Article

Antiviral Activity of Gallus Recombinant Interferon α3

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Abstract: Interferons (IFNs) were first discovered in 1957 in a nutrient fluid from chick chorioallantois membranes, where it was observed that administration of virus stimulated interferon production in many animals, tissues, and cells, within a short time. They are classified into type 1, type 2, and IFN-like cytokines, with type 1 IFN classified into IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ, and IFNτ. Clinical tests for recombinant human IFNs and bovine IFNτ have been conducted since 1981. Although infections of Highly Pathogenic Avian Influenza Virus (HPAI) have continued to cause high economic losses in poultry industry causing many deaths of poultry, few molecular experiments based on gallus (ga) IFNs have been reported since 1994 and clinical trials to test their use are limited. Here, we examined the activities of newly produced three recombinant gaIFNα3s on different species of cells. The recombinant gaIFNα3s showed significant antiviral activity in Gallus embryo fibroblast (GEF) cells, showing good potential to prevent the cytopathic effect of vesicular stomatitis virus (VSV). However, they failed to protect Wistar Institute Susan Hayflick (WISH) cells, Madin-Darby bovine kidney epithelial (MDBK) cells, and Madin-Darby canine epithelial-like (MDCK) cells. This study demonstrated the impact of species specificity on the antiviral activity of gaIFNα3 and the effect of location of fusion protein.

Keywords: interferons; recombinant protein; gallus; gallus embryo fibroblast; antiviral activity

1. Introduction

In 1957, interferons (IFNs) were first discovered from chick chorioallantois membranes which were stimulated by virus infection [1–3]. IFN is divided into three groups: type 1, type 2, and IFN-like cytokines. Among them, type 1 IFNs consist of multi-genes including IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ, and IFNτ, which are known to protect the host against viral infection through their specific cell surface receptor complex composed of IFNaR1 and IFNaR2 chains. Once type 1 IFN binds to the IFN receptor, it forms a complex with Janus Kinase (JAK), which phosphorylates signal transducers and activators of transcription (STAT), leading to STAT dimerization. The dimer and interferon regulatory factor (IRF)-9 form a trimeric interferon-stimulated gene factor-3 (ISGF-3) complex that translocates into the nucleus, where it induces the expression of genes containing interferon-stimulated regulatory element (ISRE) in the promoter: IRF-1, IRF-7, 2′,5′-oligoadenylate synthetase (OAS), and double-stranded RNA-dependent protein kinase (PKR) [4–9].

Type 1 IFNs inhibit viral gene transcription, particularly the following three genes: (1) PKR, which phosphorylates and promotes the expression of eIF-2α and suppresses translation initiation, (2) OAS-1 family and RNase L nuclease degrade RNA, (3) Mx dynamin-like GTPase (Mx) family involved in the inhibition of RNA synthesis by targeting viral nucleocapsids. However, unlike in humans or other species, the antiviral activity of gallus Mx is not well understood. Therefore, further studies are needed to clarify its activity [10–13]

Avian influenza (AI) virus is an RNA virus and a subtype of *Influenza virus* A. It is divided into two groups: Low Pathogenic Avian Influenza Virus (LPAI) and Highly Pathogenic Avian Influenza Virus (HPAI). LPAI is generally known to cause mild illnesses such as gastrointestinal disturbance, ruffled feathers, and decrease egg production or induce some disease. On the other hand, HPAI can trigger the death of more than 75% of affected poultry and has a high transmission rate. These subtypes pose major challenges to the economic development of poultry farms [14,15].

After 1978, several IFNs have been purified using reverse-phase and normal-phase high-performance liquid chromatography, with the recombinant human IFNs and bovine IFNτ adopted in clinical practice since 1981 [5]. In comparison, molecular experiments have been continuously performed on gallus (ga) IFN since 1994 [16], leading to the purification of many gaIFNs from various Aves like turkey [17], duck [18], goose [19], and red-crowned crane [20]. However, few clinical trials have been reported for the identified gaIFNs [13]. In our previous study, we demonstrated that species-specificity of IFNs should be considered when designing agents that inhibit the cytopathic effects of virus [20]. In the current study, we compared their antiviral activities using cell lines from various species.

2. Material and Methods

2.1. Preparation of Gallus Embryo Fibroblast (GEF) Cells

Fertile chicken eggs were incubated at 37 °C for 7~9 days. The fetus without a head, arms, organs, and legs was chopped and digested with trypsin-EDTA solution (Welgene, Gyeongsan, Republic of Korea) for 10 min at 37 °C, suspended and cultured in Dulbecco's Modified Eagle's medium (DMEM; Welgene) supplemented with 10% Fetal bovine serum (FBS; Welgene), 100 units penicillin, and 100 μg/mL streptomycin (Welgene).

