CHAPTER II

REVIEWS OF THE LITERATURES

Classical Swine Fever

A. Clinical Appearance and Pathogenesis

Classical swine fever (CSF), previously known as hog cholera (HC) is a highly contagious viral disease of swine. It was first noticed in Ohio, USA. in 1833. Because its high degree of contagiousness, worldwide distribution, and importance for the international trade of animals and animal products, CSF is considered to be the most important pig disease and is included in List A of notification disease of the Office International des Epizooties (OIE) (OIE, 1996). At present, CSF has a wide distribution throughout numerous geographic regions of the world. Most countries are considered free of CSF and the disease has never been reported on the mainland North America. CSF is considered endemic throughout much of Central, Eastern, South-east, and sub-continental Asia (Edwards et al., 2000). In Thailand, the first outbreak was found in Bangkok, 1950 (Kongsmak, 1980) and it has been announced as a noticeable disease on the list of the Animal Epidemics Act, B.E. 2499, Department of Livestock Development since 1956 with highly concerned as a cause of an economic loss in pig industry in Thailand. CSF is caused by the CSF virus (CSFV) of the family Flaviviridae, genus Pestivirus. The virus is enveloped and has a single-stranded, positive-sense RNA genome of about 12.5-16.5 kb which codes the production of 3 structural proteins, E1, E2, and E^{ms} (Thiel et al., 1991). The glycoprotein E^{ms} and E2 are the major envelop proteins which induce serum neutralizing antibody against the virus (Konig et al., 1995; Van Gennip, 2001). There is only one serotype but it can be divided in 3 genogroups, 1, 2, and 3 based on their genomic characteristics. CSFV group1 is represented by the Brescia and Alfort/187 strains and includes old vaccine and laboratory strains isolated until the 1980s in Europe and the United States and newer isolates from Asia, South America, and Russia. Group 2 includes almost all newer viruses isolated after 1985 in

Western and Eastern Europe and some Asian isolates. Group 3 is seemed to have limited distribution in Asia (Frias-Lepoureau and Greiser-Wilke, 2002). CSFV infection is normally restricted to swine which is the only natural reservoir. Blood, tissues, secretions, and excretions from infected animals contain high amount of virus. Transmission occurs mainly through oronasal route, although infection can occur through conjunctiva, mucous membrane, skin abrasion, and inseminations. Feeding of raw or insufficiently cooked garbage containing infective pork material is a potential source of CSFV infection. The virus primarily replicates in crypt epithelial cells of tonsil then the virus are carried by monocytes or lymphocytes to regional lymph nodes and to blood circulation. After that, the virus enter spleen where they produce a lot of progeny virion and distribute throughout the body via blood circulation (viremia) then the virus reach many target cells such as white blood cells, bone marrow stem cells, reticulo-endothelial cells, epithelial cells, vascular endothelial cells, ovarian cells, pulmonary intravascular macrophages, interstitial and alveolar macrophages (Carrasco et al., 2001; Choi and Chae, 2003; Summerfield et al., 2000). The incubation period is ranges from 2-6 days. Infected pigs may shed virus before showing clinical signs continuously or intermittently excrete virus until death. The most common macroscopic findings of CSF is hemorrhage that appeared in many parts of the body including skin, subcutaneous, serosa and mucosa of visceral organs, lungs, epiglottis, brain, and kidneys that seem to be like Turkey egg. The organ system that usually shows impressive hemorrhage is lymphoid system. Lymph nodes are swollen and hemorrhage, especially at subcapsular areas look like strawberry. The hemorrhage is caused by hemorrhagic diathesis resulted from thrombocytopenia and the defect in fibrinogen synthesis, the endothelial cell damage undoubtedly plays an important role in the genesis of the hemorrhage. Infarction of the spleen is considered to be almost pathognomonic for acute CSF. Infarctions occur as dark blebs in various sizes, raised slightly above the surrounding surface. They may appear as a single lesion or as a series, coagulescing to form a continuous border of infarct along the edge of the spleen. The infarct may also be found in tonsil, stomach, and kidney. In the case of chronic infection, button ulcer is usually found in large intestine while the hemorrhage is not prominent or absent. Clinical

