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Characterization of Swinepox Virus Secretory Polypeptides

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CHARACTERIZATION OF SWINEPOX VIRUS SECRETORY POLYPEPTIDES

by

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Swinepox virus (SPV) is the only known member of the genus *Suispoxvirus*. SPV causes a mild disease in swine characterized by slight fever, and weight loss, associated with lesions restricted to the skin. There have been few studies on SPV. Other poxviruses, such as vaccinia virus, have been well characterized and the biological activities of their secretory polypeptides have been analyzed. This study describes a major SPV secretory polypeptide with an apparent molecular mass of 35kDa. Poxvirus encoded polypeptides secreted during infection are known to exhibit immunomodulatory activities. The 35kDa SPV secretory polypeptide was tested for its ability to inhibit hIFN-γ, hTNF-α, and hIL-1β activities. Results from these experiments were inconclusive for SPV polypeptide immunomodulatory activity on these human cytokines. Unavailability of swine cytokines restricted additional studies. Since myxoma virus secretory polypeptide, which is a serine proteinase inhibitor, is shown to reduce restenosis caused after percutaneous transluminal coronary angioplasty, SPV polypeptide was tested for similar activities. SPV secretory polypeptide did not reduce restenosis and lack of evident immunomodulatory function could explain only mild porcine pathogenesis of SPV.

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INTRODUCTION

Swinepox virus is only known member of the *Suispoxvirus* genus belonging to the family *Poxviridae*. Swinepox virus is a swine pathogen that is endemic in many swine population. Infections are restricted to porcine species and are relatively mild and self-limiting (Kasza, Bohl & Jones, 1960). Also, like other poxviruses, SPV replicates within the cytoplasm of infected cells (Kasza, 1975). However, prior to our study very little was known regarding the SPV encoded secretory polypeptides.

Poxviruses have evolved multiple polypeptides which are designed specifically to inhibit the host immune and inflammatory responses (Gooding, 1992). Many of these poxvirus-encoded anti-immune polypeptides are secreted from poxvirus-infected cells, and mimic cellular receptors of cytokines or other soluble immune regulators (Moss, 1996). Cells infected with myxoma virus and Shope fibroma virus secrete virus-encoded 35kDa polypeptides, that are homologues to the ligand-binding domain of the cellular receptor for interferon-γ (Upton, Opgenorth, Traktman & McFadden, 1990). Vaccinia virus and Cowpox virus are reported to encode an IL-1β receptor homologue, which is secreted from infected cells. The IL-1β receptor homologue binds and inhibits IL-1β (Alcami & Smith, 1992; Smith & Chan, 1991; Spriggs, Hruby, Maliszewski, Pickup, Sims, Buller & Vanslyke, 1992). It has been demonstrated that Tanapox virus (TPV) encoded 38 kDa polypeptide, which is an early gene product, binds to hIFN-γ, hIL-5, and hIL2 (Essani, Chalasani,

Eversole, Beuving & Birmingham, 1994). Lucas *et al.* (1996) purified serine proteinase inhibitor (SERP-1), which was a myxoma virus encoded secreted glycoprotein, to observe effects on plaque growth after balloon angioplasty on rabbits. Plaque development was reduced by inhibition of serine proteinase activities and was characterized by decrease in macrophage infiltration immediately after the balloon angioplasty. This study attempts to identify SPV encoded secretory polypeptides, which exhibit anti-cytokine activities.

Results presented here, demonstrate that SPV secretory polypeptides do not bind to human IFN-γ, TNF-α, and IL-1β. Unavailability of swine cytokine prevented us to further these studies. These results direct to the logical conclusion that SPV encoded secretory polypeptides are highly species-specific.

Our study demonstrates that systemic SPV infection in swine does not reduce plaque formation after balloon angioplasty on the swine coronary artery. Endemic nature of SPV in swine population and a lack of established methods for measuring anti-SPV antibody titer in serum samples from swine are major problems. To elucidate true effects of SPV encoded secretory polypeptides on atherosclerotic plaque formation, either obtaining SPV-free swine population or purifying SPV encoded secretory polypeptides are required.

