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Protective efficacy of IFN-ω AND IFN-λs against influenza viruses in induced A549 cells

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Summary. – The interferon system represents one of the components of the first line defence against influenza virus infection. Interferon omega (IFN-ω) is antigenetically different from IFN-α and IFN-β and can affect patients who are resistant to these IFNs. To improve the biological characterization of IFN-ω, we compared its activity with those of type I and type III IFNs in induced A549 cells. The antiviral effect on IFN-stimulated A549 cells was most apparent after infection with avian influenza virus. IFN-ω had statistically significant antiviral activity although less than IFN-β1a, IFN-λ1, or IFN-λ2. On the other hand, IFN-ω appeared more efficient than IFN-α2. Our results also indicate that IFN-λs were more suitable against human highly pathogenic virus. In this case, IFN-λ1 and IFN-λ2 were more potent than type I IFNs.

Keywords: influenza virus; interferon; replication; antiviral effect

Introduction

Influenza A virus (IAV, the family *Orthomyxoviridae*, the genus *Influenzavirus*), a highly infectious respiratory pathogen, causes major pandemics and annual epidemics with serious health consequences. The genome contains eight segments of negative sense, single stranded RNA which encode up to 16 proteins (Wise *et al*., 2009; 2011; 2012; Jagger *et al*., 2012; Muramoto *et al*., 2013). Individual viral proteins play critical roles in species-specific pathogenicity. An important host innate immune mechanism is the production of interferons (IFNs), which can establish an antiviral state by up-regulating interferon stimulated genes that interfere with distinct steps in the viral life cycle.

IFNs are classified into subgroups: type I (IFN -α, β, ω, κ, ε, τ, ζ, δ, and ν), type II (IFN-γ), and type III (IFN-λs) (Uzé *et al*., 2007). IFNs are associated with innate immunity and especially IFN-α, IFN-β, IFN-ω and IFN-λ are produced by virus infected cells and have non-specific antiviral activity on adjacent non-infected cells (Pestka *et al*., 2004, Lopušná *et al*., 2013). These IFNs also induce anti-proliferative and anti-inflammatory responses and are involved also in adaptive immune responses (Alexopoulou *et al*., 2001; Au *et al*., 2001). Induction of IFNs by IAV depends on recognition of viral components by either cytoplasmic receptors or the toll-like receptor (TLR) system. Plasmacytoid dendritic cells use TLR7 to sense influenza virus and fibroblast and conventional dendritic cells require recognition of RNA viral genomes by the cytoplasmic RNA helicase retinoic acid-induced gene I (RIG-I) (Kato *et al*., 2007; Rehwinkel *et al*., 2010). After RNA binding, RIG-I interacts with the mitochondrial adaptor protein MAVS and initiates a signaling cascade that culminates in the activation of the transcriptional factors AP-1, NF-κB and IRF3, and the expression of IFNs. Secreted IFNs act in a paracrine and autocrine way through binding to the ubiquitously expressed receptors (IFN-αR1 and IFN-αR2 for type I IFN and IFN-λR1 and IL-10R2 for type III IFN) to induce activation of the receptor-associated tyrosine kinases JAK1 and Tyk2 and subsequent phosphorylation of the transcriptional factors STAT1 and STAT2 (Uzé *et al*., 1990; Cleary *et al*., 1994; Gad *et al*., 2009; Skorvanova and Betakova, 2013). Activated STATs form transcription factor complexes, including STAT1 homodimers and STAT1/STAT2/

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Abbreviations: IAV(s) = influenza A virus(es); IFN(s) = interferon(s)

IRF9 heterotrimers known as ISGF3 (Levy *et al.*, 1988). After assembly, ISGF3 is translocated to the nucleus where it binds to IFN-stimulated response elements (ISRE) in the promoters of various interferon stimulated genes, such as Mx1, OAS1 and IRF7. The proteins encoded by these genes mediate the antiviral activity (Sharma *et al.*, 2003).

IFN-ω may be a useful and alternative antiviral agent, in addition to IFN-α and IFN-β. Human IFN-ω is antigenetically different from human IFN-α and IFN-β and has 65% amino acid sequence homology and similar function as IFN-α (Adolf, 1987). The IFN-ω can still affect patients who are resistant to the IFN-α due to their different antigenicity and immunogenicity. Previous studies have shown that IFN-λs induce protective effect in a number of cell lines following viral infection (Kotento *et al.*, 2003; Sheppard *et al.*, 2003; Svetlikova *et al.*, 2010). We set out to improve the biological characterization of IFN-ω and IFN-λs by comparing their antiviral activity in A549 cells induced by these IFNs following infection with human and avian IAVs.

