Open

Citation: J Med Discov (2016); 1(1):jmd16004; doi:10.24262/jmd.1.1.16004

Research Article

Impacts of influenza virus upon dynamics of proteinase-inhibitory activity in chicken embryos

Valentina A. Divocha[1,*], Yaroslav A. Basarab[2]

[1]*Ukrainian Research Institute of Transport Medicine, 92, Kanatnaya, Str, Odessa, Ukraine, 65039*, [2]*Ukrainian Academy of Medical Dental 23, Shevchenko Str, Poltava, Ukraine, 36011*

This paper presents some results studying of influence of influenza A virus in development on ferments of chicken embryos. In work used influenza virus A/PR/8/34/H1N1/. Each hour were studied on 3 embryos. Not infected chicken embryos were the control. In the culled stuff defined proteinase, inhibiting, infectious activities, titer of a hemagglutinin and the protein content. Level trypsin-like proteinase and an inhibitor in 11-day chicken embryos was in balance on enough high level. At entering of influenza A virus the balance of proteinase and proteinase inhibitor was sharply broken, especially at infestation by a low dose of influenza A virus. In infested chicken embryos within the first hours there was an augmentation both proteinase, and inhibitor activity. The heavy and low doses of infestation of influenza A virus cause unequal changes of proteinase and inhibitor activity. The maximum of accumulation of an infectious influenza A virus don't depends on its dose, at a low dose accumulation time is enlarged. In chicken embryos there are a conformity between accumulation of an infectious influenza A virus and the subsequent depression proteinase activity.

Keywords: influenza A viruse, proteinase, inhibitors, chicken embryos

Journal of Medical Discovery (2016); 1(1): jmd16004; Received October 11th, Revised November 24th, Accepted November 24th, Published November 27th.

Introduction

Nowadays interaction between influenza virus and a host cell is described in numerous relevant reports as a comprehensive whole that reflects a set of internal relations between

* Correspondence: Dr. Divocha, Email:divocha09@ukr.net.

these two actively involved members. The end result of this interaction is genetically determined by the host and pathological agent. It depends on both the level of body regulation processes and the body defense ability. The balance between these preconditions is caused by the outcome of this interaction whether this may be the death of the host organism, its recovery or developing chronic infection condition.

For infiltration of influenza virus into a cell it is necessary, that the hemagglutinin (HA0) has undergone to scission on 2 subunits HA1 and HA2. Scission of HA0 of influenza virus is made by trypsin-like proteinases of a healthy cell [1-3]. This phenomenon named proteolytic activation of viruses, underlies a pathogenesis virus disease and pathogenic properties of many capsulate viruses [4, 5]. Earlier the theory of inhibition of influenza virus by means of antiproteinase agents has been stated, capable to quench a stage of proteolytic activation of influenza virus [6]. Blocking of development of influenza virus on our researches have made by two ways: by antiproteinase vaccines and by own inhibitor of trypsin-like proteinases [7, 8]. The objective of this work is to study the effects produced by influenza A viruses on enzymes (trypsin-like proteinases and inhibitors) of chicken embryos in dynamics.

Materials and Methods

Reagents

The reagents used in this study included 1.0 % solution of protamin sulphate on 0.1 M phosphate buffer pH 7.5, 20 % solution of trichloroacetic acid. 0.004% solution of oxychinoline, 100 mg of oxychinoline was diluted in 50.0 mL of ethanol with the working solution was prepared by 50 times soluted with aqua distilata initial solution just before determination, 10.0 % NaOH, 10.0 % solution of NaBrO (1.0 r Br (0.3 mL) was brought up to 100 mL with cooled to 5.0% solution NaOH), 40.0% solution of urea. All the reagents are purchased from Sigma-Aldrich (St.Louis , MO).

Virus

The influenza virus A/PR /8/34/H1N1/ used in this work was taken from the Laboratory strains museum of the D. I. Ivanovskiy Institute of Virology, Academy of Medical Sciences. The influenza virus was adapted to chicken embryos with hemagglutinin titer 1:128. 11-day chicken embryos were infected with this virus in two ways: with heavy doses of virus-containing fluid in ten fold dilution that corresponds to 20 LD_{50}, and with small doses in dilution 10^{-6} that corresponds to 1 $LD_{50.}$ We focused on the role of proteinases and their inhibitors in the emergence of influenza infection.