2.2. Antiviral Assay Inhibiting Cytopathic Effect of VSV

Three recombinant gaIFNα3 proteins (gaIFNα3-1, -2, and -3) were purchased from CcCaM (Seoul, Republic of Korea). gaIFNα3-1 was purified using a eukaryotic system, whereas gaIFNα3-2 and -3 were purified using a prokaryotic system. Among the prokaryotically-purified proteins, gaIFNα3-2 contains a 6x His-tag at its C-terminal site, while gaIFNα3-3 has a 6x His-tag at its N-terminal site. To test their antiviral activities, different cell lines, including Wistar Institute Susan Hayflick (WISH) cells, Madin-Darby bovine kidney epithelial (MDBK) cells, Madin-Darby canine kidney epithelial-like (MDCK) cells, and GEF cells were adopted as the in vitro. WISH, MDBK, and MDCK were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). WISH was cultured in Modified Eagle's medium (MEM; Welgene) supplemented with 10% FBS, 100 units of penicillin, and 100 μg/mL streptomycin. MDBK and MDCK were cultured in DMEM supplemented with 10% FBS, 100 units penicillin, and 100 μg/mL streptomycin under water-saturated 5% CO_2 at 37 °C. The GEF cells were prepared as described the above section.

These prepared cells were seeded in a 96-well plate in a 100% monolayer status confluence. After one day, the cell's media were suctioned, and gaIFNα3s and huIFNα2a were added to the cells at a 3-fold serial dilution. After incubation for 6 h, the vesicular stomatitis virus (VSV; Indiana strain) was added to infect the cells. Once cells in the negative control line, infected with VSV, died as confirmed through microscopic observation, the media were removed, and the cells were stained with crystal violet solution for visualization.

2.3. RT-PCR

2 mL volume of MDBK, MDCK, WISH, and GEF cells were seeded in 6-well plates (1×10^6 cells/well) and incubated with gaIFNα3-2 and huIFNα2a at various concentrations depending on the species of cells for 6 h and harvested using RNAiso (Takara, Kusatsu, Japan) for RNA extraction. 2 μg of RNA from each cell type was reverse-transcribed with 1 μL of M-MuLV reverse transcriptase (Cosmo Genentech, Seoul, Korea) in 20 μL reaction volume following the manufacturer's instructions. The PCR products were loaded onto 1%-agarose gel and identified through electrophoresis under an ultra-violet (UV) trans-illuminator. We performed PCR of MX-1 (accession NO. NM_204609) using the following primer sequences. Sense primer: 5′-CAGAACATGAACAATCCACGG-3′; reverse primer: 5′-AATTAGTGTCAGGTCTGG GAC-3′, OASL (OAS like; accession No. NM_001397447) with sense primer: 5′-AATGGAGCTGGGCGTGAG-3′; reverse primer: 5′-AGAGGGTGAGGCTGAGGG-3′. The details of the primers of other species were described previously [21,22].

3. RESULTS

3.1. Inhibition of the Cytopathic Effect of VSV by gaIFNα3

The anti-cytopathic effect of gaIFNα3-1 and huIFNα2a against VSV was determined in GEF cells (Figure 1). Notably, gaIFNα3-1 strongly inhibited the cytopathic effect of VSV, whereas huIFNα2a failed to protect chicken GEF cells even at 10 times high concentration (1000 ng/mL). Subsequently, we compared three gaIFNα3s, gaIFNα3-1, gaIFNα3-2, and gaIFNα3-3, which have different expression vectors. Unlike others, gaIFNα3-2 was

effective in blocking the cytopathic effect of VSV at a concentration of 0.33 ng/mL (Figure 2). Next, we tested the efficacy of gaIFNα3-2 on human WISH cells, bovine MDBK cells, and canine MDCK cells. As shown in Figure 3, gaIFNα3-2 failed to protect the cells of other species from VSV-induced cytopathic effect, unlike huIFNα2a, even at high concentrations (1000 ng/mL). However, huIFNα2a provided effective protection on human WISH cells, bovine MDBK cells, and canine MDCK cells, with the lowest activity in canine MDCK cells.

