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Effect of soluble porcine aminopeptidase N on antibody production against porcine epidemic diarrhea virus

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A few members of coronavirus group I which includes porcine epidemic diarrhea virus (PEDV) use porcine aminopeptidase N (pAPN) as a cellular receptor. Cellular receptors play an important role in virus attachment and entry. However, the low permissiveness of PEDV to APN-expressing porcine cell lines has made it difficult to elucidate the role of pAPN *in vitro*. The purpose of this study was to prove whether the treatment of soluble pAPN could enhance the antibody production against PEDV in guinea pigs, rabbits and sows. The animals (20 guinea pigs, 8 rabbits and 20 sows) were divided into 4 groups. Group A was injected intramuscularly (IM) with soluble pAPN at one hour before intramuscular infection of PEDV on the same site, group B for IM simultaneous injection of pAPN and PEDV, and group C for IM injection of PEDV only. Group D served as a control of pAPN treatment or PEDV infection. Antibody production against PEDV was compared among groups at regular intervals. The results suggested that pAPN could enhance the antibody production against PEDV in guinea pigs and rabbits which are free of pAPN, however, the effect of pAPN treatment in sows was not clearly elucidated.

Key words: Porcine epidemic diarrhea virus, porcine aminopeptidase N, immune responses

Introduction

Aminopeptidase N (APN) acts as a cellular receptor of coronavirus group I, such as transmissible gastroenteritis virus (TGEV) [1,7,25], feline infectious peritonitis virus (FIPV) [22] and human respiratory coronavirus (HCoV)-229E [27]. Porcine epidemic diarrhea virus (PEDV) is a member of the coronavirus group [23,24], and causes watery diarrhea, dehydration and high mortality in suckling pigs [2,17]. Similar to transmissible gastroenteritis virus (TGEV), PEDV replicates in the enterocytes present in the villi of small intestine [5,6] and is cultured in Vero cells [10,12,13], porcine bladder and kidney cells [20], and swine cell line KSEK6 and IB-RS-2 cells [11]. In some viruses like human immunodeficiency virus (HIV), soluble cellular receptors can enhance the infectivity of the virus [19]. Recently, soluble porcine APN (pAPN) could enhance PEDV infectivity in Vero cells [16]. However, it has not been reported whether the treatment of soluble pAPN is helpful for enhancing the antibody production against PEDV *in vivo*. This work was carried out to determine whether the antibody production against PEDV would be enhanced by soluble pAPN treatment in guinea pigs and rabbits as nonpermissive hosts and sows for a permissive host.

Materials and Methods

Cell and virus

Continuous Vero cell line (ATCC, CCL-81) was regularly maintained in α-minimum essential medium (α-MEM) supplemented with 5% fetal bovine serum and antibiotics. The cell passaged PEDV, KPEDV-9 strain [14] was kindly provided by the Green Cross Veterinary Product Co. Ltd. (Suwon, Korea). The virus was propagated in Vero cells with virus replication medium of α-MEM supplemented 0.02% yeast extract, 0.3% tryptose phosphate broth and 2 μg of trypsin (T-VM).

Animals

Guinea pigs: Four groups of 5 guinea pigs (200 g body

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Table 1. Experimental design for soluble porcine aminopeptidase N treatments

Group	pAPN treatment[1]	Guinea pigs		Rabbits[2]		Sows[3]	
		No. of animal	Dose of pAPN	No. of animal	Dose of pAPN	No. of animal	Dose of pAPN
A	1 hour before PEDV[4] infection	5	10 μg	2	10 μg	5	10 μg
B	Simultaneous PEDV	5	2.4 pg	2	24 pg	5	24 ng
C	PEDV only	5	-	2	-	5	-
D	Control	5	-	2	-	5	-

[1]The animals were treated with pAPN intramuscularly.
[2]The rabbits were boosted with the same protocol of the first injection at 3 weeks.
[3]The sows were treated with the same protocol at 5 and 2 weeks before farrowing.
[4]The volume of PEDV was 1 ml (titer, $10^{4.5}$ $TCID_{50}$/ml).

