FULL PAPER *Virology*

Suppression of influenza virus infection by the orf virus isolated in Taiwan

Fong-Yuan LIN[1,2,3], Yeu-Yang TSENG[2], Kun-Wei CHAN[4], Shu-Ting KUO[5], Cheng-Hsiung YANG[6], Chi-Young WANG[1], Masaki TAKASU[7], Wei-Li HSU[2]# and Min-Liang WONG[1]#*

[1]*Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan*
[2]*Graduate Institute of Microbiology and Public Health, College of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan*
[3]*Department of Beauty Science, MeiHo University, Neipu, Pingtung County, Taiwan*
[4]*Department of Veterinary Medicine, National Chiayi University, Chiayi, Taiwan*
[5]*Animal Health Research Institute, Council of Agriculture, Tamsui, Taiwan*
[6]*Formerly at the Livestock Disease Control Center of Taichung County, Taichung, Taiwan*
[7]*Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193, Japan*

(Received 18 December 2014/Accepted 26 March 2015/Published online in J-STAGE 7 April 2015)

ABSTRACT. Orf virus (ORFV), a member of parapoxvirus, is an enveloped virus with genome of double-stranded DNA. ORFV causes contagious pustular dermatitis or contagious ecthyma in sheep and goats worldwide. In general, detection of viral DNA and observing ORFV virion in tissues of afflicted animals are two methods commonly used for diagnosis of orf infection; however, isolation of the ORFV in cell culture using virus-containing tissue as inoculum is known to be difficult. In this work, the ORFV (Hoping strain) isolated in central Taiwan was successfully grown in cell culture. We further examined the biochemical characteristic of our isolate, including viral genotyping, viral mRNA and protein expression. By electron microscopy, one unique form of viral particle from ORFV infected cellular lysate was demonstrated in the negative-stained field. Moreover, immunomodulating and anti-influenza virus properties of this ORFV were investigated. ORFV stimulated human monocytes (THP-1) secreting proinflammatory cytokines IL-8 and TNF-α. And, pre-treatment of ORFV-infected cell medium prevents A549 cells from subsequent type A influenza virus (IAV) infection. Similarly, mice infected with ORFV via both intramuscular and subcutaneous routes at two days prior to IAV infection significantly decreased the replication of IAV. In summary, the results of a current study indicated our Hoping strain harbors the immune modulator property; with such a bio-adjuvanticity, we further proved that pre-exposure of ORFV protects animals from subsequent IAV infection.

KEY WORDS: cell culture, immunomodulating, influenza virus, orf virus, plaque assay

doi: 10.1292/jvms.14-0663; *J. Vet. Med. Sci.* 77(9): 1055–1062, 2015

Orf virus (ORFV), a member of genus parapoxvirus, has a double-stranded DNA genome of approximate 138 kilo-base pairs [10]. The ovoid-shaped viral particles with a crisscross textures have an average size of 260 nm × 160 nm [6]. It can cause contagious pustular dermatitis or contagious ecthyma in sheep, goats, cattle and other ruminants, manifesting extensive vesicles, papules and scabs and distributing at lips, gums, muzzles and udders [4, 29, 33]. Moreover, lesions around mouths and lips of kids and lambs make them reluctant to suck, and their mortality can be as high as 93% under secondary complications [12]. In severe outbreaks, nearly 100% morbidity can be reached, but the mortality for adult animals was often less than 20%. Veterinarians, farmers and inspectors may get this disease through small wounds in hands during milking, feeding and slaughtering [21, 34].

Traditional methods of diagnosis of ORFV infection include clinical symptoms, histopathology examination, serology surveillance and electron microscopy. DNA-based techniques, such as restriction endonuclease analysis, PCR and loop-mediated isothermal amplification (LAMP), were used to facilitate a rapid and accurate diagnosis. And, these also provide molecular characterization and phylogenetic analysis of viral genes [5, 28, 39].

Isolation and propagation of biologically active viruses are essential for virological research and is also a prerequisite for the development of vaccines and anti-viral drugs. In the case of ORFV, primary cells derived from kidney cortex of fetal lambs and cell lines, CSL503 and ovine testis (OT), originated from lung and testes of older lamb, respectively, have been used for the isolation and propagation of virus [32]. The strains of viruses and types of cells determined the outcome of an adaption. For example, Nara strain and OKA strain were adapted in testes and kidney cells as well as lung cells, respectively [33]. Results from capripoxvirus showed that despite tedious procedures for preparations, primary cells were more susceptible to the infection of field isolates than cell lines and the titer of progeny virus from primary cells was consistently higher than that from the ovine testis cell line [2]. However, this was not likely to be the case for those laboratory strains, which had been adapted to cell lines and exhibited stable titers in serial passages. Therefore, the choice of primary cells or cell lines for the culture of ORFV mostly depended on the research purposes.

*CORRESPONDENCE TO: WONG, M.-L., Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan. e-mail: mlwong@dragon.nchu.edu.tw
#: The two authors contribute equally to this study

Since 2006, several outbreaks of orf were found in the central Taiwan, and studies focused on viral DNA sequencing and phylogenetic analysis were reported. [5, 6]. Although the morbidity of these cases were only approximately 2–6% and mild lesions with the mortality of less than 0.8% were found in lambs, more and more cases indicated Taiwan had become an endemic area of orf. Differential diagnostic techniques based on the heterogenic variations in the C-terminals of ATPase genes have been developed for those strains in Taiwan [5]. The initial goal of the present study is to grow ORFV found in Taiwan in cell cultures. One interesting aspect of ORFV biology is the ability to modulate immune response [11]. Hence, the cytokine stimulating activity of our isolate and its effect on following influenza virus replication in cultured cells and animal model were also studied.

MATERIALS AND METHODS

Preparation of primary goat testis cells: One two-week old lamb (Nubian breed) was sacrificed, and tissue including his testis and ears was removed. The use and experimental protocols were approved by the Committee on the Ethics of Animal Experiments of National Chung Hsing University (approval numbers: 101-40). The testis cells derived from removed testis and fibroblasts from ears were cultured in 1× RPMI 1640 medium with 10% fetal bovine serum (FBS). When cells grew to full confluency, cells were trypsinized and subcultured in 1:1 or 1:2 ratios.