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courses of the disease mainly depend on the virus and host factors as the age of the animals. The virulence of the virus and the time of infection are of greatest importance. The disease may show many types of signs; peracute (sudden death), acute, chronic, and clinically inapperance (Van Oirschot, 1980). In acute infection, the most common form of infected pigs show severe depression, weakness, anorexia, high fever (>40^{o}C), conjunctivitis, mucopurulent ocular discharge, constipation followed by diarrhea. Some of them may show nervous signs, unsteady gait, ataxia or convulsion. Pigs may suddenly die within a few days with some signs however the pigs will die within 1-2 wks after infection. Chronic form is usually subclinical, due to infection with moderate or low virulent strains. The pigs show clinical signs resembling of acute form, intermittent pyrexia and diarrhea more than 30 days but the virus titer is low while the antibody is increasing. CSFV in circulation are destroyed and limited in some lymphoid tissues, ileum, kidney, or salivary glands. The occurrence of virus and antibody may result by the deposition of antigen-antibody complexes in the kidney, leads to glomerulonephritis. At the late stage of infection, the virus will multiply and spread throughout the body as viremic condition again. The pigs will suffered and die by bacterial or other microbial complications. CSFV can cross the placenta and infect pig fetuses leading to abnormalities of the fetuses such as mummification, edema, stillbirth, neonatal death, and congenital tremors. The outcomes of transplacental infection depend on gestation period and virulence of the virus (Van Oirschot and Terpstra, 1977). Clinical courses of the disease in Thailand have a wide variety because there are many genogroups of virus which had a different characteristic (Pachariyanon et al., 2000; Pinyochon et al., 1999). Recently, it was reported that CSF in Thailand has been changed from acute form to chronic form. Because of subclinical or chronic infection with non-specific signs and lesions, the infected pigs with virus shedding are usually moved from farm to farm. Thus purchasing of weaning piglets from different breeding farms to fattening units or transfer to markets are common modes of transmission of CSF in our country.

B. Effect of CSFV on lymphoid tissues and white blood cells

In CSFV infection, lymphoid tissues are pathologically affected as proliferation or depletion. Competition between the proliferation and the depletion of lymphoid tissues is relevant to decisive importance for the outcome of CSF infection. The severity of destroying lymphoid tissues depends on virulence, amount of the virus, and the age of pigs. The primary affected site in the lymphoid tissues where the viral antigen (Ag) was found after inoculation with high virulent or low virulent CSFV was germinal centers. The virus Ag still remained in that area, even though the lymphoid cells in the follicle had completely been destroyed (Narita et al., 2000). It is revealed that various strains of CSFV cause the various outcomes on the number of peripheral blood mononuclear cell (PBMC), especially subpopulations of T and B-lymphocytes. But the major population affected by CSFV in most studies was B-cells which had been decreased since the 2nd day post-infection (dpi) with a faster decreasing in a higher virulent strain (Gisler et al., 1999; Markowska-Daniel et al., 1999; Narita et al., 1996; Pauly et al., 1998; Shimizu et al., 1995; Soos et al., 2000; Summerfield et al., 2001(a)). Very interestingly, cells that do not affected by CSFV, monocyte, is a major target population of infection in circulation (Lee et al., 1999; Summerfield et al., 1998; Susa et al., 1992). The results indicate that the decreasing of white blood cells might not be due to a direct effect of infection but could be a result of suppression of hematopoiesis, change in the distribution of leukocytes within different compartments of the immune system, and apoptosis. The mechanisms of immune deficiency caused by apoptosis of lymphocytes has been described previously for a number of virus infections in pig such as porcine reproductive and respiratory syndrome virus (PRRSV) (Choi and Chae, 2002), influenza viruses (Hinshaw et al., 1994), transmissible gastroenteritis virus (TGE) (Sirinarumitr et al., 1998), porcine rubulavirus (Rodriguez-Ropon et al., 2003). CSFV also destroy infected cells and induce apoptosis that appear in both PBMC and bone marrow hematopoietic cells, which are detected by measure the decreasing of mitochondrial transmembrane potential of ($\Delta\Psi_m$), a particular early marker for apoptosis, and reduction of DNA content. The result of such studies indicated that the apoptosis rapidly occurred in 1-2 dpi before viremia (3dpi), and most of infected cells did not