Viral allantoic fluid were withdrawn in 15 min, 30 min, 1 h, 6 h, 34 h, 48 h, 72 h, 96 h and 122 h after the inoculation of the virus. We examined 3 embryos hourly (in each moment of time). Materials selected were used

to evaluate proteinase, inhibitory, and infectious activity of hemagglutinin and protein. The control group included non-infected chicken embryos, which were treated with the technique described above. Trypsin-like proteinase activity was evaluated by the method of K.Veremeenko modified by S.V. Vovchuk [9]. Regarding the determination of trypsine-like proteinases activity by protamin hydrolysis, qualitative reaction to arginin, forming at the hydrolysis of protamin and histons, and which does not precipitate with 20% CCl3COOH, forms the basis the method.

Procedure of determination

To the mixture, containing 0.2 mL of protamin sulphate and 0.5 mL 0.1 M phosphate buffer pH 7.5, 1 mL of enzymatic solution was added. The samples were incubated 150 min on the water bath at 38℃. The reaction was stopped by 0.9 mL of 20.0% CCl3COOH. The content of the tube was mixed and centrifugated 15 min at 6000 R.P.M. 10 mL of supernatant was got over into ice-cooled tubes. 1.0 mL of oxychinolone solution, 1.0 mL of 10% NaOH and 0.2 mL of NaBrO was added in succession. The tubes were 6 agitated and in 15 min 1.0 mL of 40.0% urea was added. 1.0 mL of cooled distillated water was added and in 5 min the activity was checked at 508 mL (blue-green light filter). To the contral sample 0.8 mL of 20% CCl3COOH was added to 0.8 mL of protamin sulphate and phosphate buffer before 0.5 mL 0.1 M of phosphate buffer pH 7.5; 0.1 mL of the solution under study; 0.8 mL CCl3COOH (20%) 0.2 mL 1% solution of protamin sulphate on phosphate buffer we added to the control sample.

Spectrophotometer was maintained by the control to reagents – instead of 1.0 mL of supernatant liquid 1.0 mL of 10.0% of CCl3COOH was added in the sample. The further determination was similar to that in the experimental sample.

Enzymes activity was determined with the following formula:

Homogenate of lungs

$A= \Delta Emg \bullet 1.6 \bullet 5/(0.1 \bullet T \bullet 174.2)$

Blood serum

$A= \Delta Emg \bullet 1.6 \bullet 100/(0.1 \bullet T \bullet 174.2)$

Total formula

$A=\Delta Emg \bullet n \bullet 2/(0.1 \bullet T \bullet k)$ (modified by S.V.Vovchuk)

In these, the symbols indicate: A – activity of enzyme in u/mL. A unit of activity is equal to an amount of enzyme causes formation of 1 micromol of agrinin per 1 min of incubation; ΔE – extinctions difference between experimental and control sample; n – dilution of enzymatic solution; 1.6 – the total volume of sample; 2 and 5 – recalculation for the whole mixer under incubation; 100 – calculation on 100 mL of serum; 0.1 – volume of enzymatic solution;

T – time of incubation, min; 174.2 – molecular mass of arginin; K – coefficient of conversion of extinction amount to micromol of arginin.

For conversation of extinction to arginin's mcmol we plotted a calibrated curve against standard solution of arginin. The study of proteinase inhibitors of allantoic fluid was carried by casein technique elaborated by A. P. Levitsky [10].

Method of determination of inhibitor activity

Determination of proteinases inhibitors in lungs homogenates, blood serum, and allantoic liquid was done by caseine's method offered by A. P. Levitsky. To put 0.2 mL of supernatant into new glass tubes. To add 2 mL of reagent A and 2 mL of Follin's reagent. Contact – 30 min at room temperature. Analyze at spectrophotometer.