Virus inoculation and adaptation to cell culture: Samples were collected from ORFV affected animals in central Taiwan (Hoping) in 2009. Half gram of collected scab from mouth of infected goats was soaked and homogenized in 5 m*l* TE buffer (50 mM Tris-HCl and 10 mM EDTA, pH 8.0) with additional antibiotic. After centrifugation at 3,500 rpm for 10 min, the supernatant was transferred, filtrated through a 0.45 μm filter and stored as a crude viral stock. The homogenate was subsequently inoculated into the primary goat testis cells. During viral absorption, the cells were gently shaken forward and backward every 15 min. After 1 hr, the unattached virus was removed by washing the cells twice with pre-warmed 1× PBS, and the cells were maintained in complete medium 1× RPMI 1640 with 2% FBS. The cell morphology was checked daily for 7 days. If no cytopathy was found, another run of blind passage and the detection of orf viral DNA were conducted. Briefly, the cell lysate was prepared by three freeze-thaw cycles. Then, 10 microliter of cell lysate was incubated with 4-fold volume of QuickExtract solution (DNA Extraction Soln. 1.0, EPICENTRE, Madison, WI, U.S.A.) at 65°C for 6 min and then 98°C for 2 min, followed by detection of viral DNA by PCR (the method was described in the following section). The PCR-positive cellular lysates were sonicated for three cycles (10 sec each cycle at 4°C). Subsequently, the lysate was added into fresh primary testis cells for further incubation for 7 days, and cytopathy was examined by microscopy. Continued passages were repeated until viral cytopathy was obvious.

Detection of viral DNA by nested PCR: During blind passages, the presence of viral DNA was examined by the nested PCR using 2 sets of B2L gene specific primers: outer primer set OVB2LF1 and OVB2LR1 [17], and inner primer set OVB2LF2 and OVB2LR2 (sequences are listed in Table 1) for detecting existing viral DNA. The expected size of PCR products obtained from the first and second round amplification is 1.2 kbp (OVB2LF1 and OVB2LR1; full length B2L gene) and 889 bp (OVB2LF2 and OVB2LR2), respectively. In the first round of nested PCR, the PCR condition started with a denaturation step at 94°C for 4 min, followed by 29 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing) and 72°C for 1 min (extension), and the reaction was ended by a final extension at 72°C for 7 min. One microliter of the first round PCR product was used for the next round of PCR which was followed the same conditions, except with 25 amplification cycles. PCR products were analyzed by 1% agarose gel electrophoresis with Health safe nucleic acid stain. For detection of viral gene during the plaque purification, the PCR was conducted using the inner set of B2L-specific primers (OVB2LF2 and OVB2LR2) following the same PCR condition with a 35 amplification cycles.

Table 1. List of the primers

Primer	Sequence
OVB2LF1	5′ − TCCCTGAAGCCCTATTATTTTTGTG − 3′
OVB2LR1	5′ − GCTTGCGGGCGTTCGGACCTTC − 3′
OVB2LF2	5′ − GCAGCTTCTGCTGCAACCTGAG − 3′
OVB2LR2	5′ − AAGGCGTGGTAGCGGTAGTG − 3′
FP	5′ − GTGTTGATCATCGAAGACTCGGTG − 3′
RP1	5′ − GTCGCCCTTGTCGCCCTTAGTCTC − 3′
RP2	5′ -CCGCCGTCAGAGTCGACGTCGCCC T- 3′

PCR for strain classification of isolated ORFV: After three runs of plaque purification, the picked plaques were selected for identification of viral strains by single-step PCR [5]. Viral DNA for PCR was prepared by the DNA Extraction Soln. 1.0 (EPICENTRE). PCR was conducted with three primers (FP, RP1 and RP2; Table 1) targeting the C-terminus of the ATPase gene. The PCR program proceeded with an initial denaturation at 94°C for 5 min, 35 cycles of brief denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 7 min. The amplified PCR products were analyzed by electrophoresis on 2% agarose gel.

Restriction enzymes digestion: The primary goat testis cells were seeded on the 10-cm cell culture dish (approximate 2×10^6 cells) and were infected with 0.1 MOI (multiplicity of infection) of ORFV. The infected cells were trypsinized and collected when 80% infected cells showing cytopathy (~24 hr). Cells were harvested by brief centrifugation and washed with 1 x PBS twice. The total DNA was obtained by following a method previously described [20]. For each reaction, 5 μg of extracted DNA was digested with 20 units of a restriction enzyme at 37°C for 2 hr. The mixture was analyzed by electrophoresis at 70 volts in a 0.7% agarose gel.

Examination of viral gene expression by RT-PCR: The primary goat testis cells (3.5×10^5) were infected with 10 MOI of ORFV and incubated at 37°C with 5% CO_2. Total

cellular RNA was extracted from the infected cells following the instruction of the RNeasy Mini Kit (Qiagen, Limburg, Netherlands). After RNA quantification, one microgram of RNA was treatment with RQ1 RNase-Free DNase (Promega, Madison, WI, U.S.A.) to eliminate DNA contamination. Reverse transcription was conducted with 0.5 μg of RNA, and the cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen,Waltham, MA, U.S.A.). Subsequent PCR was performed with OVB2LF2 and OVB2LR2 primers. The condition of PCR was a first denaturation at 94°C for 4 min, following with 35 cycles of brief denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 7 min.

Western blot analysis: The primary goat testis cells were infected with 1 plaque forming unit (PFU) of ORFV and maintained with 1 × RPMI 1640 medium containing 2% FBS at 37°C with 5% CO_2. The cellular lysate was prepared by rupturing cells with sample buffer after twice washing with PBS. Following boiling at 100°C for 6 min, proteins were separated by the SDS-15% PAGE and transferred onto a PVDF membrane (Amersham, GE Healthcare, Buckinghamshire, U.K.). After blocking with TBST (20 mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.6) containing 5% skim milk, the membrane was incubated with 1: 2,000 diluted mouse polyclonal anti-OV20.0 antibody generated from mice immunized with purified OV20.0 recombinant protein at 4°C overnight. Then, the membrane was washed 3 times with TBST. The secondary antibody, the horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, Suffolk, U.K.), was added and incubated for 1 hr at room temperature. The signal was then developed using an enzyme-linked chemiluminescence system (ECL, Amersham, GE Healthcare).