weight) were allocated as shown in Table 1. In group A, 1 ml of the soluble pAPN (Sigma, USA) at the concentration of 10 μg/ml was injected IM. After 1 hour of pAPN injection, 1 ml of PEDV ($10^{4.5}$ $TCID_{50}$/ml) was infected at the same inoculation site. Group B was injected simultaneously with 1 ml of PEDV ($10^{4.5}$ $TCID_{50}$/ml) containing soluble pAPN at the concentration of 2.4 pg/ml. Group C was infected with 1 ml of PEDV ($10^{4.5}$ $TCID_{50}$/ml) only without pAPN and group D was a control of T-VM. All guinea pigs were raised for 6 weeks after inoculation. Blood samples were collected weekly from each guinea pig for serum neutralization (SN) test.

Rabbits: Four groups of 2 rabbits (1.5 Kg body weight) were allocated as indicated in Table 1. Group A of rabbits was treated as the same protocol described in the group A of guinea pigs. Group B was injected simultaneously with 1 ml of PEDV ($10^{4.5}$ $TCID_{50}$/ml) containing soluble pAPN at the concentration of 24 pg/ml. Group C was injected with 1 ml of PEDV ($10^{4.5}$ $TCID_{50}$/ml) only without pAPN treatment, and group D was a control of T-VM. Booster injection was performed by the same protocol of described above at 3 weeks later. All rabbits were raised for 6 weeks after inoculation. Sera were taken every week for serological analysis.

Sows: Twenty commercial pregnant sows (Landrace × Yorkshire), regardless of parity number, were employed to investigate whether the antibody production against PEDV would be enhanced by soluble pAPN from a commercial swine farm. All sows were housed separately in a stall and fed with a commercial feed. Four groups of five sows each were allocated as shown in Table 1. Commonly, inoculation route was IM and the titer of PEDV was $10^{4.5}$ $TCID_{50}$/ml. Group A of sows was treated as the same protocol described in the group A of guinea pigs. Group B was injected simultaneously with 1 ml of PEDV containing soluble pAPN at the concentration of 24 ng/ml. Group C was injected with 1 ml of PEDV only without pAPN treatment, and group D was a control of T-VM. Two inoculations were carried out by the same protocol described previously at 5 weeks and 2 weeks before farrowing. Blood samples were collected from each sow before each injection and at farrowing. Colostrum was also collected from each sow at farrowing. Antibody titers against PEDV in sera and colostrum were examined by SN test, and indirect ELISA (isotype IgG or IgA).

Serum neutralization test

The SN test was carried out by the microtiter method using Vero cells as described previously [20]. Colostrum was centrifuged at 12,000 × g for 30 minutes and clear supernatant was collected for SN test. Sera and colostrum were heated at 56°C for 30 minutes before use. Serum or colostrum diluted serially in two-fold were mixed with an equal volume of PEDV (200 $TCID_{50}$). The mixture was incubated for 1 hour at 37°C and 0.1 ml of virus-serum mixture was inoculated into each well of Vero cell monolayers. Following adsorption for 1 hour at 37°C, the inocula were removed and the monolayers were washed three times with T-VM. Then, 0.1 ml of T-VM was added to each well and the cultures were incubated for 5 days at 37°C. The antibody titer was expressed as the reciprocal of the highest serum dilution that inhibited cytopathic effect (CPE).