Materials and Methods

Cells and viruses. A549 and MDCK (ATCC CCL) cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Influenza viruses A/PR/8/34 [H1N1] and A/chicken/Germany/27 [H7N7] were cultured in 10-day-old fertile hen's eggs.

Antiviral activity assay. Confluent monolayer of A549 cells (in 24-well plates) was pre-incubated for 24 hr with 0, 10, 20, and 40 ng/ml of recombinant human IL-29/IFN-lambda 1, recombinant human IL-28A/IFN-lambda 2, recombinant human IL- 28B/IFN-lambda 3, recombinant human IFN-omega (R&D System) or 0U, 50U, 100U, 200U, 400U, and 800U of recombinant human IFN-alpha 2b, recombinant human IFN-beta 1a (R&D System). The cells were washed once with phosphate buffered saline (PBS) and then infected with influenza A/PR/8/34 [H1N1] or A/chicken/Germany/27 [H7N7] virus at a multiplicity of infection (MOI) of 0.5 plaque forming units (PFU) per cell for 1 hr at room temperature. After adsorption, cells were washed three times with PBS and then cultured in serum-free MEM at 37°C. At 24 hr post infection, cells were scraped and centrifuged at 500×g for 2 min. Viral titers in supernatants were determined on MDCK cells by plaque assay.

Plaque assay. Confluent MDCK monolayers propagated in 24-well plates were infected with a serial 5-fold dilution of supernatant from scraped cells. Following adsorption, cells were washed with PBS and overlaid with 0.5% carboxymethyl-cellulose in MEM. After 72 hr, cells were fixed in 10% PBS-buffered formalin and plaques were visualized by staining with crystal violet.

Statistical analyses. Significant differences in the virus titer between the control group (untreated cells) and IFNs pre-incubated cells were calculated using the unpaired Student's *t*-test. P values <0.05 were considered significant. Statistical analysis was performed using Graph-Pad Prism software (http://www.graphpad.com/quickcalcs/ttest1.cfm).

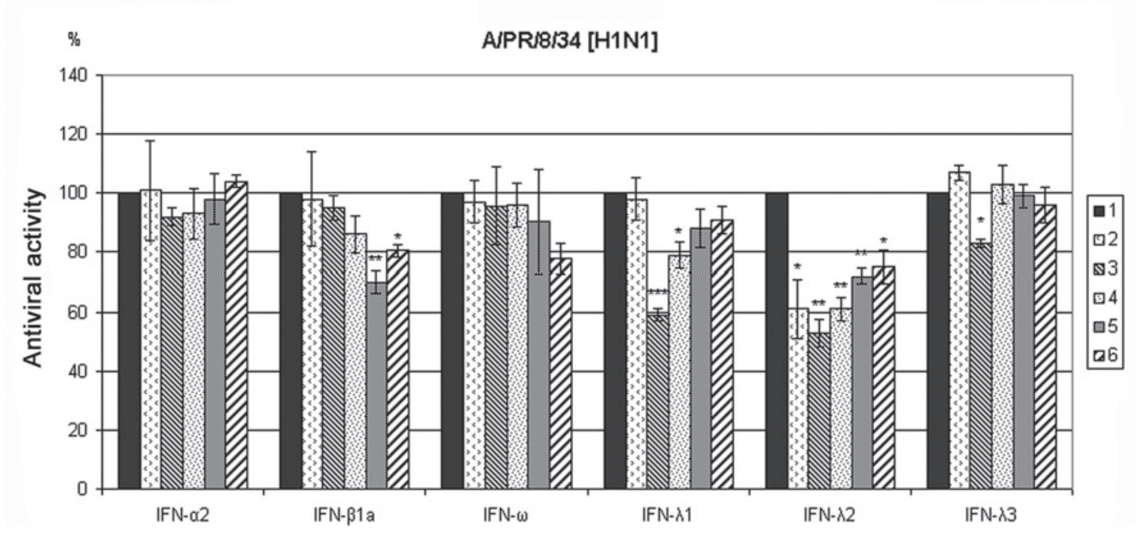

Fig. 1

Antiviral activity of IFN-α2, IFN-β1a, IFN-ω, IFN-λ1, IFN-λ2, and IFN-λ3 in IFN induced A549 cells infected with A/PR/8/34 [H1N1]
The column bars represent the average results with standard deviation from three experiments performed on different occasions. 100% (1) represents infected cells without IFN. IFN-α2 and IFN-β1a were used in concentration of 50 U (2), 100 U (3), 200 U (4), 400 U (5) and 800 U (6). IFN-ω and IFN-λs were used in concentration 2.5 ng/ml (2), 5 ng/ml (3), 10 ng/ml (4), 20 ng/ml (5) and 40 ng/ml (6). *Statistical significance (*P <0.05; **P <0.02; ***P <0.01 by unpaired Student's t-test).