Electron microscopy: Electron microscopy was used to examine the morphology of isolated virus particles. The plaque purified virus was inoculated into the goat primary testis cells. The cells were maintained at 37°C with 5% CO_2 and observed the CPE formation. When 80% of the infected cells show severe CPE, the cells and medium were collected by scraping off the attached cells with tip. After brief sonication on ice, the cell debris was removed by centrifugation. The cell supernatant was transferred to a new tube and processed for negative-strain electron microscopy by staining with 2% phosphotungstic acid (PTA).

Cytokines expression in ORFV-infected THP-1 cells: Total 1×10^6 THP-1 cells kindly provided by Professor S.S. Chiou in Graduate Institute of Microbiology and Public Health, National Chung Hsing University were seed in 6 well multiple plates within 1 m*l* of RPMI 1640 medium containing 10% FBS. Subsequently, 10 MOI of ORFV was inoculated into the cells and incubated with the human monocyte THP-1 cells. Forty-eight hr later, the supernatant was collected by brief centrifugation and transferred to a new tube which was then kept at −80°C. The cytokine expression in the culture medium was examined by using the Human IL-8 and tumor necrosis factor alpha (TNF-α) ELISA kit (KOMA BIOTECH INC., Seoul, Korea) performed following the manufacturer's procedure. A set of standard cytokines, provided within the kit, with known concentrations were analyzed in parallel for establishment of standard curve. Finally, the OD value of each sample was obtained from reading the plate at 450 *n*m wavelength. Concentrations of the cytokines were estimated by comparing with the standard curve. The mean value of the results was obtained from three independent experiments and was then assessed by T-test. *P*-value ($P<0.05$) indicated the significant difference between two groups of samples.

Effect of ORFV on subsequent influenza virus infection in cells: Goat fibroblast primary cells were infected with ORFV at MOI of 1, and the cell media were collected at 0, 6, 12 and 24 hr post-infection (hpi). A549 cells were pre-treated with the ORFV infected cell medium for 24 hr. After removal of the cell medium and PBS washing, the A549 cells were infected with 1 MOI of influenza virus (PR8 strain) [7]. Cellular lysate of infected human lung carcinoma A549 cells obtained from ATCC (CCL-185) was harvested at 12 hpi and resolved by SDS-PAGE electrophoresis followed by Western blot analysis using IgY antibody against influenza virus NP generated from chickens immunized with purified NP recombinant protein. The overall procedures of Western blot analysis followed the description in the previous section. Densitometric quantifications of each band indicating NP expression level were carried out using National Institutes of Health IMAGEJ software version 1.43 (http://rsb.info.nih.gov/ij).

Effect of ORFV on subsequent influenza virus infection in mice: The inhibitory activity on influenza virus infection by ORFV was further tested in mice. Six weeks old, female BALB/C mice received UV-inactivated 2×10^5 plaque formation unit (PFU) of isolated ORFV, Hoping strain (n=4) or PBS (n=4, as negative control) by intramuscular (IM) or subcutaneous (SC) routes. Two days after infection, the mice were intranasally challenged with 1×10^4 influenza virus (PR8 strain). Seven days post infection, blood was taken from each mouse for determining the concentrations of IL-6 and TNF-α in serum by ELISA kits (BioLegend; San Diego, CA, U.S.A.) performed following the manufacturer's procedure. Subsequently, the mice were scarified, and the lungs of the mice were collected. The whole lung tissue was homogenized in 1 m*l* of DMEM without FBS. After brief centrifugation, the supernatant was used for measurement of the influenza virus titer on MDCK cells. After two to three days post-infection, the infected MDCK cells were fixed in methanol and stained with crystal violet. The number of influenza viral plaque was calculated. The biostatic analysis was performed by using the *t*-test model.

RESULTS

Viral infection and adaptation to the primary testis cells: The crude virus stock was serially diluted and inoculated into the primary goat testis cell. Cells were observed daily for appearance of CPE, and the presence of viral DNA was determined by PCR in each run (1 week interval). After 3–5 rounds of blind passages, stronger bands were amplified in the second round of nested PCR, although no apparent CPE was observed. In subsequent cycles of blind passages, ORFV was adapted and enriched in primary goat testis cells; viral