Indirect ELISA

The indirect ELISA was carried out in 96-well microtiter plate (Nalge Nunc International, USA). For antigen coating, the PEDV ELISA and mock-infected cell antigens were prepared as described previously [3]. The antigens were diluted in coating buffer (50 mM carbonate buffer, pH 9.6). Alternate 8-well rows of the plate was coated with 100 μl of diluted PEDV antigen per well (0.1 μg/well) in coating buffer and incubated overnight at 4°C. The antigen was then poured off and washed 5 times with phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (Sigma, USA). Subsequently, remaining free binding sites on the surface were blocked with 200 μl of 5% rabbit serum (Gemini Bioproducts, USA) in PBS for 1 hour at 37°C. Two

positive and negative reference each and sample swine sera were diluted 1 : 50 in PBST. Each serum was transfered to 2 wells (100 µl/well) each and the plates incubated for 1 hour at 37°C. Then, HRP-conjugated goat anti-pig IgG antibody (KPL, USA), which was diluted 1 : 2,000 in PBST, was added to each well (100 µl/well). For the assay of IgA antibody in sera and colostrum, HRP-conjugated goat anti-pig IgA antibody (1 : 250, KPL, USA) was used. After incubating further for 1 hour at 37°C, plates were washed 5 times with PBST, and 100 µl of ABTS substrate (KPL, USA) were added to each well. After incubation for 20 minutes at room temperature, the reactions were stopped by adding 0.5 M H_2SO_4 and optical density was measured at 405 nm. The corrected OD of each serum and colostrum was calculated as follows.

Corrected OD = OD of a test serum on the PEDV antigen - OD of a test serum on the mock-infected cell antigen.

Statistical analysis

To compare the difference between the control group and the other groups during the entire period of experiment, ANOVA test was used using Microcal Origin 6.0 (Microcal Software, USA).

Results

Comparison of neutralizing antibody production against PEDV in animals

Immune responses in guinea pigs: The SN antibody production of guinea pigs was depicted in Fig. 1. All of the guinea pigs in the groups A, B, and C developed SN antibodies against PEDV from 1 week after PEDV inoculation. However, the T-VM inoculated guinea pigs as a control (group D) remained seronegative during the experiment. In guinea pigs pre-treated with pAPN at the concentration of 10 µg per guinea pig (group A), SN titers were constant and significantly higher than group C until 6 weeks after PEDV inoculation ($p < 0.01$). The guinea pigs inoculated simultaneously with PEDV and pAPN responded dramatically to the PEDV infection as shown in Fig. 1. The maximum SN titers in guinea pigs of group B were $2^{5.2}$ at 1 and 2 weeks after PEDV infection and then slowly decreased. However, PEDV antibody titers of group B were significantly higher than those of group C until 5 weeks after PEDV inoculation ($p < 0.01$). Significant difference in SN titers between group A and B showed only at 1 and 2 weeks after PEDV inoculation ($p < 0.01$). Group C which was inoculated with PEDV only showed the highest titer at 2 weeks ($2^{2.8}$) and slowly declined. Therefore, the titers were as low as $2^{2.4}$ folds when compared to the maximum titer of group B guinea pigs.

Immune responses in rabbits: The SN antibody production of rabbits was depicted in Fig. 2. All of the rabbits in the groups A, B, and C developed SN antibodies against PEDV from 1 week after PEDV inoculation. However, the T-VM inoculated rabbits as a control (group D) remained seronegative during the experiment. In rabbits pre-treated with pAPN at the concentration of 10 µg per rabbit (group A), SN titers were significantly higher than group C after boosting at 3 weeks after PEDV infection ($p < 0.01$). The rabbits infected simultaneously with PEDV and pAPN responded dramatically to the PEDV infection as shown in Fig. 2. The maximum SN titers in rabbits of group B were $2^{8.0}$ at 5 weeks after PEDV infection and then slowly decreased. However, PEDV antibody titers of group B were significantly higher than those of group C after boosting at 3 weeks after PEDV infection ($p < 0.01$). Significant difference in SN titers between group A and B showed from 4 weeks after PEDV infection ($p < 0.01$). Group C which was infected with PEDV only showed the highest titer at 4 weeks ($2^{5.0}$) and slowly declined. Therefore, the titers were as low as $2^{3.0}$ folds when compared to the maximum titer of group B rabbits.