Calculate of inhibiting activity (IA)

The formula for calculation the inhibiting activities in serum and tissues were listed below:

Serum:

$IA=(\Delta Etr-\Delta E0n)\cdot 0.2\cdot n/\Delta E$(g/l; mg/mL)

Tissue:

$IA=(\Delta Etr-\Delta E0n)\cdot 200\cdot 21\cdot m/(\Delta Etr\cdot 1000)$

In the formula, the symbols indicate: ΔEtr - extinction of the sample with trypsin; n - dilution of the solution with serum; 0.2 – trypsin's concentartion, mg/mL; m – dilution of inhibitor's solution; 200 – the amount of trypsin in 1 mL (200 mkg); 21 – ratio of tissue's of tissue's charge to extragent, weight 100 mg. per 2 mL; 1000 – recalculation coefficient against 1 g of tissue; IA-content of inhibitor per 1 kg; 1 - a unit corresponding to 1 gr of crystalline trypsin; E0n - extinction of the sample with the mixture trypsin + inhibitor.

Infectious virus titre of allantoic fluid was assessed by infecting 9-10-day-old chicken embryos and expressed as lg EID50/mL. Hemagglutination assay was performed by the conventional procedure, and the Lowry protein assay was used for determining the total level of protein in a solution [11].

Method of definition of infectious activity.

In the beginning made dilution of influenza virus A from 10^1 to 10^8. 3 chicken embryos used for each dilution. In each embryos have led on 0.2 mL of influenza virus. Infested embryos put on contact in a thermostat at 37℃ at 48 hours. Then embryos have been supplied in a refrigeration cabinet at 4℃ at 18 hours. Embryos have been dissected and virus-containing allantoic fluid (VCAF) is exhausted in vials. Infectious titer of influenza virus A defined in hemagglutination assay. On what dilution (10^6) it concluded determination in # (4+,4 crosses)(or we can accept in one embryos three crosses) in 3 embryos and there was an infectious titer of influenza virus.

Definition of hemagglutination activity of influenza virus.

At the heart of a hemagglutination reaction lays the phenomenon of coagglutination of erythrocytes at adsorption on them of viruses. Initial allantoic virus was diluted from 1:2 to 1:128. For the control took chicken erythrocytes, washed them 3 times an isosmotic solution and centrifuged during 15 minutes at 3000 rpm on whizzer OPN-8, dilute to 1% erythrocytes - ready to experiments. In special plastic plates with the small cavities have added on 0.2 mL of an isotonic solution, then on 0.1 mL of influenza virus, on 0.1 mL of erythrocytes of 1% and again on 0.1 mL of an isotonic solution, have admixed and have left for 30 minutes on contact. Positive considered result of reaction at which erythrocytes evenly covered all bottom of the small cavity. At negative reaction erythrocytes in the form of a small disk or "button" settled down in the small cavity centre. The link http://www.virology.ws/2009/05/27/influenza-hemagglutination-inhibition-assay is similar. Last column we use for control of erythrocytes.

Results

The balance of proteinase and inhibitor activity in chicken embryos without infection

Proteinase and inhibitory activity in infected chicken embryos was observed to be highly balanced (Fig. 1). Its rippling occurred not only during the embryonic development (in this case - 11-16 day), but during the day (inhibitory activity in particular) as well.

Alterations in proteinase and inhibitory activity in chicken embryos with virus infection

When chicken embryos were infected with influenza virus A/PR/8/34 in heavy doses (20 LD50), proteinase activity became increasing in 15 min and reached its maximum in 30 min (Fig. 2). In an hour after inoculation of the virus it went back to its initial level, and since that time it began swift falling. In 24 h proteinase post-infection activity could not be determined. It was not detected in the samples in 48 hours after infection, but passing through 48 h term this figure began to rise again, up to the 72nd hour.

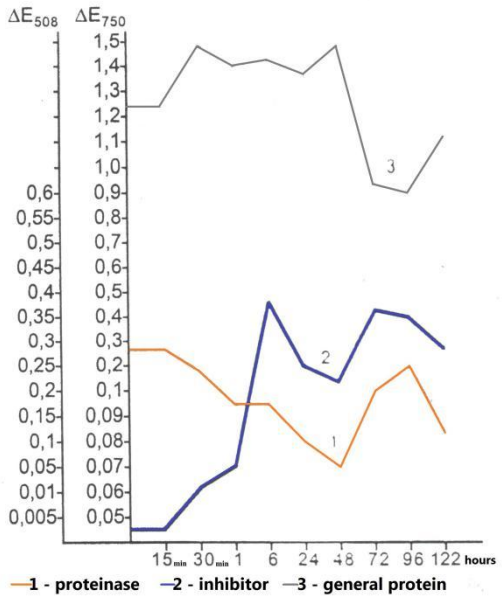

Fig. 1 Proteinase and inhibitory activity in uninfected

chicken embryos (control). ΔE508 - a proteinase axis. ΔE750 - a protein and inhibitor axis. Time axis - minutes, hours.