REVIEW OF LITERATURE

General Characteristics of Swinepox Virus

Swinepox virus (SPV) is classified in the genus *Suispoxvirus* of the family *Poxviridae*. SPV is a large enveloped DNA virus and appears oval or brick shape (Moss 1996). The non-infectious SPV genome is a 175 k bp linear double stranded DNA (Massung & Moyer, 1991). The DNA of SPV is terminally cross-linked and contains terminal inverted repetitions (TIRs) similar to the genere *Orthopoxvirus* and *Leporipoxvirus* (Massung & Moyer, 1991). Structural components of SPV include a core, lateral bodies, surface protein, and membranes; these are all common *Poxviridae* properties. Also, like other poxviruses, SPV replicates within the cytoplasm of infected cells (Kasza, 1975) and produce a cytopathic effect (CPE) with characteristic intracytoplasmic inclusions and vacuoles in the cytoplasm (Kasza, 1975).

Historical Background

The earliest reports of Swinepox are found in the early European literature. Spinola (1842), Bollinger (1877), Peiper (1901), and Szanto (1906) described swinepox and associated it with vaccinia virus (VV). Swinepox was first described in North America in 1929 (McNutt, Murray & Purwin, 1929). In 1940, it was reported that both VV and SPV caused swinepox, and that SPV was swine specific

(Manninger, Csontos & Salyi, 1940). A further study was conducted to ensure that the pox in swine was caused by either SPV or VV (Schwarte & Biester, 1941). In 1960, SPV was isolated and cultivated from swine tissue cultures.

Epidemiology

Swine can be infected with both SPV and VV under both experimental and natural conditions, and the natural infection is maintained in the herd indefinitely due to the method of raising pigs and the characteristics of poxviruses. Since new stock continuously replace adult pigs, these young animal provide a susceptible host for continuous infection. However little attention has been paid for elimination of SPV due to the low rate of pig mortality. Also infected swine are a constant source of SPV because resistance of the poxvirus in dried crusts contribute to a continuous herd infection (Kasza, 1975). Unless effective viricidal agents are used, SPV can survive for at least 1 year in dried form (Kasza, 1975).

Transmission of SPV is by direct contact. Thus, there is no evidence for transmission by other infection routes. Skin injury is necessary for infection. Normally, SPV is transferred from one pig to another pig by lice which cause skin injury and generate and SPV portal of entry. Other blood-sucking insect vectors may also play a similar role in transmission of SPV. Younger pigs in a herd are more susceptible to infection, because older pigs may acquire immunity from a previous SPV infection.

Clinical Signs, Gross Lesions, and Pathogenesis

The incubation period of SPV infection is 3 to 6 days and the course of infection is 1.5 to 3.5 weeks (Kasza, 1975). SPV infected pigs under experimental conditions show a slight elevation of body temperature 2 to 4 days post-inoculation (Kasza, 1975). Swine consume less food than usual during the temperature elevation (Kasza, 1975). After 7 days post-infection, small red skin lesions become visible and further develop into papules which are abundant on the face, ears, inside the legs, and on the abdomen. The papules further develop into pustules within a few days. Pustle centers turn dry, are scabbed, and surrounded by an inflamed area that appear umbilicated. Most of the dried crusts are scrubbed off twenty-one days post-infection and only small, white, and discolored spots are visible (Kasza, 1975). The only organs, besides the skin, that develop macroscopic lesions are inguinal lymph nodes (Kasza, 1975).