apoptosis. Infected animals showed activation of Fas on T-lymphocytes which increase sensitivity to apoptosis by mechanism of Fas-mediated activation-induced cell death (Fas-ADCC). In addition, lymphocytes from the infected animal decrease responsiveness to mitogen stimulation (Gómez-Villamandos et al., 2001; Summerfield et al., 1998, 2000, and 2001a). Not only PBMC are destroyed by cytolysis and apoptosis but also lymphocytes in lymphoid tissues such as lymph nodes, thymus, and spleen are affected (Sato et al., 2000). How dose CSFV affect a wide variety of cells throughout the body? It was suggested that E^{ms}, an extra envelope glycoprotein (gp) in pestivirus, which has RNase activity and secrete into the extracellular environment. Consequently may affect the lymphoid tissues throughout the body and plays an important role in induction apoptosis of pestiviruses (Bruschke et al., 1997). This mechanism is also found in Human immunodeficiency virus (HIV) infection which release gp120 and induce apoptosis of lymphocytes (Everett and McFadden, 1999). Infected monocytes and macrophages, the most preferably target cells of CSFV, secrete a lot of prostaglandin E2 (PGE-2) and interleukin-1 (IL-1) which induce fever, affected blood coagulation and endothelia of the infected animal (Knoetig et al., 1999). Most infected monocytes and macrophages usually do not die, thus they will be a good place for virus replication. The decline apoptosis of CSFV infected monocytes and macrophages have never been studied but some mechanisms studied in other viruses suggested that very rapid replication of virus before apoptosis usually found in most RNA virus, or cryptic infection that dose not induce apoptosis of infected cells may be mechanisms that viruses survive in the cells (Koyama et al., 2000).

C. Diagnosis

Clinical signs and lesions seen at post-mortem in pigs infected with CSF are highly variable and the pathologic picture of acute and subacute CSF is that of a septisemic disease such as salmonellosis, therefore diagnosis of the disease should be confirmed by laboratory investigation. Laboratory methods are aimed at the detection of the virus or specific antigens, or detecting antibodies against the virus. As the virus specific antibody response takes 2-3 weeks to develop and blood samples should be

collected 3 weeks or later after the suspected contact. Virus neutralization tests (VN) is performed in cell cultures using a constant virus with varying-serum method. As CSFV is non cytopathic, any non-neutralised virus must be detected. The fluorescent antibody virus neutralization test and the neutralizing peroxidase-linked assay (NPLA) are commonly used techniques. The peroxidase system has the advantage that the results can be read by the naked eye and shows a good correlation to other tests (Jensen, 1981; Terpstra et al., 1984). Detection of the virus or viral antigen can be done by several methods such as immunological method, isolation of virus or molecular diagnosis. Fluorescent antibody test (FAT) is a rapid test that can be use in cryostat sections of tissue samples such as tonsil, spleen, kidney, lymph nodes, or distal part of ileum. In acute suspected cases, tonsillar tissue is the best while chronic cases, the ileum are frequently positive. Isolation of virus in cell culture is a sensitive but it is time-consuming. The isolation of CSFV is best performed in PK-15 cells and then examined by FAT or immunoperoxidase test (Terpstra, 2000). Molecular diagnosis using reverse transcription-polymerase chain reaction (RT-PCR) is rapid and more sensitive than other diagnostic methods. Using RT-PCR with restriction endonuclease enzymes can discriminate between field viruses and virus vaccine strains (Harding et al., 1994; Vilcek and Belak, 1998). To make a good diagnosis, especially in outbreak of the disease, combination of such methods should be done for example, the late outbreak in The Netherlands in 1998, the diagnosis was achieved through the combine of a direct immunofluorescene and peroxidase assay with tissue samples, isolation the virus from heparinized blood for suspected animals and using serological methods such as ELISA or VN for healthy pigs (De Smith et al., 1999).