Discussion

Inhibitory activity varied more significantly. Its decline occurred in 48 hours after the infection. It was not detected at the 72nd and 96th hours after the inoculation of the virus, and only in 122 hours it renewed and reached the initial values. At high doses of infection loading we observed ripplings of protein. The growth of inhibitory and proteinase activity during the first 30 minutes after the infection increased the total amount of protein. The fading of proteinase and inhibitory activity in 48 h after the infection resulted in lowered total protein. Further the growth of proteinase

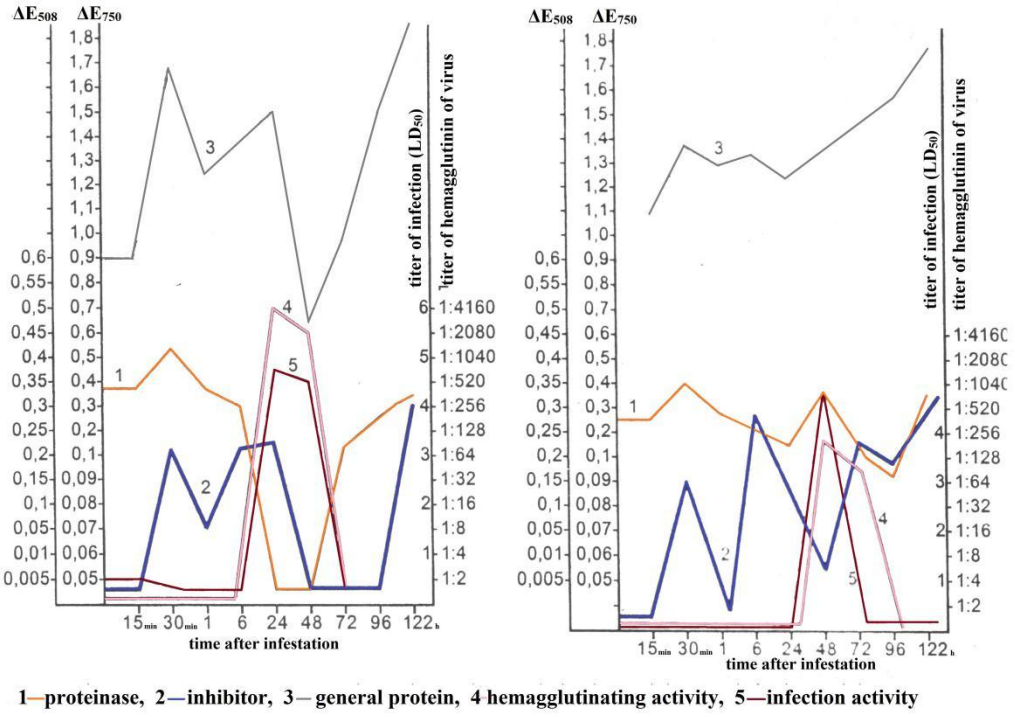

Fig. 2. Changes in proteinase and inhibitory activity in chicken embryos infected with influenza A virus taken in heavy (1) and low (2) doses. ΔE508 - a proteinase axis. ΔE750 - a protein and inhibitor axis. Time axis - minutes, hours.

activity occurred in parallel with the growth of total protein, i.e. total protein amount increased due to the amount of proteinase and inhibitors.

When chicken embryos were infected with low doses of influenza A virus (dissolved in 10-6 that corresponds to 1 LD50) we observed growing of proteinase and inhibitory activity already in 30 min after the infection (Fig. 2).

The second wave of the growth of proteinase activity was registered during the maximum accumulation of infectious influenza A virus (in 48 h after infection). During this period, hemagglutination activity was observed to accumulate reaching its maximum. Inhibitory activity in low dose infection decreased by 4 digits compared with the control.