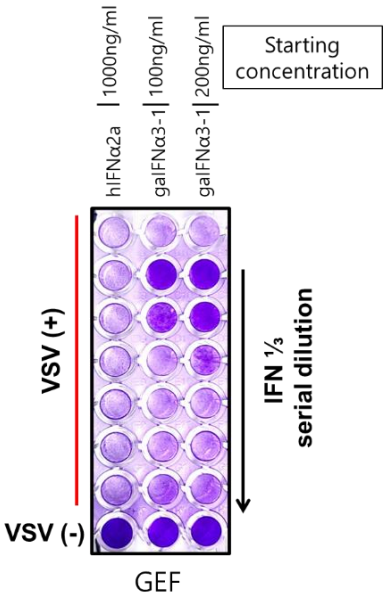

Figure 1. Antiviral activity of gaIFNα3-1 in GEF cells. The GEF cells were treated with recombinant huIFNα2 and gaIFNα3-1 proteins and then infected with VSV to examine the IFNs' antiviral activity. Plates were stained with crystal violet solution to be visualized. For cells in the control, the wells at the bottom row were not treated with the proteins, and the top rows were only infected by VSV.

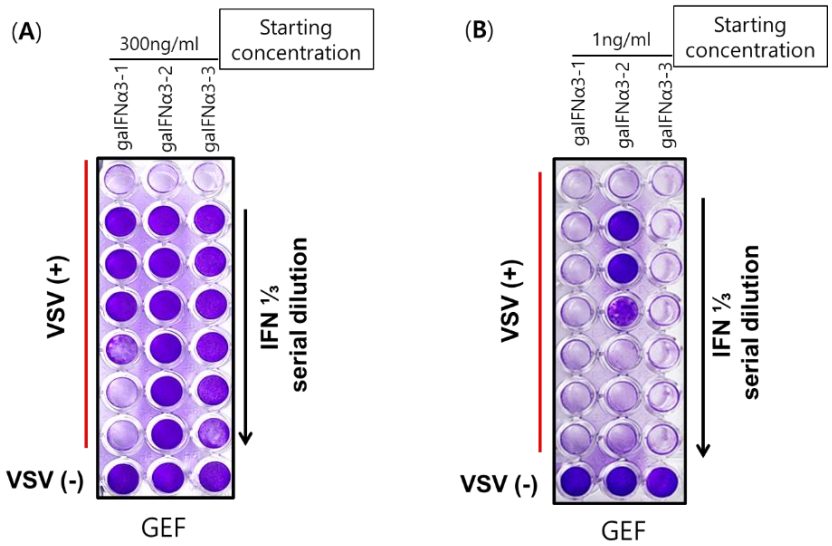

Figure 2. Comparison of gaIFNα3s purified using different expression vectors. (**A**) GEF cells were treated with three gaIFNα3s at the same concentration (300 ng/mL). gaIFNα3-1 is a C-terminal fusion recombinant protein from CHO cells. gaIFNα3-2 is a C-terminal fusion recombinant protein from *E. coli*. gaIFNα3-3 is an N-terminal fusion recombinant protein from *E. coli*. (**B**) To determine the limit of antiviral activity of the three gaIFNα3s, cytopathic tests were repeated from the 1 ng/mL.