Microscopic Lesions

The earliest microscopic changes are detectable three days post-infection. Hydropic degeneration in the stratum spinosum causes the epidermis to focally thicken. Vacuoles in the nuclei are observed in a few cells. This papular stage progresses and the epidermis increases in thickness due to hydropic degeneration and epithelial hyperplasia. At this stage, cytoplasmic inclusions and nuclear vacuoles are evident. Infiltration of dermis with immune cells and dilatation of blood vessels are observed. Subsequent small vesicles are visible in the epidermis

due to cell rupture and convergence. In the pustular stage, extensive necrosis is detected in the basilar layer which is often characterized by infiltration of neutrophiles and a lesser number of eosinophiles and lymphocytes. At this stage, small vesicles are still visible in the stratum spinosum. Vacuoles in the nuclei and cytoplasmic inclusions are present at the border of the necrotic area. When dried crusts are formed, both cellular necrosis and crust formation are the most abundant feature of the lesion. At the same time, epidermis regeneration is in progress. Electron microscopic examination revealed that viral replication in the skin of experimentally inoculated pigs is limited to the cytoplasm of epidermal cells (Kasza, 1975).

Kinetics of SPV Infection

SPV is characterized by slow growth in tissue culture (Kasza, 1975). CPE is not evident until approximately four days post-infection when Porcine Kidney Cells (PK-15 cells) are infected with SPV (Massung & Moyer, 1991a). In contrast, CPE is evident prior to 24 hours post-infection with *Orthopoxviruses* such as vaccinia virus (Moss, 1996). Pulse labeling of infected PK-15 cells and Coomassie-stained total protein patterns fail to detect expression of SPV viral proteins prior to 24 hours post-infection (Massung & Moyer, 1991b). By 32 hours post-infection, however, SPV viral proteins are clearly detected and their production continues to increase through 48 hours (Massung & Moyer, 1991b). On the other hand, using immunoprecipitation technique, early viral protein

synthesis is detected by 4 hours post-infection and late viral proteins are evident as early as 12 hours post-infection (Massung & Moyer, 1991b). It has been reported (Massung & Moyer, 1991b) that the pattern of viral protein synthesis prior to 32 hours post-infection is disguised because SPV ineffectively shuts off host protein synthesis prior to 24-32 hours post-infection. Massung *et al.* (1991a) designed an experiment to measure the accumulation of replicated viral DNA. The result revealed that DNA accumulation differs significantly between SPV and vaccinia over 48 hours post-infection. The accumulation of replicated SPV DNA is first detectable at 12 hours post-infection and does not peak until 48 hours (Massung & Moyer, 1991a). On the other hand, the accumulation of replicated vaccinia DNA is detectable at 6 hours post-infection and peaks at 24 hours post-infection. Messenger RNA (mRNA) transcription analysis of SPV also corresponds with DNA analysis and exhibit reduced mRNA synthesis compared to mRNA synthesis in vaccinia virus. Kinetics of DNA, mRNA, and protein synthesis directed by SPV reveals that SPV is unusual among well-characterized mammalian poxviruses. Tanapox virus is also a slow growing virus and CPE is not evident in TPV infected tissue culture cells until 3 to 4 days post-infection (Knight, Novembre, Brown, Goldsmith, & Esposito, 1989).

Viral Genome

Poxviruses are large, double-stranded DNA viruses which develop in the host cell cytoplasm. Poxvirus genomes are approximately 130 to 280 kbp and all

poxvirus isolate characterized have covalently cross-linked termini. The results of pulsed-field electrophoresis analysis reveal the SPV genome to be 175 kbp with 4,300 bp TIRs (Massung, Jayarama & Moyer, 1993). Complete DNA sequence analysis of the Copenhagen strain of vaccinia virus revealed a conserved, common pattern of genomic organization composed of a conserved central core and variable near terminal regions (Goebbel, Johnson, Perkus, Davis, Winslow & Paoletti, 1990). According to the analysis, the core region of the vaccinia virus DNA encodes the enzymatic and structural components necessary for the viral cytoplasmic life cycle and the near terminal regions of the poxvirus DNA encode products necessary for the wide range of pathogenic effects and host specificity. Inspired by this finding, Massung *et al.* (1993) examined the nucleotide sequence of fragments from several regions of the SPV genome including a 2.85 kb fragment from the potentially conserved part and two fragments, 14.2 kb and 3.6 kb, within the presumed variable near-terminal regions which tend to be unique for a given poxvirus. These latter fragments were found to encode 25 open reading frames (ORFs) including proteins predicted to be secreted or membrane bound in SPV infected cells. A few unique SPV ORFs which might be involved in immune modulation, cell attachment and pathogenesis were detected (Massung, Jayarama & Moyer, 1993). Comparison of amino acid sequences between SPV and vaccinia revealed that a few polypeptides encoded within the near terminal regions of the vaccinia virus DNA, which determine virulence and host range, were lacking within the same region in SPV. Functional homologues

of vaccinia growth factor, complement binding protein, and ORFs responsible for determining host range were not found in near terminal regions of SPV.