D. Prevention and control

Control strategies of CSF in the EU countries, USA and several other countries are stamping out all infected herds and perform annual routine serological survey, which is extremely expensive. Vaccination in those countries is prohibited or under regulation. The ban of routine vaccination is introduced, because of the difficulty to distinguish between naturally infected animals and those immunized with

conventional vaccines. However, the prophylactic vaccination is still carried out in many parts of the world where the disease is endemic such as South-east Asia, including Thailand where the vaccination against CSF has been practically used. Effective vaccines against CSF are based on live virus that has been attenuated by passage through a suitable host species such as rabbit (Lapinized vaccine), subunit vaccines, or DNA vaccines that have been proved to have a good protection (Terzic et al., 2003; Yu et al., 2001). It is found that the vaccine induces a stable and long lasting immunity. In Thailand, CSF vaccine has been produced at Veterinary Biologics Division, Department of Livestock Development (DLD), Pakchong, Nakhornratchasima since 1975, using lapinized Chinese strain obtained from the Veterinary Institute of Hungary. Recently GPE$^-$ vaccine, new live CSFV tissue culture vaccine, has been developed and proved to have high potency against CSFV virulent strain. One vaccination at an appropriate time (7-9 wk of age), when the interference from maternal antibody has been down, is adequate for protect the animal from disease. The recommendation can be made in herd free of CSF but cannot be applied without risk in situations where CSF is endemic, so dual or more vaccination in very young pigs is the common strategy in our country. In addition, in the endemic area, especially the farm where CSF persists, pigs will have an irregular antibody profiles (Geerts et al., 1995; Morilla and Carvajal, 2002). As previous studies, vaccination of pigs with high maternal antibody titer ($\geq$ 32) can cause low active antibody response to vaccination (Parchariyanon et al., 1994; Suvintarakorn et al., 1993) and the pigs might be infected with virulent CSFV and show clinical illness (Suradhat and Damrongwatanapokin, 2003).

Apoptosis

A. Mechanism

Apoptosis is an important mechanism for the development and homeostasis of living animals, serves as a precise regulation of cell numbers, and defense mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes, viral infected cells, and tumor cells (Krammer et al., 1994).

Apoptosis is thought to be responsible for numerous physiologic and pathologic events that cells die in a controlled manner, in response to specific stimuli, apparently following an intrinsic program. It occurs in developmentally regulated cell death in the embryo including implantation and organogenesis, in deletion of auto-reactive T-cell clones during thymic maturation, cell deletion in proliferating cell populations such as intestinal crypt epithelia, and following removal of specific growth factors such as lymphocytes deprived of IL-2, endometrial cells breakdown during the menstrual cycle, ovarian follicular atresia in the menopause and regression of the lactating breast after weaning. Apoptosis is also found in cells attacked by cytotoxic T-lymphocytes (CTL) and natural killer cells (NK) and in lymphoid cells exposed to moderate doses of ionizing radiation (Strasser and Bouillet, 2003; Thompson, 1995; Williams and Brady, 2001). In the case of apoptosis, dead cells are rapidly removed, and any leakage of their noxious and possibly dangerous contents is avoided. In contrast to necrosis, a pathological form of cell death that results from overwhelming cellular injury, cell swell and lyse, thereby releasing cytoplasmic material which often triggers an inflammatory response to surrounding tissues (Figure 1). The morphologic features of apoptosis occur in three phases. In the first, the most characteristic change of apoptosis is condensation of nuclear chromatin in to crescentic caps beneath the nuclear wall, nucleolar disintegration, and reduction in nuclear size. Shrinkage of total cell volume, increase in cells density, compaction of cytoplasmic organelles, and dilatation of endoplasmic reticulum are observed. In the second phase, there is budding and separation of nucleus and cytoplasm into multiple, small, membrane-bound apoptotic bodies. In the third phase, there is progressive degeneration of residual nuclear and cytoplasmic structures.