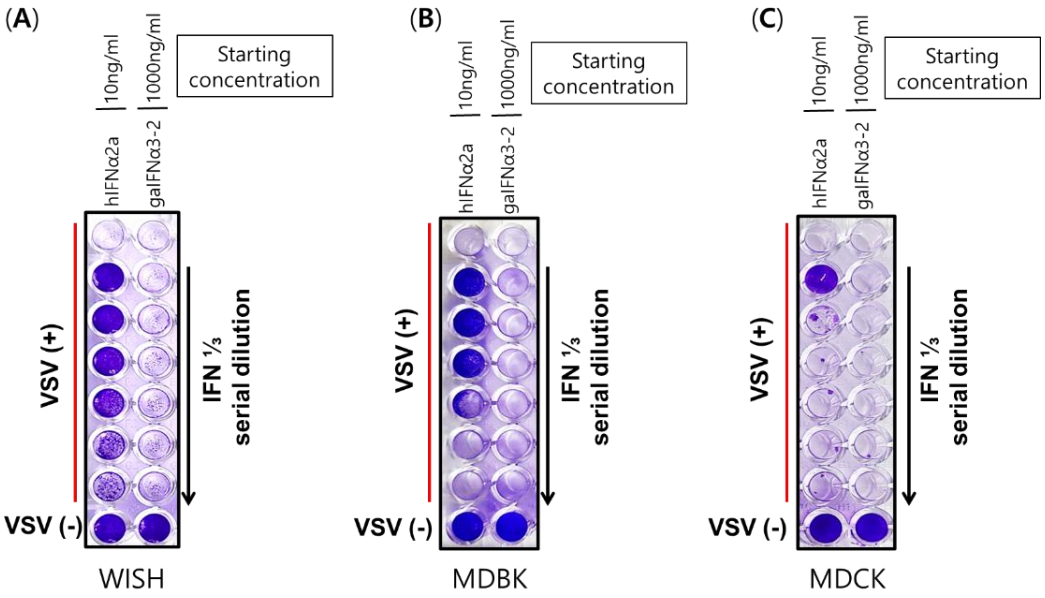

Figure 3. Antiviral activity of galFNα3-2 in cells from various species. The WISH (**A**), MDBK (**B**), and MDCK cells (**C**) were incubated with galFNα3-2 and control hIFNα2 at a 3-fold serial dilution. After 6 h, the cells were infected with VSV and then were stained and evaluated.

3.2. Species-Dependent Regulation of Antiviral Genes by galFNα3-2

Subsequently, we performed RT-PCR to determine the production of interferon-stimulated gene (ISG) and explore the antiviral mechanism of galFNα3-2. The results showed that the expression of Mx-1 and OASL genes was higher in chicken GEF cells treated with a very low concentration of aviIFNα3-2 at 6 h compared to the negative control (Figure 4A). Even at the concentration of 0.1 ng/mL, its induced significant increase in the expression of antiviral genes Mx-1 and OASL. However, in human WISH cells, bovine MDBK cells, and canine MDCK cells, galFNα3-2 at 1 μg/mL, a concentration 10,000-fold higher than the concentration treated with GEF cells, failed to upregulate any the levels of ISGs. Human IFNα2a effectively induced the expression of antiviral genes in WISH and MDBK, but did not increase ISGs expression in MDCK (Figure 4B–D). Analysis of gene expression of the control β-actin revealed that the observed differences in ISGs expression were not due to experimental variation (Figure 4).

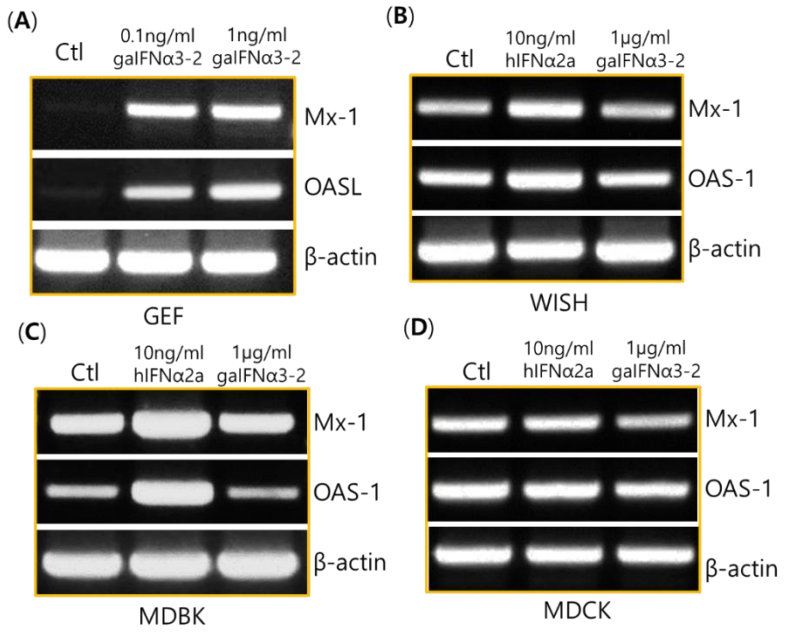

Figure 4. Recombinant galFNα3 induced various antiviral-associated genes in various species cells. The GEF cells (**A**), WISH cells (**B**), MDBK cells (**C**), and MDCK cells (**D**) were incubated with galFNα3-2 and control hIFNα2.

The concentration of gaIFNα3-2 and control hIFNα2 is indicated on the top. After 6 h, the cells were harvested, and total RNA was extracted for RT-PCR of Mx-1, OAS, and β-actin for normalization. Ctl, control.

4. Discussion

Three types of recombinant gaIFNα3 proteins were purchased from CcCaM (Seoul, Korea). Two recombinants gaIFNα3-2 and -3 were produced in *E. coli* and their concentrations were verified repeatedly and used to examine the antiviral activity of the recombinant gaIFNα3 in cell lines from different species. GaIFNα3-1, which was produced in CHO (Chinese Hamster Ovary) cells, blocked VSV in GEF cells, whereas human IFNα2a failed to protect GEF cells from VSV infection (Figure 1). Subsequently, a comparison of the activities of three gaIFNα3 types shown in Figure 2 revealed that gaIFNα3-2 had the highest antiviral activity among the three gaIFNα3s. In contrast, gaIFNα3-2, despite demonstrating the highest antiviral activity, did not inhibit the cytopathic effects of VSV in MDBK, WISH, and MDCK (Figure 3). Furthermore, the RT-PCR results of ISGs presented in Figure 4 were consistent with the findings regarding the antiviral activity of IFNs shown in Figure 3. These data suggested that the antiviral activity of IFNs was species-specific.