Results

Inhibition of A/PR/8/34 [H1N1] replication in the cells pre-incubated with IFNs

A549 cell were stimulated with different concentration of IFNs. Subsequently, cells were infected with A/PR/8/34 [H1N1] virus and infected cells were scraped 24 hr later. The IFN-ω insignificantly decreased the viral titre to 78% (Fig.1). The further increasing of IFN concentration did not result in better inhibitory activity. Only minor changes in plaque number were observed in the A549 cells treated with the IFN-α2. The IFN-β1a significantly inhibited virus replication and reduced the virus titer to 70% (P <0.02). The IFN-λ2 was more potent than IFN-λ1 and IFN-λ3. The best inhibitory effect was observed with 5 ng/ml of IFN-λs. Pre-incubation of A549 cells with IFN-λ2 decreased the virus titer to 55% (P <0.02). IFN-λ1 reduced virus titer to 59% (P<0.01) and IFN-λ3 inhibited virus replication only to 83% (P <0,05) (Fig.1).

Inhibition of A/chicken/Germany/27 [H7N7] replication in the cells pre-incubated with IFNs

Antiviral activity of IFNs was also checked with avian strain [H7N7]. In this case, the best inhibitory activity was observed with IFN-β1a. The virus titer was reduced to 17% (P <0.01) (Fig.2). IFN-ω decreased the virus titer to 32% (P <0.01) and IFN-α2 to 46% (P <0.02). IFN-λ1 and IFN-λ2 were more potent than IFN-λ3. Antiviral activity of IFN-λ1 is comparable with activity of IFN-β1a. The least efficient IFN-λ3 inhibited virus replication to 52% (P <0.02). All IFNs exerted antiviral activity against virus in a dose-dependent manner, with the optimal concentration of IFN ranging from 10 to 20 ng/ml.

Discussion

The results presented here compare the antiviral activity of IFN-ω with antiviral activities of IFN-α/β and IFN-λs against human (A/PR/8/34 [H1N1]) and avian (A/chicken/Germany/27 [H7N7]) IAV in A549 cells. The A549 lung epithelial cells produce a high yield of MxA protein in response to IFN and thereby are suitable for antiviral assays (Files *et al.*, 1998). IAV induces only a weak cytokine response in these cells and this response can be enhanced by pre-treated cells with IFNs (Veckman *et al.*, 2006).

The antiviral effect on IFN-stimulated cells was most apparent on A549 cells infected with avian IAV. IFN-ω significantly inhibited replication of IAV and inhibition was observed in a dose-dependent manner, with optimal concentration of 10 ng/ml. Among type I IFNs, IFN-ω exhibited better reduction of virus titer (32%) than IFN-α2 (46%). Its activity was two times lower than activity of IFN-β1a and was a little bit lower than activities of IFN-λ1