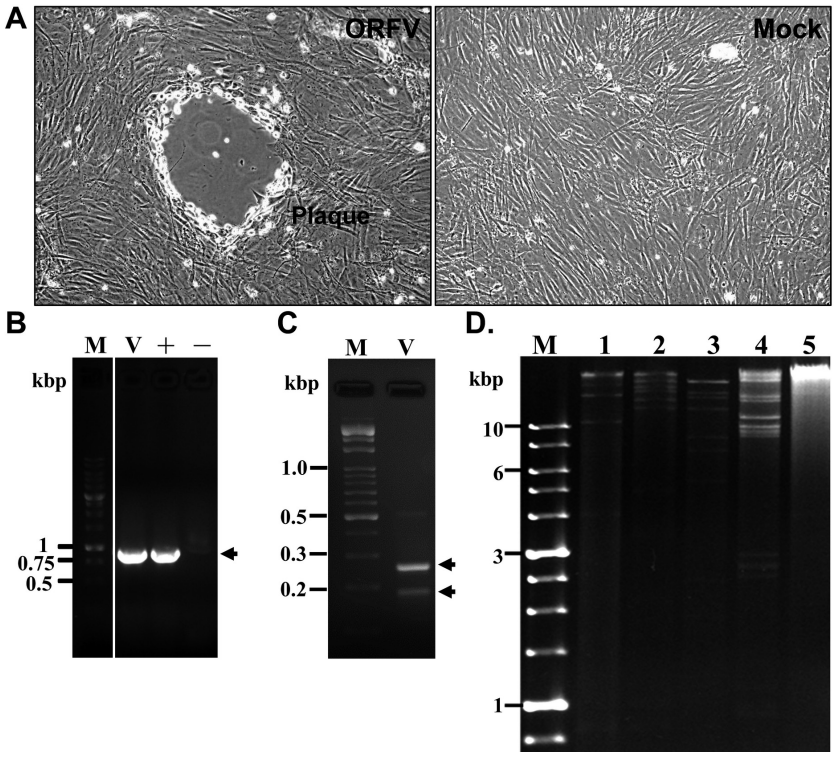

Fig. 1. Cytopathic effect on primary goat testis cells caused by cellular lysate containing ORFVs. (A) The infected primary goat testis cells showed rounding, shrinking and detachment, and eventually formed a viral plaque (200 × magnification). Mock is the image of uninfected cells. (B) Detection of orf viral DNA by PCR. The isolated plaque (v) showed the expected sizes (~900 bp, as indicated by the arrow) of amplified products of partial B2L gene. The positive control (+) was the virus-infected cell lysate in PCR; the negative control (–) was done without any DNA template. M is DNA size markers. (C) Identification of isolated ORFVs by the single-step PCR. As the PCR amplification yielded two DNA fragments with characteristic sizes (180 and 254 bp, as indicated by arrows), it indicated the isolated ORFV is the Hoping strain (Chan *et al.*, 2009). (D) Patterns of orf viral DNA after restriction enzymes digestion. Lane 1: DNA treated with EcoR I; lane 2: treatment with BamH I; lane 3: treatment with Hind III; lane 4: treatment with Kpn I; lane 5 is the uninfected cell, and the stained DNA is smearing after Kpn I digestion. M is the DNA size markers.

DNA can be detected in the first round of the nested PCR.

Plaque purification of the ORFV: With continued viral passages, the infected primary goat testis cells began to show the cytopathic effect (CPE) and form a viral plaque. The CPE in primary goat testis cells was local and limited on the area of affected cells after four to five days after infection (Fig. 1A) that is consistent with one previous study that parapoxviruses form plaques in primary bovine testis cells [22]. After three times of plaque purification, the purity of isolated ORFV was examined by PCR using primers targeting viral B2L gene. The size of PCR product is proximate 900 bp (Fig. 1B), and subsequent DNA automated-sequencing confirmed the nucleotide identity of ORFV B2L gene (data not shown).

Identification of isolated ORFV: A single-step PCR developed in our laboratory that shows distinct amplification patterns of three ORFV strains in Taiwan was used for identifying the isolated viruses [5]. The Nantou and Taiping strain could amplify 180 and 217 bp product, respectively, and 2 different length fragments (180 and 254 bp) can be produced at the same time in the primary cells from goats. Results showed our purified ORFV was the Hoping strain (Fig. 1C). The nucleotide sequences of the Hoping strain were further confirmed by automated DNA sequencing (data not shown). Furthermore, the restriction enzyme digestion pattern of viral DNA of the Hoping strain was also confirmed (Fig. 1D). In comparison with the smearing DNA of uninfected sample (lane 5 in Fig. 1D), all the DNA of virus-infected samples treated with restriction enzymes showed characteristic cutting patterns.

Viral gene expression examined by RT-PCR and immunoblotting: To examine viral gene expression in the primary goat testis cell, the viral RNA was detected by RT-PCR. Despite the weaker expression, the transcripts of B2L can be detected at the early stage of infection (2–3 hpi), and it was largely synthesized after 12 hpi (Fig. 2A). Moreover, the ORFV gene expression was verified by Western blotting by using the mouse polyclonal anti-OV20.0 antibody. Results of Fig. 2B demonstrated the viral OV20.0 protein (25 kDa) was produced after 6, 12 and 24 hpi. These data indicated that genes of isolated ORFV can be actively expressed in the primary goat testis cells.

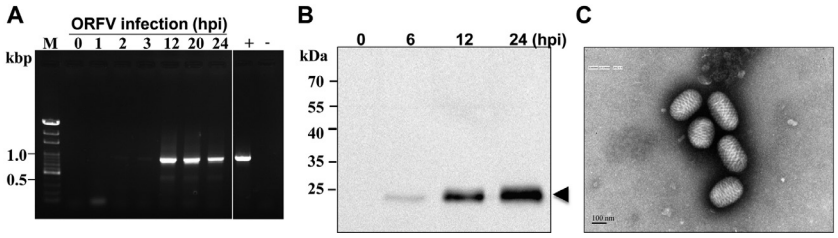

Fig. 2. Detection of the viral B2L gene expression and viral OV20.0 protein in infected primary goat cells. (A) RNA was extracted from cells infected with ORFV at 0, 1, 2, 3, 12, 20 and 24 hpi (lanes 1–7) for reverse transcription (RT) − PCR detection. The transcription of B2L appeared at 2 and 3 hr post-infection and then was saliently increased at 12, 20 and 24 hpi. The RT was conducted without reverse transcriptase (−). + indicates a positive control in which DNA template is derived from virus lysate. (B) The expression of OV20.0 (the ortholog of vaccinia virus E3 protein) was observed at 6 hr, 12 hr and 24 hr after infection; the expected molecular weight of OV20.0 is 25 kDa indicated by the arrowhead. Lane 1 is mock infection. Electron micrograph of orf viruses (C) prepared from primary goat testis cells. The characteristic morphology of orf virus was observed, and viral particles showed ovoid-shape with a spiral crisscross pattern (bar=100 nm).

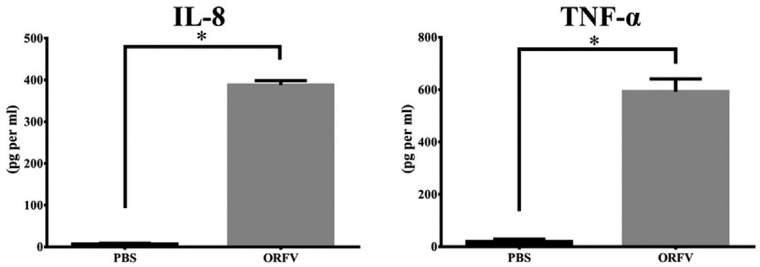

Fig. 3. Expression of IL-8 and TNF-α in ORFV treated human monocyte THP-1 cells. To examine whether our ORFV can affect the cellular cytokine levels, THP1 cells were incubated with 10 MOI of ORFV for 48 hr. The cell medium was collected for examination of the cytokine IL-8 and TNF-α expressions by using the ELISA kit (KOMA BIOTECH INC.) The results demonstrated that IL-8 and TNF-α expression level significantly increased in cells infected with ORFV, compared with that of PBS control. The column of each group was the mean (+/–SD) of three independent experiments. Statistical analysis was performed using unpaired T-test, and P value <0.05 (shown with a star symbol) indicates the statistical significance.