Virus Entry

There are many conflicting theories explaining poxviruses entry into cells (Moss, 1996). This controversy is due to the existence of infectious intracellular matured virions (IMV) and infectious extracellular enveloped virions (EEV) (Moss, 1996). It is not yet known if there is a cell surface receptor for viral attachment. Electron microscopic examination reveals that vaccinia IMV is fused to membranes by pinocytosis (Dales & Kajioka, 1964) and to the plasma membranes (Chang & Mets, 1976). A study with lysomotoropic agents suggests that the plasma membrane is a fusion site (Moss, 1996). Another study fusion (Doms, Blumenthal & Moss, 1990) using a fluorescent assay revealed that a neutral pH and temperature-dependent process is required for vaccinia IMV cell. Using antibody neutralization studies, several vaccinia membrane proteins have been shown to be important in cell surface attachment and/or penetration (Oie & Ichihashi, 1981). Several vaccinia gene products bind with high affinity to the surfaces of a variety of cells (Lai, Gong & Esteban, 1990). Although recent studies (Moss, 1996) indicate that a vaccinia virus-encoded protein, vaccinia growth factor (VGF), has the ability to bind to the cellular epidermal growth factor (EGF) receptor, the general importance of the EGF receptor for vaccinia virus entry is not conclusive.

Neutral pH is known to be required for EEV fusion to cell surface (Moss, 1996). The mechanisms of entry of EEV into host cells have not been elucidated. It has been suggested (Moss, 1996) that the entry mechanisms of EEV differ from that of IMV since EEV infectivity is not neutralized by antibody to IMV.

Viral Polypeptides

Poxvirus contains large variety of polypeptides. Approximately 30 bands are clearly resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of vaccinia virus purified from infected cells and disrupted with sodium dodecyl sulfate and reducing agent (Moss, 1996). Also, even more polypeptides are detected with two-dimensional gel electrophoresis. Little is known about the polypeptides produced upon SPV infection. Immunoprecipitation and immunoblot revealed that at least 8 major polypeptides (97kD, 65kD, 48kD, 35kD, 32kD, 18kD, 15kD, and 12kD) are present (Ouchi, Fujiwara, Hatano, Yamada & Nii, 1992). Neither structural nor secretory proteins due to SPV infection have not been characterized at all.

Although characterization of isolated SPV secretory proteins has not been done, DNA sequence studies have revealed putative properties of some SPV secretory proteins. The near terminal regions of SPV DNA (Massung, Jayarama & Moyer, 1993) may encode membrane-bound or secreted viroceptors that bind to various immune components (based on the presence of foreseen hydrophobic leaders and internal transmembrane domains). DNA analysis revealed that three

SPV open reading frames (ORFs) encode polypeptides that are homologues to a serine proteinase inhibitor (serpin), an interferon gamma receptor, and a G protein-coupled receptor (Massung, Jayarama & Moyer, 1993). The SPV K1R ORF showed high degree of homology (32.1% identity in 343 amino acid) of the cowpox virus 38 kDa protein, a serpin homologue which is shown to inhibit the cellular interleukin 1β converting enzyme (Upton, 1992; Massung, Jayarama & Moyer, 1993). The SPV ORF C6L is homologous to the human interferon-γ receptor (Massung, Jayarama, & Moyer, 1993). The SPV ORF C6L is also homologous to myxoma virus and Shope fibroma virus interferon-γ receptor homologues (Upton, 1992; Massung, Jayarama & Moyer, 1993). These DNA base sequence studies also revealed the SPV ORFs C3L and K2R to be members of the G-protein coupled receptor family (Massung, Jayarama & Moyer, 1993).