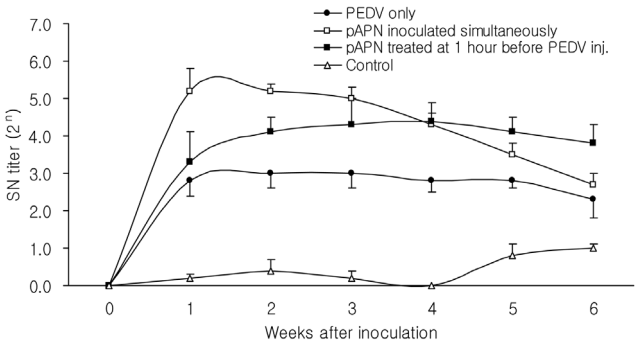

Fig. 1. Neutralizing antibody titers against PEDV in guinea pigs. Titers were described in mean ± S.E.

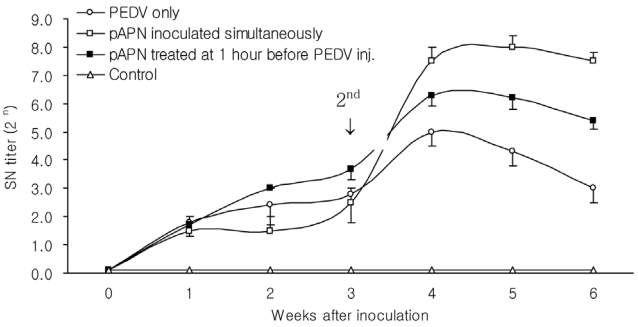

Fig. 2. Neutralizing antibody titers against PEDV in rabbits. The rabbits were boosted at 3 weeks. Titers were described in mean ± S.E.

Immune responses in sows: The SN antibody production of sows was depicted in Fig. 3. All of the sows in the groups A, B, and C developed SN antibodies against PEDV, even though the significant differences of SN antibody titers

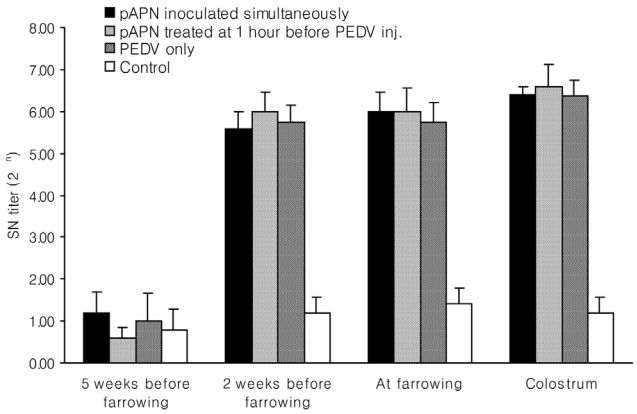

Fig. 3. Neutralizing antibody titers against PEDV in sows. The sows were treated at 5 and 2 weeks before farrowing. Titers were described in mean ± S.E.

among the groups were not observed ($p < 0.01$). However, the T-VM inoculated sows as a control (group D) remained seronegative during the experiment. In the SN test, the average antibody titers were $2^{6.00}$ in group A, $2^{5.60}$ in group B, and $2^{5.75}$ in group C before boosting at 2 weeks before farrowing, respectively. At farrowing, the antibody titers of colostrum were increased to $2^{6.60}$ in group A, $2^{6.40}$ in group B, and $2^{6.38}$ in group C, respectively, suggesting no significant difference among the groups.

Comparison of Ig isotype titers against PEDV in sows: In the indirect ELISA for detection of IgG isotype against PEDV, corrected ODs of sow sera from groups A, B, and C were 0.365, 0.372 and 0.313 at farrowing, respectively. However, IgG titers both sera and colostrum did not show the significant differences among the groups (Fig. 4A). In detecting IgA isotype against PEDV, corrected Ods of sow sera from groups A, B and C were 0.293, 0.283 and 0.261 at farrowing, respectively. However, IgA titers both sera and colostrum did not show the significant differences among the groups in this experiment (Fig. 4B).