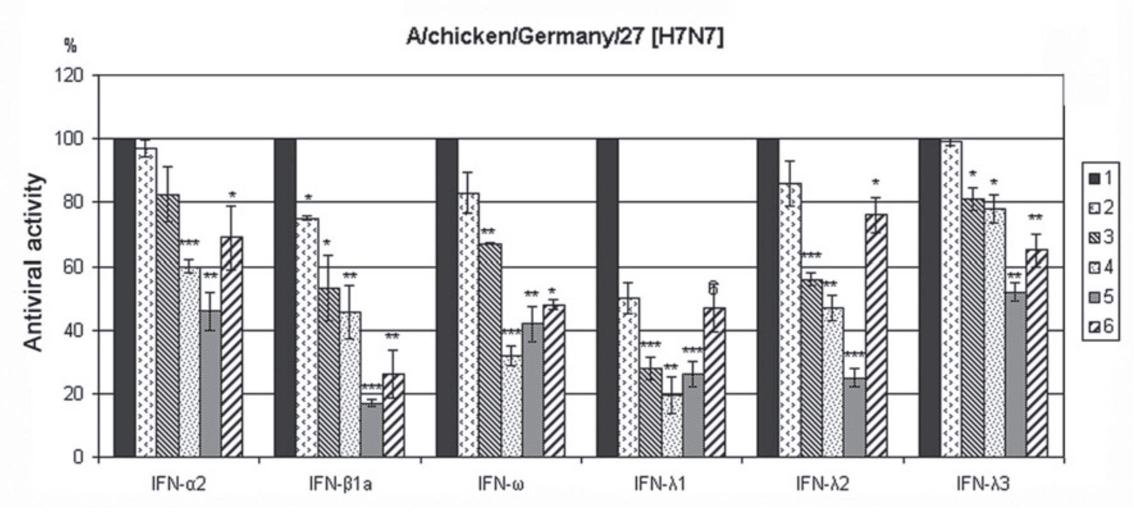

Fig. 2

Antiviral activity of IFN-α2, IFN-β1a, IFN-ω, IFN-λ1, IFN-λ2, and IFN-λ3 in IFN induced A549 cells infected with A/chicken/Germany/27 [H7N7]

The column bars represent the average results with standard deviation from three experiments performed on different occasions. 100% (1) represents infected cells without IFN. IFN-α2 and IFN-β1a were used on concentration of 50 U (2), 100 U (3), 200 U (4), 400 U (5) and 800 U (6). IFN-ω and IFN-λs were used in concentration 2.5 ng/ml (2), 5 ng/ml (3), 10 ng/ml (4), 20 ng/ml (5) and 40 ng/ml (6). *Statistical significance (*P <0.05; **P <0.02; ***P <0.01 by unpaired Student's *t*-test).

and IFN-λ2. Previous studies have shown the protective potential of human exogenous IFN-ω against pandemic 2009 A [H1N1] influenza viruses *in vitro* and in guinea pigs (Xu *et al*., 2011).

The IFN-α2 and IFN-ω only slightly influence the replication of A/PR/8/34 [H1N1]. The most active IFN-β1a and IFN-λs significantly reduced the virus titer to 70% and 60%, respectively. Our previous studies have acknowledged the antiviral role of IFN-λs *in vitro* and *in vivo* (Svetlikova *et al*., 2010; Svancarova *et al*., 2015a,b). Some viruses encode NS1 proteins that are more efficient in suppressing the host antiviral response. The NS1 protein of the highly pathogenic 1918 virus blocked the expression of IFN-regulated genes more efficiently than the NS1 from influenza A/WSN/33 (Geiss *et al*., 2002).

Antiviral effect of IFN-β1a was reduced 4 times in the cells infected with human virus compared to avian virus. On the other hand, IFN-λ1 and IFN-λ2 reduced their antiviral activity in the cells infected with human virus compared to the cells infected with avian virus only 1.8 and 2.8 times, respectively. IFN-λs and IFN-α have cell-specific effects in regard to STAT signaling, interferon stimulated genes (ISGs) expression, and cytokine and chemokine induction. Type I IFNs receptor complex consists of two chains, IFN-αR1 and IFN-αR2. IFN-λs bind to a distinct membrane receptor, composed of IFN-λR1 and IL-10R2 (Skorvanova and Betakova, 2013). The IFN-λ receptor has a more limited tissue distribution than the IFN-α receptor (Kotenko *et al*., 2003; Sheppard *et al*., 2003). Treatment with IFN-λ has limited effects on some types of cells in terms of induction of both ISG expression and on pro-inflammatory mediator release, partly due to the restricted distribution of the IFN-λ receptor and the lower levels of expression observed compared with the IFN-α receptor (Dumoutier *et al*., 2004; Freeman *et al*., 2014). This correlates with clinical observations of fewer related adverse events for IFN-λ vs. those typically associated with IFN-α (Freeman *et al*., 2014; Mihm *et al*., 2014). Taken together, better antiviral effect of type III IFNs than type I IFNs might be explained by lower induction of ISG expression and pro-inflammatory mediators what can lead to lower inhibition of RIG-I pathway by NS1 protein. Of course, the role of alternative pathway cannot be excluded. Differences in the replication characteristics and antivirus signaling responses among the different viruses were observed (Sutejo *et al*., 2012).

Peg-IFN-λ1 is currently undergoing clinical development for the treatment of viral hepatitis (Duong *et al*., 2014). Recombinant human IFN-ω-Fc fusion protein represents a useful and promising and alternative antiviral agent especially for the treatment of chronic viral disease, such as hepatitis C virus infection (Li *et al*., 2011). Accordingly to our results, the IFN-ω should be suitable as antiviral agent against some avian strains and IFN-λ1s should be used against human influenza viruses.

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