Virus Host Interactions

Inhibition of Host Macromolecular Synthesis

Utilization of host biosynthetic processor is a requirement for all viruses. Therefore, poxviruses are believed to inhibit host DNA, RNA, and protein synthesis upon infection. Infection of tissue culture cells with *orthopoxviruses* shows cytopathic effects, changes in membrane permeability, and inhibition of DNA, RNA, and protein synthesis (Moss, 1996). Several factors may be involved for the switch from host to viral protein synthesis. The comparative contribution

of each factor may rely on the virus multiplicity, cell type, time of analysis, and use of metabolic inhibitors (Moss, 1996). The surface tubules, the B1R protein kinase, and the F17R phosphoprotein in vaccinia virus particles inhibit host protein synthesis in the absence of viral gene expression (Mbuy, Morris & Bubel, 1982; Beaud, Sharif, Topamass & Leader, 1994; Person-Fernandez & Beaud, 1986). Also, small poly (A)-containing RNA molecules may play an inhibitory role during viral infection (Cacoullos & Bablanian, 1991). Synthesis and processing of host cell RNAs are also inhibited on initiation of vaccinia virus protein synthesis (Moss, 1996).

Stimulatory Effects on Cell Growth

It is common for poxviruses to exhibit hyperplasia and tumors in the host skin. These consequences of poxvirus infection are well documented in fowlpox virus, Shope fibroma virus, Yaba virus, and molluscum contagiosum infections (Moss, 1996). Secretion of poxivirus encoded growth factors is believed to contribute to hyperplasia. Vaccinia virus growth factor (VGF) also shows homology to epidermal growth factor (EGF) and transforming growth factor α (Blomquist, Hunt & Barker, 1984). The secreted VGF can bind to the EGF receptor of hosts and stimulate cell growth. Although these effects of VGF are not observed in tissue culture cells, a rapid proliferation of ectodermal and endodermal cells of the chicken embryo chorioallantoic membrane does occur

(Moss, 1996). Vaccinia virus lacking VGF has diminished virus replication *in vivo* and an attenuated pathogenecity (Moss, 1996).

Viral Defense Molecules

Vertebrates have developed a highly sophisticated immune system to suppress virus propagation. Soon after introduction of virus, non-specific immune factors such as complement, interferons, and natural killer cells suppress virus invasion. Cytotoxic T cells and virus specific antibodies neutralize and inactivate viruses. Some viral (virulence factor) proteins secreted from poxvirus-infected cells have the ability to inhibit host immune responses (Barinaga, 1992). Immune evasion properties are known for a wide spectrum of viruses. Those include herpes simplex virus type 1, adenoviruses, and retroviruses such as HIV (Gooding, 1992). The concept of *virokine* or *viroceptor* has been introduced for viral proteins responsible for immune evasion (Gooding, 1992).

Viral Protein – SERPIN Superfamily

Host cell cytokines IL-1α and IL-1β are responsible for inhibiting early stages of viral infections and to regulate the host inflammatory process. Several poxviruses have developed strategies to inhibit actions of IL-1. The cowpox virus gene responsible for encoding immune evasive protein has sequence motifs common to the serine proteinase inhibitor (SERPIN) superfamily (Palumbo, Pickup, Frederickson, McIntyre & Buller, 1989). *In vitro* studies have revealed

that this gene product, SPI-2 or crmA, inhibits the IL-1β converting enzyme which is an atypical cysteine proteinase (Ray, Black, Kronheim, Greenstreet, Sleath, Salvesen & Pickup, 1992). By inhibiting intracellular conversion of the precursor of IL-1β to the active form, crmA can function as a viral anti-inflammatory molecule. Cells infected with myxoma virus secrete SERP-1 (a related but distinct SERPIN homolog), since a deletion mutant of myxoma virus lacking the SERP-1 gene results in a significant attenuation of the virus, it is possible that SERP-1 interferes with inflammation (Macen, Upton, Nation, & McFadden, 1993). SERP-1 is also known to inhibit, *in vitro*, a number of serine proteinases such as plasmin, urokinase, tissue plasminogen activator, and a constituent of the complement component cascade (Macen, Upton, Nation & McFadden, 1993).