Electron micrograph observation: Morphological confirmation of orf virions in the infected goat testis cells was achieved with electron microscopy. The electron micrograph results demonstrated the presence of ovoid-shape virions with a spiral crisscross pattern (Fig. 2C).

Detection of cytokines produced in THP-1 cells with ORFV: The ORFV infection elicits expression of proinflammatory cytokines, such as several interleukins (ILs) and TNF-α, which contributes to the immune regulation of ORFV and has been demonstrated in many studies [8, 9, 15, 26, 37]. It is important to explore whether ORFV (Hoping strain) harbors the immunostimulating activity. The human monocyte cell line, THP-1 cell, was infected with our local isolate at MOI of 10 for 48 hr. The cell medium was collected for detection of IL-8 and TNF-α cytokine production. As the result shown in Fig. 3, compared with a mock control, infection of ORFV indeed caused the rise of the IL-8 and TNF-α expressions in THP-1 cells.

ORFV inhibited influenza virus replication in A549 cells: As an immune modulator, ORFV has been shown to act as an inhibitor to prevent other virus infection and also to work as a tumor killer [3, 16, 19, 31]. The primary goat fibroblast cells were infected with 1 MOI of ORFV. The ORFV infected cell medium, of which the infectivity of ORFV was under detection, was collected at 6, 12 and 24 hpi, and overlaid onto A549 cells. After 24 hr treatment, the A549 cells were then infected with influenza virus PR8 strain for 12 hr. A significant decrease of viral NP protein expression was observed in cells pre-treated with ORFV infected cell medium (Fig. 4). Noticeably, increase of inhibitory strength coincided with the length of ORFV infection; the longer ORFV infection, the stronger inhibitory effect was observed. It indicates ORFV (Hoping strain) can prevent influenza virus infection in A549 cells.

Inhibition of subsequent influenza virus replication in mice inoculated with UV-inactivated ORFV: To further investigate the inhibitory activity on influenza virus infection by UV-inactivated ORFV, 6 weeks old female BALB/C mice were inoculated with either 2×10^5 PFU of UV-inactivated ORFV (n=4) or PBS (n=4, as a negative control) by IM and SC routes for 2 days and subsequently infected with influenza virus. As shown in Fig. 5A, in mice groups received the inactivated ORFV via both IM and SC routes, the titer of influenza viruses significantly decreased (Fig. 5A). The cytokine profile was also determined in mice inoculated with

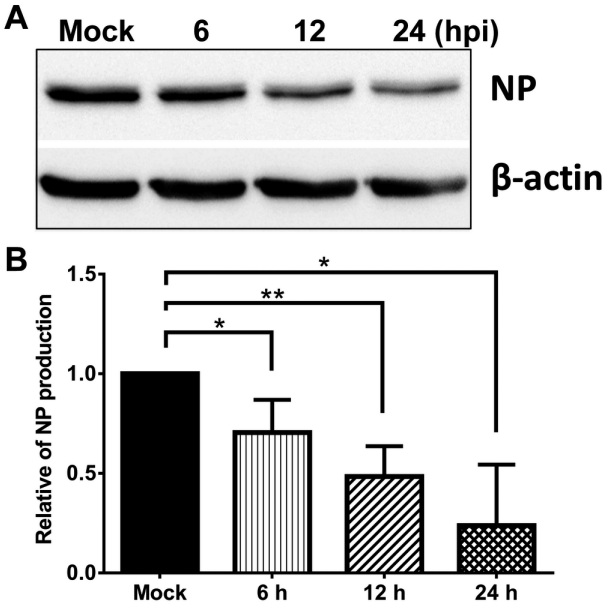

Fig. 4. Pre-treatment of ORFV-infected cell medium prevents A549 cells from type A influenza virus (IAV) infection. The primary goat testis cells were infected with ORFV (Hoping strain). At two hr post infection (hpi), the unattached virus was removed by washing with PBS. The cells were maintained in complete RPMI 1640 medium with 10% FBS. At 6, 12 and 24 hpi, the medium was collected and used for treatment of human A549 cells. After 24 hr treatment, the A549 cells were infected with 1 MOI of IAV (PR8 strain). At 12 hpi, expression of viral nucleoprotein (NP) of the IAV was analyzed by immunoblotting (panel A), and the quantitative analysis of NP production was shown in panel B. The column of each group was the mean (+/–SD) of three independent experiments. The results were analyzed by the T-test. The *P* value <0.05 (shown with a star symbol) indicates the difference between 2 groups is statistically significant.

ORFV. Elevated IL-6 and TNF-α expressions in mice with the inactivated ORFV were noticed; however, only increased IL-6 was statistically significant (Fig. 5B).

DISCUSSION

ORFV, an epitheliotropic parapoxvirus, causes proliferative dermatitis in goats and sheep, and persistence infection usually occurs in an outbreak farm. Although molecular identification by PCR and phylogenetic analysis of viral DNA purified from the animal tissues are common, the isolation of ORFV from the field and production of virions in cell culture are still formidable tasks [1]. The isolation of parapoxviruses from cows, goats and sheep and serows as well as from ruminant animals (musk ox and Sichuan takin) of a zoo was claimed [13, 14, 18, 22, 24, 30, 36, 38]; two of these studies investigated diseases of goats [18, 30]. So far, the most studied ORFV strains are NZ2, IA82, SA00 and D1701 which were isolated from New Zealand, north America and Germany [10, 25, 29]. In this work, a new ORFV strain was isolated from current outbreak in Taiwan, and the immunoregulation activity of this Asian strain was demonstrated for the first time.