DNA fragmentation is usually associated with ultrastructural changes in cellular morphology in phase 1. The large histone associated DNA fragments of 300 kb and 50 kb are first produced by endonucleolytic degradation of higher-order chromatin structural organization. These large DNA fragments are visible on electrophoresis gels. The activation of Ca^{+2} and Mg^{+2} dependent endonuclease activities further shortens the

fragments by cleaving the DNA at linker sites between nucleosomes. The ultimate DNA fragments are multimers of about 180 bp nucleosomal unit, oligonucleosome or mononucleosome. These multimers appear as the familiar "DNA ladder" seen on standard agarose electrophoresis gels while the DNA of normal cells had high molecular weight and failed to enter agarose gels (Adrends et al., 1990).

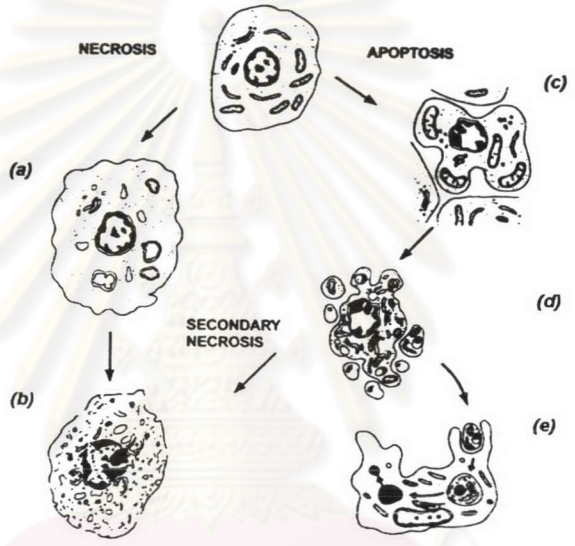

Figure 1 Diagram illustrating morphological change in necrosis and apoptosis. a) cell and micro-organelles swelling, b) plasma membrane rupture, c) cytoplasmic shrinkage and chromatin condensation, d) cytoplasmic blebbing and formation of apoptotic bodies, e) the apoptotic bodies are engulfed by phagocytes (Karolinska Institute of Environmental Medicine, 2005).

Gene involved apoptosis might encode ligands and their receptors, or a cascade of signaling molecules which might either stimulate or block apoptosis. Genes that regulate apoptosis in *Caenorhabditis elegans*, the classical model used for study of apoptosis, are 2 groups of genes (*ced*-3 and *ced*-4, *ced*-9) that balance each activity of promoting and inhibiting the apoptosis (Steller, 1995). The genes directly implicated in

lymphoid apoptosis are p53, c-myc, several members of the bcl-2 family, and the gene encoding APO-1/Fas (CD95). Activation of those genes by many regulatory stimuli such as TNF, IL-1, IL-6, IL-10 and Fas ligands (FasL) or increase expression of their receptors on cell membrane found in many virus infections, induce or sensitize the cell to apoptosis (Nagata and Golstein, 1995). The signals from Fas-FasL interaction induce apoptosis via caspases, a family of cysteine acid proteases which cleave and activate downstream effectors caspases (caspases 3, 6, 7) and induce fragmentation of nuclear chromatin (Figure 2).