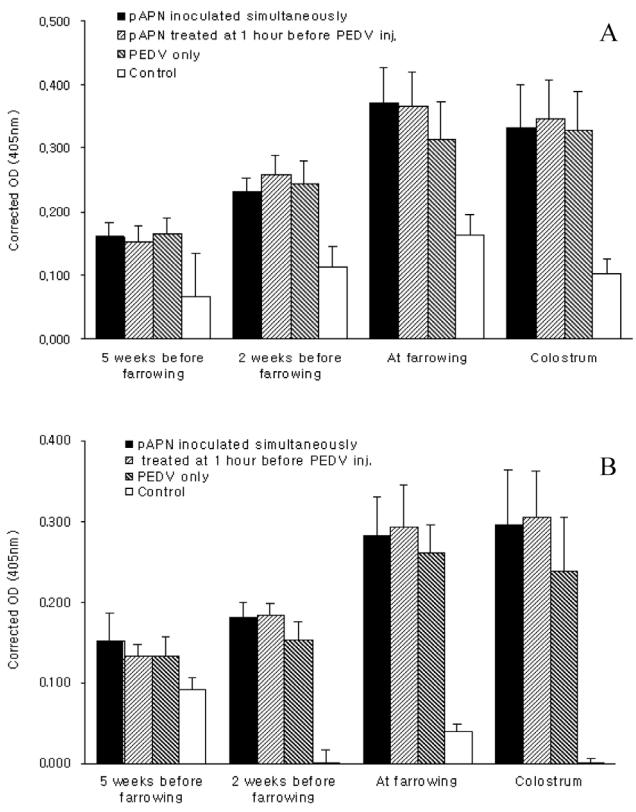

Fig. 4. Antibody titers against PEDV in sows by indirect ELISA. The sows were treated at 5 and 2 weeks before farrowing. Absorbance were described in mean ± S.E. A; IgG, B; IgA.

Discussion

As the first step in viral infection, viruses attach to specific receptors on the surface of cells. This specific interaction with cells determines, to a large extent, the host-range specificities and tissue tropism of viruses [4,9,21]. Characterization of the host-virus interaction ultimately requires isolation of the cellular receptor and the virus attachment protein. While several virus attachment proteins have been identified [8,15,18], little is known about the nature and properties of cellular receptors against PEDV.

The present study demonstrated that the treatment of soluble pAPN which is a cellular receptor of coronavirus group I could enhance the antibody production against PEDV in guinea pigs and rabbits. Single inoculation of PEDV with pAPN increased significantly SN titers in guinea pigs up to four weeks after inoculation. However, the SN titers of rabbits had no difference between three groups after the first inoculation, although the difference was observed after the second inoculation. These results support that the pre-treated or simultaneously treated pAPN may play a role in PEDV attachment and entry in the muscle cells of experimental animals. Thus, the increased entry of PEDV into the muscle cells might induce higher antibody production against PEDV when compared to PEDV inoculation only without pAPN treatment. However, these results could be explained that pAPN treatment may play a role of simple potent adjuvant leading to macromolecules which easily bound to antigen presenting cells in experimental animals. In addition, as molecular mechanisms of dendritic cell-induced T cell activation, pAPN appeared to be involved in activation of naïve T cells (CD13) [26].

Sows did not make difference even though pAPN was treated before or simultaneous inoculation of PEDV. This phenomenon was observed regardless of the methods of serological test; SN test and indirect ELISA. However, the ELISA practically could measure all of the possible subclasses of immunoglobulin against PEDV. It is thought that no difference in sows would be due to the improper

concentration of pAPN treatment when compared to body weights of animals used. On the other hand, it could be due to a reason why sows hold enough distribution of pAPN in the enterocytes of small intestine villi [5,6]. Therefore, the antibody production against PEDV in pigs may be enhanced with the aid of higher concentration of pAPN than that in experimental animals.

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