The primary cells from goats, sheep or cattle source have been used to isolate parapoxviruses [18, 22, 24, 38], and subsequently, some cell lines were found to be susceptible to ORFV infection, for examples, Madin-Darby ovine kidney (MDOK) cells and Madin-Darby bovine kidney (MDBK) cells [13, 14, 23, 30]. The primary testis cells of goat, the original host of ORFV, were used to isolate the virus from the field samples. After several rounds of plaque purification, our local ORFV was purified in homogeneity as indicated by PCR amplifications and by electron microscopy; the typical ovoid-shaped virus particles with particular surface pattern of ORFV were demonstrated. On the basis of PCR genotyping, we confirmed the resulted purified orf viruses were the Hoping strain, one of the three strains of ORFV identified in Taiwan.

Inactivated ORFV has been recognized as an immunomodulator [3]. Previously, immune stimulation effects of several strains of ORFV were demonstrated. Anziliero *et al*. reported that the inactivated ORFV particles (Iowa strain) mediated activities against infections of different microbes and transient induction of certain innate immune mechanisms by ORFV administration likely contributing to the immunostimulant effects [11]. Weber *et al*. showed treatment of ORFV (D1701 strain) protects mice from acquiring and recurrent of herpes simplex virus type 1 infection [40]. More recently, the effect of ORFV particles on inhibition of viral infection was further demonstrated in different models: Paulsen *et al*. replication of hepatotropic pathogens, i.e. hepatitis B virus and hepatitis C virus, was significantly reduced in a mouse model or *in vitro*, respectively [31]; and such an anti-viral activity can be observed in 2 different viral strains (D1701 and NZ2). Moreover, D1701 strain, a highly attenuated viral strain with reduced pathogenicity [27], is the main component of the commercially available immunomodulator. Considering the immunoregulatory effect might be variable between strains with different virulence, and hence, in this study, we set out experiments to explore the effect of Hoping strain on subsequent influenza virus infection that poses a severe threat to humans and animals and is considered a major worldwide public health problem.

Our results indicated that the production of proinflammatory cytokines, such as IL-6 and TNF-α, was significantly increased in the sera of mice pre-treated with ORFV compared with that in the sera of mice receiving the PBS before IAV infection (Fig. 5B). TNF-α and IL-8 were also higher in medium of ORFV treated human monocyte THP-1 cells (Fig. 3) that indicates an important role for ORFV in modulating production of multiple inflammatory cytokines. This is in line with previous findings; TNF-α contributes to resolving influenza virus infection in the host respiratory tract [37], and IL-6 promotes viral clearance and reduces mortality to influenza virus infection via enhancing neutrophil survival from influenza virus-induced death [11].

Recently, Rohde *et al.* generated 2 ORFV recombinants expressing the hemagglutinin (HA) or nucleoprotein (NP) of the highly pathogenic avian influenza virus (HPAIV, strain H5N1), and via IM route immunization of two doses (10^7

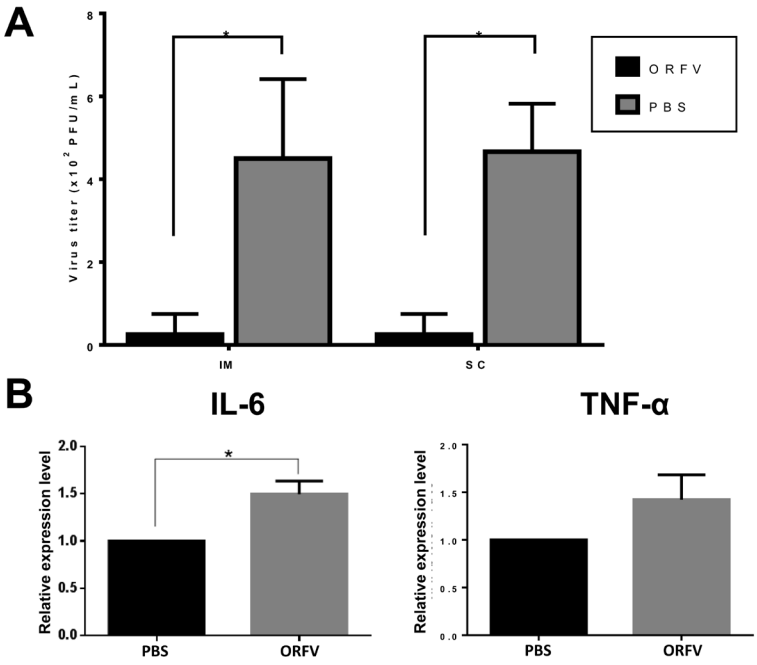

Fig. 5. Infection of ORFV suppresses the subsequent IAV infection in mice. ORFV was administrated intramuscularly (IM) or subcutaneously (SC) to BALB/c mice (6 weeks old, n=4) at dosage of 2×10^5 PFU before IAV infection. One group was administrated with as a control (n=4). Two days later, all groups of mice received a dosage of 1×10^4 PFU IAV via intra-nasal route. Mice were observed to check any illness symptoms. Seven days post infection, all the mice were sacrificed, and their lungs were removed for titration of the virus loads (A), and IL-6 and TNF-α expressions in blood were evaluated by ELISA (BioLegend) (B). Values display the means (+/–SD) of each group. Statistical analysis was performed using unpaired T-test with Welch's correction. *P* value<0.05, shown with a star symbol, indicates statistical difference between 2 groups.

PFU of each) of HA-expressing ORFV protects mice from lethal HPAIV infection [35]. The present study showed that without expressing any influenza specific antigen, treatment of ORFV significantly inhibited expression of NP protein in cells, and also, one dose (10^5 PFU) of ORFV immunization is able to reduce the influenza viral load in mouse lung. Based on the cytokine expression profile, it appears ORFV infection elevates levels of proinflammatory cytokines (IL-6 or IL-8). However, further studies are required to elucidate the mechanisms of suppressing influenza virus infection. In summary, our findings indicate the immunostimulating effect of ORFV.