When infecting a large dose of influenza A virus in 24 hours after the infection we observed sharp suppression of proteinase activity, and then in 48 hours the suppression of inhibitory activity due to maximal increase of the influenza virus concentration and its hemagglutinin. At the same time, inoculation with low dose influenza A virus did not inhibit proteinase activity, and inhibitory activity was nearly unchanged. Maximum titre accumulation of infectious and hemagglutinin activity was observed a day earlier under inoculation with heavy doses of influenza A virus (24 hours) compared with the low doses (48 hours).

Thus, proteinase and inhibitory activity in uninfected chicken embryos was always observed to be well balanced. The virus inoculation dramatically upset the balance between proteinase and proteinase inhibitors, especially in the low dose inoculation of the virus. Proteinase and inhibitory activity in case of virus inoculation in any concentration increased within the first 30 min after the inoculation. When the virus concentration in a dose introduced was initially high, further it dramatically lowered in 24 hours. From the 24^{th} to the 48^{th} hour after the inoculation, it began to grow. Inhibition of proteinase and inhibitory activity can occur due to maximum accumulation of the virus and hemagglutinin activity. Inhibitory activity became suppressed a day later compared with the suppression of proteinase activity (in 48 h after the virus inoculation).

Studying the changes in proteinase and inhibitory activity in chicken embryos infected with heavy and small doses of influenza virus A/PR/8/34 we have found out the changes similar to those observed in white mice [12] In the first 30 min after the inoculation we saw the growth of proteinase and inhibitory activity. Under the influence of doses with high virus concentration beginning with the 6^{th} h after the inoculation the proteinase and inhibitory activity was getting suppressed and reached its maximum in 24 h.

During this period neither proteinase nor inhibitory activity was observed. M. S. Ewasy and. P. Sabina have proven that in the process of replication of different influenza virus strains there is observed the marked suppression of proteinase activity in allantoic fluid, when the yield of the infectious virus reaches its maximum. In 72 h after the inoculation with virus, proteinase activity started growing and reached the initial values in 122^{nd} h. Complete suppression of inhibitory activity delayed until the 48^{th} h and lasted till the 96^{th} h, and at the 122^{nd} h reached the initial values.

During the period of inhibitor blockage (48-96 h) proteinase activity was growing rapidly. This points out the presence of

different inhibitors. The first peak of inhibitor performs the body defense function to prevent the infection entering. The second peak of inhibitor was slightly suppressed slightly, although during this period the concentration of infectious virus reached its maximum level. Under the low dose of virus inoculation the second peak was unchanged. The third peak was sharply suppressed with large dose and little changed under the influence of small doses. As the key role of the third peak of inhibitor is to preserve the infectivity of the virus by protecting it from proteolytic degradation, the interference with synthesis of inhibitor may be of great therapeutic value.

Heterogeneity of serine proteinase inhibitors that are induced by influenza viruses, is confirmed with other authors. Thus, an allantoic fluid inhibitor differed from trypsin inhibitor examined in the 17-day amniotic fluid and ovomucoid in egg-white of chicken embryos [13] Proteinase inhibitors of allantoic fluid demonstrated properties similar to the properties of subtylisin inhibitors described for ovoinhibitors and ovomacroglobulin of chicken embryos [14] for the filtrate of Streptomyces sybtilisin inhibitor culture [15,16]. Proteinase inhibitors in the cycle of reproduction of adenoviruses, vesicular stomatitis virus and herpes simplex virus are virus specific inhibitors that block critical stages in the metabolism of infected cells [17-19]. Our studies suggest that virus-induced inhibitors discovered during the first hours after the infection block the activity of proteinases of the host cell, providing the protection of influenza A virus proteins from proteolytic hydrolysis. When virus-induced inhibitors become exhausted, proteinase begins to break down hemagglutinin and infectious virus titer grows, therefore in 6 h after the infection it is recommended to introduce inhibitor to block proteinase activity.