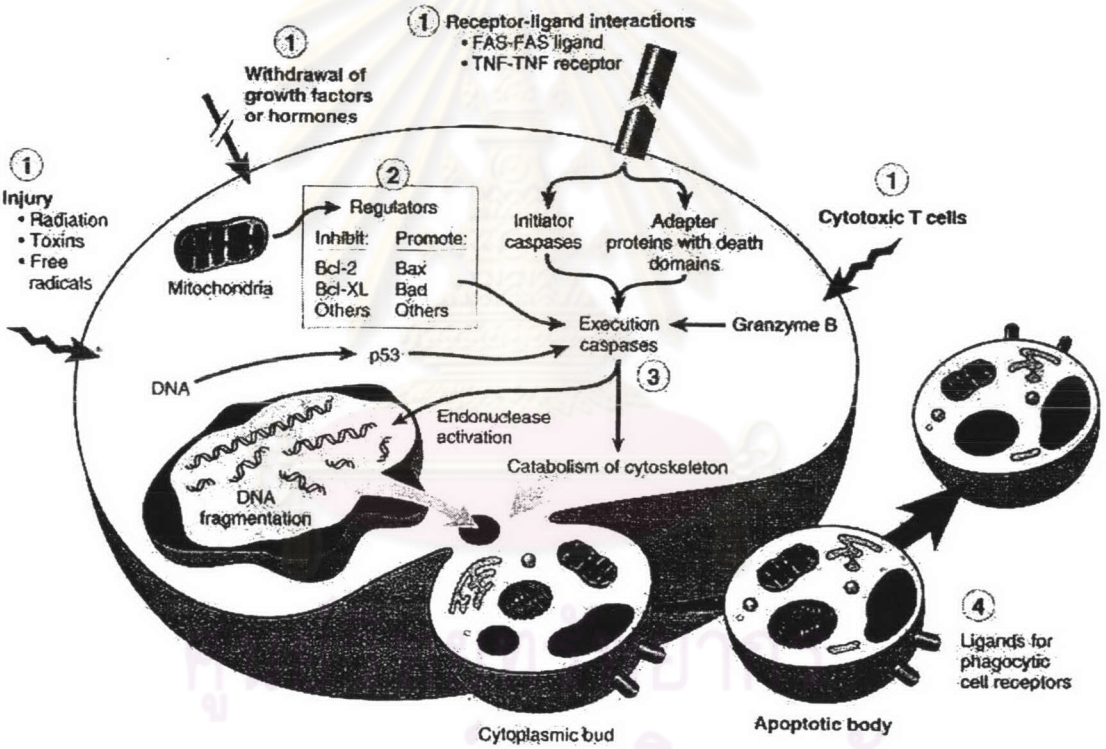

Figure 2 Regulation of apoptosis (Castleman, 2003).

B. Detection

There are several changes in the cells during apoptosis as described above thus many methods are developed to detect the apoptosis from initial stage to the end, for example, detect caspases, bax, or bcl-2 activity, detect mitochondrial

transmembrane potential which are the events at beginning of apoptosis or detect cleaved caspase3 (Allen and Willingham, 2002). DNA ladder, fragmentation of DNA at the late stage is detected by agarose gel electrophoresis. In general, it is difficult to distinguish *in situ* cells undergoing apoptosis by light microscopy so the method of TdT-mediated dUTP-biotin nick end labeling (TUNEL) has been developed by Gavrieli et al. (1992). The method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of fragmented DNA, ensuring a synthesis of a polydeoxynucleotide polymer. After the exposure of nuclear DNA on histological sections by proteolytic treatment, TdT was used to incorporate biotinylated deoxyuridine at sites of DNA breaks. The signal was amplified by avidin-biotin peroxidase, enabling conventional histochemical identification by light microscopy (Figure 3).

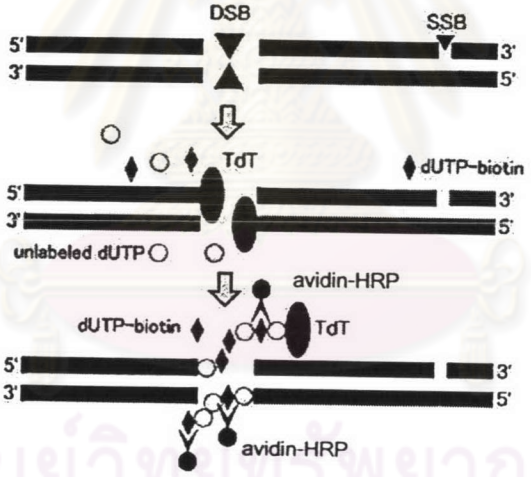

Figure 3 TUNEL diagram (modified from Tsutsumi, 2005., www.info.fujita-hu.ac.jp).

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