ACKNOWLEDGMENTS. We thank Dr. Hiroshi Sentsui (Department of Veterinary Medicine, Nihon University, Fujisawa, Kanagawa, Japan) for helpful suggestion and for sharing his experience in parapoxvirus research. The project was supported in part by the Ministry of Science and Technology (NSC101-2321-B-005-005, NSC102-2321-B-005-001, MOST 103-2321-B-005-001) and by the Ministry of Education, Taiwan, R.O.C. under the ATU plan.

REFERENCES

1. Abu Elzein, E. M. and Housawi, F. M. 1997. Severe long-lasting contagious ecthyma infection in a goat's kid. *Zentralbl. Veterinarmed. B.* **44**: 561–564. [Medline]
2. Babiuk, S., Parkyn, G., Copps, J., Larence, J. E., Sabara, M. I., Bowden, T. R., Boyle, D. B. and Kitching, R. P. 2007. Evaluation of an ovine testis cell line (OA3.Ts) for propagation of capripoxvirus isolates and development of an immunostaining technique for viral plaque visualization. *J. Vet. Diagn. Invest.* **19**: 486–491. [Medline] [CrossRef]
3. Büttner, M. 1993. Principles of paramunization. Option and limits in veterinary medicine. *Comp. Immunol. Microbiol. Infect. Dis.* **16**: 1–10. [Medline] [CrossRef]
4. Büttner, M. and Rziha, H. J. 2002. Parapoxviruses: from the lesion to the viral genome. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **49**: 7–16. [Medline] [CrossRef]
5. Chan, K. W., Hsu, W. L., Wang, C. Y., Yang, C. H., Lin, F. Y., Chulakasian, S. and Wong, M. L. 2009. Differential diagnosis of orf viruses by a single-step PCR. *J. Virol. Methods* **160**: 85–89. [Medline] [CrossRef]
6. Chan, K. W., Lin, J. W., Lee, S. H., Liao, C. J., Tsai, M. C., Hsu, W. L., Wong, M. L. and Shih, H. C. 2007. Identification and phylogenetic analysis of orf virus from goats in Taiwan. *Virus Genes* **35**: 705–712. [Medline] [CrossRef]
7. Chen, T. Y., Chen, D. Y., Wen, H. W., Ou, J. L., Chiou, S. S., Chen, J. M., Wong, M. L. and Hsu, W. L. 2013. Inhibition of enveloped viruses infectivity by curcumin. *PLoS ONE* **8**: e62482. [Medline] [CrossRef]
8. Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M., Ho, Y. F., Shen, T. S., Ko, J. Y., Lin, J. T., Lin, B. R., Ming-Shiang, W., Yu, H. S., Jee, S. H., Chen, G. S., Chen, T. M., Chen, C. A., Lai, M. K., Pu, Y. S., Pan, M. H., Wang, Y. J., Tsai, C. C. and Hsieh, C. Y. 2001.

Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.* **21**: 2895–2900. [Medline]
9. Chuang, S. E., Kuo, M. L., Hsu, C. H., Chen, C. R., Lin, J. K., Lai, G. M., Hsieh, C. Y. and Cheng, A. L. 2000. Curcumin-containing diet inhibits diethylnitrosamine-induced murine hepatocarcinogenesis. *Carcinogenesis* **21**: 331–335. [Medline] [CrossRef]
10. Delhon, G., Tulman, E. R., Afonso, C. L., Lu, Z., de la Concha-Bermejillo, A., Lehmkuhl, H. D., Piccone, M. E., Kutish, G. F. and Rock, D. L. 2004. Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. *J. Virol.* **78**: 168–177. [Medline] [CrossRef]
11. Dienz, O., Rud, J. G., Eaton, S. M., Lanthier, P. A., Burg, E., Drew, A., Bunn, J., Suratt, B. T., Haynes, L. and Rincon, M. 2012. Essential role of IL-6 in protection against H1N1 influenza virus by promoting neutrophil survival in the lung. *Mucosal Immunol.* **5**: 258–266. [Medline] [CrossRef]
12. Gumbrell, R. C. and McGregor, D. A. 1997. Outbreak of severe fatal orf in lambs. *Vet. Rec.* **141**: 150–151. [Medline] [CrossRef]
13. Guo, J., Rasmussen, J., Wunschmann, A. and de La Concha-Bermejillo, A. 2004. Genetic characterization of orf viruses isolated from various ruminant species of a zoo. *Vet. Microbiol.* **99**: 81–92. [Medline] [CrossRef]
14. Guo, J., Zhang, Z., Edwards, J. F., Ermel, R. W., Taylor, C. Jr. and de la Concha-Bermejillo, A. 2003. Characterization of a North American orf virus isolated from a goat with persistent, proliferative dermatitis. *Virus Res.* **93**: 169–179. [Medline] [CrossRef]
15. Hergenhahn, M., Soto, U., Weninger, A., Polack, A., Hsu, C. H., Cheng, A. L. and Rosl, F. 2002. The chemopreventive compound curcumin is an efficient inhibitor of Epstein-Barr virus BZLF1 transcription in Raji DR-LUC cells. *Mol. Carcinog.* **33**: 137–145. [Medline] [CrossRef]
16. Hofstetter, R., Mayr, A. and von Bernuth, G. 1981. [Echocardiographically determined ventricular function in newborns with and without treatment of the mother with fenoterol during pregnancy (author's transl)]. *Z. Geburtshilfe Perinatol.* **185**: 227–230. [Medline]
17. Hosamani, M., Bhanuprakash, V., Scagliarini, A. and Singh, R. K. 2006. Comparative sequence analysis of major envelope protein gene (B2L) of Indian orf viruses isolated from sheep and goats. *Vet. Microbiol.* **116**: 317–324. [Medline] [CrossRef]
18. Hosamani, M., Yadav, S., Kallesh, D. J., Mondal, B., Bhanuprakash, V. and Singh, R. K. 2007. Isolation and characterization of an Indian ORF virus from goats. *Zoonoses Public Health* **54**: 204–208. [Medline] [CrossRef]
19. Hsu, C. H., Chuang, S. E., Hergenhahn, M., Kuo, M. L., Lin, J. K., Hsieh, C. Y. and Cheng, A. L. 2002. Pre-clinical and early-phase clinical studies of curcumin as chemopreventive agent for endemic cancers in Taiwan. *Gan To Kagaku Ryoho* **29** Suppl 1: 194–200. [Medline]
20. Inoshima, Y., Morooka, A., Murakami, K. and Sentsui, H. 2000. Simple preparation of parapoxvirus genome DNA for endonuclease analysis. *Microbiol. Immunol.* **44**: 69–72. [Medline] [CrossRef]
21. Inoshima, Y., Murakami, K., Yokoyama, T. and Sentsui, H. 2001. Genetic heterogeneity among parapoxviruses isolated from sheep, cattle and Japanese serows (Capricornis crispus). *J. Gen. Virol.* **82**: 1215–1220. [Medline]
22. Kanou, Y., Inoshima, Y., Shibahara, T., Ishikawa, Y., Kadota, K., Ohashi, S., Morioka, K., Yoshida, K. and Yamada, S. 2005. Isolation and characterization of a parapox virus from sheep with popular stomatitis. *Jpn. Agric. Res. Q.* **39**: 197–203. [CrossRef]
23. Klein, J. and Tryland, M. 2005. Characterisation of parapoxviruses isolated from Norwegian semi-domesticated reindeer (Rangifer tarandus tarandus). *Virol. J.* **2**: 79. [Medline] [CrossRef]
24. Kottaridi, C., Nomikou, K., Lelli, R., Markoulatos, P. and Mangana, O. 2006. Laboratory diagnosis of contagious ecthyma: comparison of different PCR protocols with virus isolation in cell culture. *J. Virol. Methods* **134**: 119–124. [Medline] [CrossRef]
25. Lu, H. F., Yang, J. S., Lai, K. C., Hsu, S. C., Hsueh, S. C., Chen, Y. L., Chiang, J. H., Lu, C. C., Lo, C., Yang, M. D. and Chung, J. G. 2009. Curcumin-induced DNA damage and inhibited DNA repair genes expressions in mouse-rat hybrid retina ganglion cells (N18). *Neurochem. Res.* **34**: 1491–1497. [Medline] [CrossRef]
26. Mayr, A. 1981. Virus diseases in dogs and cats. New trends in the microbial spectrum and in treatment. *Tierarztl. Prax.* **9**: 103–116. [Medline]
27. Mayr, A., Herlyn, M., Mahnel, H., Danco, A., Zach, A. and Bostedt, H. 1981. [Control of ecthyma contagiosum (pustular dermatitis) of sheep with a new parenteral cell culture live vaccine]. *Zentralbl. Veterinarmed. B.* **28**: 535–552. [Medline] [CrossRef]
28. Mercer, A., Fleming, S., Robinson, A., Nettleton, P. and Reid, H. 1997. Molecular genetic analyses of parapoxviruses pathogenic for humans. *Arch. Virol. Suppl.* **13**: 25–34. [Medline]
29. Mercer, A. A., Ueda, N., Friederichs, S. M., Hofmann, K., Fraser, K. M., Bateman, T. and Fleming, S. B. 2006. Comparative analysis of genome sequences of three isolates of Orf virus reveals unexpected sequence variation. *Virus Res.* **116**: 146–158. [Medline] [CrossRef]
30. Musser, J. M., Taylor, C. A., Guo, J., Tizard, I. R. and Walker, J. W. 2008. Development of a contagious ecthyma vaccine for goats. *Am. J. Vet. Res.* **69**: 1366–1370. [Medline] [CrossRef]
31. Paulsen, D., Urban, A., Knorr, A., Hirth-Dietrich, C., Siegling, A., Volk, H. D., Mercer, A. A., Limmer, A., Schumak, B., Knolle, P., Ruebsamen-Schaeff, H. and Weber, O. 2013. Inactivated ORF virus shows antifibrotic activity and inhibits human hepatitis B virus (HBV) and hepatitis C virus (HCV) replication in preclinical models. *PLoS ONE* **8**: e74605. [Medline] [CrossRef]
32. Pye, D. 1989. Cell lines for growth of sheep viruses. *Aust. Vet. J.* **66**: 231–232. [Medline] [CrossRef]
33. Pye, D. 1990. Vaccination of sheep with cell culture grown orf virus. *Aust. Vet. J.* **67**: 182–186. [Medline] [CrossRef]
34. Robinson, A. J. and Mercer, A. A. 1995. Parapoxvirus of red deer: evidence for its inclusion as a new member in the genus parapoxvirus. *Virology* **208**: 812–815. [Medline] [CrossRef]
35. Rohde, J., Amann, R. and Rziha, H. J. 2013. New Orf virus (Parapoxvirus) recombinant expressing H5 hemagglutinin protects mice against H5N1 and H1N1 influenza A virus. *PLoS ONE* **8**: e83802. [Medline] [CrossRef]
36. Sentsui, H., Murakami, K., Inoshima, Y., Shibahara, T. and Yokomizo, Y. 1999. Isolation of parapoxvirus from a cow treated with interferon-gamma. *Vet. Microbiol.* **70**: 143–152. [Medline] [CrossRef]
37. Seo, S. H. and Webster, R. G. 2002. Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. *J. Virol.* **76**: 1071–1076. [Medline] [CrossRef]
38. Suzuki, T., Minamoto, N., Sugiyama, M., Kinjo, T., Suzuki, Y., Sugimura, M. and Atoji, Y. 1993. Isolation and antibody prevalence of a parapoxvirus in wild Japanese serows (Capricornis crispus). *J. Wildl. Dis.* **29**: 384–389. [Medline] [CrossRef]
39. Tsai, S. M., Chan, K. W., Hsu, W. L., Chang, T. J., Wong, M. L. and Wang, C. Y. 2009. Development of a loop-mediated isothermal amplification for rapid detection of orf virus. *J. Virol. Methods* **157**: 200–204. [Medline] [CrossRef]
40. Weber, O., Siegling, A., Friebe, A., Limmer, A., Schlapp, T., Knolle, P., Mercer, A., Schaller, H. and Volk, H. D. 2003. Inactivated parapoxvirus ovis (Orf virus) has antiviral activity against hepatitis B virus and herpes simplex virus. *J. Gen. Virol.* **84**: 1843–1852. [Medline] [CrossRef]