New pathways of type 1 IFNs are increasingly investigated, with the JAK-STAT signaling pathway being the most commonly involved in antiviral activity [4]. The two representative associated genes are OAS-1 and Mx-1. In other species, the OAS family comprises multiple proteins: the human and canine OAS families each contain 4 genes (OAS1, OAS2, OAS3, and OASL), while the bovine OAS family contains 5 genes (OAS1X, OAS1Y, OAS1Z, OAS2, and OASL). In contrast, the chicken OAS family expresses only 1 protein: OASL. IFNs-induced OAS activates Ribonuclease-Latent (RNase L), which recognizes double-stranded RNA, ultimately preventing viruses from replicating [8,23–26].

Although the mechanism of Mx and antiviral activity of gallus Mx is not well understood, studies have demonstrated that Mx inhibits several types of viruses by inhibiting RNA synthesis [27–31]. In this study, we demonstrated that gaIFNα3 and human IFNα2a induced the expression of cellular antiviral factors such as OASL and Mx-1 as confirmed by RT-PCR (Figure 4). These results were consistent with the inhibitory effects of different IFN against the VSV-induced cytopathic effects (Figure 3). This suggests that gaIFNα3 and type 1 IFNs used similar pathways to inhibit the cytopathic effect of the virus.

This study showed that two gaIFNα3 expressed in the prokaryotic system had higher antiviral activity than gaIFNα3 expressed in the eukaryotic system. gaIFNα3-2 had approximately 100-fold higher antiviral activity and gaIFNα3-3 had about 9-fold higher activity than gaIFN3α3-1 (Figure 2). Since gaIFNα3-2 possesses 6x His-tag at its C-terminal site and gaIFNα3-3 contain it at its N-terminal site, we postulate that the N-terminal site of gaIFNα3 is more involved in its antiviral activity compared to the C-terminal region. Usually, when *E. coli* is used to facilitate the expression and purification of recombinant proteins, it may be aggregated in the body. This aggregation can decrease the stability, folding, and activity of recombinant proteins, considering that *E. coli* has limited capacity to perform post-transcriptional modifications such as glycosylation. However, because this system is cost-, time-, and manipulation-efficient, it may serve as a useful method if the purified recombinant proteins exhibit adequate activity [32,33].

When the chickens infected with HPAI die, they leave traces of contamination in their environment. Therefore, to control avian influenza, vaccination has been proposed to alleviate and eliminate the virus. However, vaccination is costly and will impose a high economic burden on farms. Moreover, diversification and viral mutation may occur in response to vaccination [14,15,34]. Although we used only VSV in this study to demonstrate the antiviral activity of gaIFNα3s, gaIFNα3 can provide protection against various viruses, including avian influenza viruses, regardless of strain. This is because antiviral mechanisms are generally considered similar [35,36]. Therefore, predicting which strain might cause the next pandemic may not be necessary. The cost of purifying gaIFNα3-2 could be lowered so as not to burden the farmhouse if suitable conditions are found.

In summary, we examined the antiviral activity of gaIFNα3s purified from domestic chicken. The three gaIFNα3s expressed by different cellular systems exerted antiviral activity in chicken GEF cells, but not in human WISH cells, bovine MDBK cells, and canine MDCK cells. Among the three types, gaIFNα3-2 purified using a prokaryotic system with a C-terminal 6x His-tag had the highest activity. If stability and activity are assessed in further in vivo studies, gaIFNα3-2 may serve as a treatment for avian influenza with lower cytotoxicity and cost.

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Abbreviations

CHO: Chines Hamster Ovary; ga: Gallus; GEF: Gallus embryo fibroblast; hu: Human; HPAI: Highly pathogenic avian influenza virus; JAK-STAT: Janus kinase-signal transducer and activator of transcription; IFN: Interferon; ISG: Interferon-stimulated gene; MDBK: Madin-Darby bovine kidney epithelial; MDCK: Madin-Darby canine kidney epithelial-like; Mx-1: MX Dynamin Like GTPase 1; OAS: OAS1 2′-5′-oligoadenylate synthetase; VSV: Vesicular stomatitis virus; WISH: Wistar Institute Susan Hayflick.

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