Conclusions

The level of trypsin-like proteinase and inhibitor in 11-day-old chicken embryos was quite well ballanced. In infected chicken embryos during the first hours we observed an increase of both proteinase and inhibitory activity. Large and small infecting doses of influenza A virus cause different changes in proteinase and inhibitory activity. Heavily concentrated doses during the maximum accumulation of infectious and hemagglutinin activity completely suppress proteinase activity and cause insufficient changes in inhibitory activity. Maximum accumulation of infecting influenza A virus does not depend on the dose, while at the low dose the time of viral accumulation increases. In chicken embryos, there is correspondence between the accumulation of infectious influenza A virus and subsequent decrease in proteinase activity. We used these researches for reception of an antiviral drug from a flu virus - an inhibitor of trypsin-like proteinases and working out of antiproteinase vaccines.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was funding by Ministry of Healthcare of Ukraine according to the next programs: Governmental registration No. 0198U007840, No. 0101U007760, No.0108U008481.

References

1. Murakami M., Towatari T., Ohuchi M. et al. Mini-plasmin found in the epithelial cells of bronchioles triggers infection by broad-spectrum influenza A viruses. Purification and characterization. Eur. J. biochem, 2001: 268(10): 2847-2855.

2. Bottcher E., M. Matrosovich T., Beyerle M. et. al. Ptoteolytic activation of influenza virus by serine proteases TMPRSS2 and HAT from human airway epithelium. J. Virol, 2006: 80 (19): 9896-9898.

3. Divocha V.O. Cells components associated with flu virus. Odessa Med. J., 1998: 2 (46):8-10.

4. Zhirnov O. P, Pojarkov N.A., Malyshev N.A. Anti-virus and antiphlogistic targets: prospects of new use. Pulmonology, 2009: 3:109-118.

5. Veremeyenko BN Enzymes in Otolaryngology. Health Protection, 1980:147-149.

6. Zhirnov O. P, Ovcharenko A.B, Bukrinskaja A.G., Zhdanov V.M. Ingibitor of proteases blocks lessimination of flu virus in an organism of infested animals. Report AH the USSR, 1983:270:1483-1485.

7. Divocha V. A, Mihalchuk V. N, Gozhenko A.I. Biochemistry-viral substantiation of antiproteinase therapy of a flu. АПТМ: 2 (12: 118-123).

8. Divocha V. A. Prospect of creation of antiviral agents on the basis of inhibitors of proteases. Theses of reports: 2003: 2 (12: 29).

9. Vovchyk C Method for determining proteolytic enzymes for hydrolysis of protamine and histone. Biochemical research methods of breeding material. Odessa, 1979:59-66.

10. evitsky AP, Vovchyk S, Pylneva PN et al. The use of trypsin inhibitor and chymotrypsin indicator in the selection of maize and barley. Metod. Recommendations. Odessa, 1984: 214 p.

11. Lowry OH Rosebrough NS, Farr AL et al Protein measurement with the Folin phenol reagent. J. Biol. Chem.1951; 193: 265-275.

12. Divocha VA, Gozhenko AI, Mykhalchyk VN Biological basis antiproteinases treatment of influenza. ATV: Lugansk, 2011. – 315 p.

13. Evasihin ME, Sebino LA Inhibition subtilisin allantoic fluid, infection with influenza virus. Acta virology.1986; 30:187-191.

14. Kilamonto T., Nakashima H., Jkai A. Han egg white ovomacroglobulin has protease inhibitory. J. Biohemistry.1982; 92:1679-1682.

15. Takemae N, Ruttanapumma R, Parchariyanon S at al. Alterations in receptor-binding properties of swine influenza viruses of the H1 subtype after isolation in embryonated chicken eggs. J Gen Virol. 2010; 91:938-948.

16. Stevens J, Chen LM, Carney PJ et al. Receptor specificity of influenza A H3N2 viruses isolated in mammalian cells and embryonated chicken eggs. J Virol. 2010; 84(16):8287-8299.

17. Solovey NV, Protasevich TS Topical therapy of acute upper respiratory tract infections: old problem, new

opportunities. Intern. reviews: clinical. Practice and Health. 2016; 1:35-43.

18. Korovin AN, Kuhanova MK Kochetkov SN Search herpes virus replication inhibitors: 30 years after acyclovir. Biotechnologia acta. 2013; 6(4):78-85.

19. Hutornenko AA, Dalin AA, Chernyak BV Dehydrotate dehydrogenase role in the induction of apoptosis by inhibition of complex III mitochondrial respiratory chain. Acta naturae. 2014; 1(20):74-81.