATCACGGACTACAGAA-3′	5′-TCTGTGGACGTGTCATAGAT-3′
ISG15	5′-CAATGGCCTGGGACCTAAA-3′	5′-CTTCTTCAGTTCTGACACCGTCAT-3′

macrophages (BMDM, 2×10^6/well of a 6-well tissue cell culture plate). BMDMs were isolated from leg bones of 7-week-old B6.A2G-*Mx1* mouse and then differentiated by growth for 7 days with M-CSF (25 μg/ml, R&D) according to the previous study (Yuk et al., 2009). BMDMs were treated with media only, 1 mg/ml HM-γ-PGA, 500 U of IFN-β, or 1 MOI of H5N2 virus and harvested at 0, 6, 12, 24, and 36 hpt. Total mRNA was extracted using the RNeasy Mini Kit (Qiagen, USA) from 0.03 g lung tissue harvested for immunoblotting or treated cells in each well of 6-well tissue cell culture plate. The extracted mRNAs were then amplified by RT–PCR using target-specific primers listed on Table 1. As shown in Fig. 3B, the mRNA level of Mx1, TNF-α, IFN-α and IFN-β increased after HM-γ-PGA treatment of both normal C57BL/6 and B6.A2G-*Mx1* mouse strains, and the stimulation in mRNA expression was similar to the levels seen in the IFNs treated animals. Interestingly, mRNA levels remained high in the HM-γ-PGA treated

group out to 36 hpt, but levels had decreased in all of the other treatment groups. In addition, the determined mRNA expression levels of several ISGs in B6.A2G-*Mx1* BMDMs increased following HM-γ-PGA or IFN-β treatment and HM-γ-PGA treatment yielded a longer lasting stimulation (Fig. 3C), as describe for the lung tissue. H5N2 virus-treated BMDM showed an early increase and later decrease of mRNA expression. These results suggested that induced IFNs by HM-γ-PGA stimulate the Mx1 gene as well as other ISGs, and this pathway appears to have a crucial role in protecting mice against influenza virus infection *in vivo*. Moreover, according to the previous study, HM-γ-PGA is engulfed into immune cells by Toll-like receptor 4-mediated endocytosis (Lee et al., 2009). After endocytosis, the signal is transduced by MyD88-dependent or independent pathways, and NF-κB and IFNs are produced as a result of the signal cascade. Together with this previous finding, a certain function of HM-γ-PGA to induce type I IFNs has been demonstrated again through this study.

In conclusion, this study has shown an antiviral function of HM-γ-PGA against influenza virus through the stimulation of type I IFN and Mx1 protein both *in vitro* and *in vivo*. Therefore, HM-γ-PGA, a safe natural substance, may have potential as a prophylactic treatment against influenza virus and possibly other IFN/Mx-sensitive RNA virus infections.

Acknowledgements

The authors thank Dr. Peter Staeheli of University of Freiburg for providing B6.A2G-*Mx1* mice, BioLeaders Corporation (Daejeon, Korea) for providing HM-γ-PGA, and Dr. Jung Jae U of University of Southern California for providing the GFP-expressing PR8 virus.

This study was supported in part by National Agenda Project grant from Korea Research Council of Fundamental Science and Technology and KRIBB Initiative program (Grant No. KGM0821113), and the Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (Grant No. 110057-03, 111106-02), and the Korean Institute of Oriental Medicine by the Ministry of Education, Science and Technology (MEST), Korea (Grant No. K12050). The study was also financially supported by the research fund of Chungnam National University in 2010.

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