IL-1 and TNF Receptor Homologues

Vaccinia virus and Cowpox virus encode an IL-1β receptor homologue which is secreted from infected cells and the IL-1β receptor homologue binds and inhibits IL-1β (Alcami & Smith, 1992; Smith & Chan, 1991; Spriggs, Hruby, Maliszewski, Pickup, Sims, Buller & Vanslyke, 1992). Schreiber *et al.* (1994) have identified a myxoma virus tumor necrosis factor (TNF) -receptor homologue (T2), which inhibits TNF α. Tanapox virus also secretes a soluble TNF receptor homologue which binds to human TNF α (Paulose and Essani, unpublished data).

Inhibitors of Interferon

Type I and type II interferons play a major role in host defenses against poxviruses (Karupiah, Frederickson, Holmes, Khairallah & Buller, 1993). Two examples of interferon regulation have been well identified. First, the double stranded RNA dependent protein kinase (PKR) pathway directs the phosphorylation of the eukaryotic translation initiation factor eIF-2α which results in inhibition of protein synthesis (Moss, 1996). Second, vaccinia virus encoded A18R protein, which is a DNA dependent ATPase, enhances degradation of mRNA and rRNA which is interceded through the 2-5A/Rnase L pathway as a result of abnormal transcription (Bayliss & Condit, 1993). Poxviruses also utilize secretory proteins to protect themselves against interferons. Cells infected with myxoma virus and Shope fibroma virus secrete virus-encoded 35 kd proteins that are homologus to the ligand-binding domain of the cellular receptor for interferon γ (Upton, Opgenorth, Traktman & McFadden, 1992). These proteins selectively bind and inhibit interferon γ. Vaccinia and variola viruses are also known to encode secretory proteins that are interferon γ receptor homologues (Massung, Esposito, Liu, Qi, Utterback & Knight, 1993; Moss, 1996).

Complement Regulatory Protein

Complement components are capable of enhancing antibody mediated neutralization of viruses. Several poxviruses have developed means of

modulating classical and alternative pathways of complement activation. The major secretory protein of vaccinia virus, VCP, binds the C4b fragment of complement component C4 and thus inhibits complement fixation directed by the classic compliment pathway (Kotwal, Issacs, McKenzie, Frank & Moss, 1990). The protein also inhibits the alternative pathway of complement fixation (Moss, 1996).

Cell Adhesion Molecules

The molecular basis of cellular adhesion in vertebrates is regulated by cell adhesion molecules (CAMs). Intercellular adhesion molecules (ICAMs) are expressed on activated endothelial cells, where they bind to integrins on the surface of white blood cells and aid in adherence of these blood cells at sites of inflammation (Edelman & Crossin, 1991). ICAM-1 also binds lymphocyte function associated protein 1 (LFA-1) on stimulated T cells.

The majority of T and B cells continuously recirculate between the cardiovascular system and the lymphatic system. In a lymph node, lymphocytes migrate from the blood stream to small lymphatic vessels. This event depends on specific interactions between the lymphocyte surface and the surface of specialized endothelial cells lining small veins in a lymph node. Many cell types in the cardiovascular system interact with these endothelial cells, but only lymphocytes adhere and migrate out from the blood stream to the lymphatic vessels. This migration is supported by varieties of homing receptors on

lymphocytes and by the ligands for these receptors on endothelial cells. A cell adhesion protein called E-Selectin supports migration of lymphocytes from the blood stream to lymphatic vessels. This homing receptor, which is present on most lymphocytes, binds specific ligands on the endothelial cells lining venules in lymph nodes. E-selectin binding causes the lymphocyte to attach weakly to the endothelial cells and to roll slowly on the surface. Rolling continues until a stronger adhesion mechanism is applied. A member of integrin family of CAMs on the lymphocyte surface mediates strong adhesion. Upon strong adhesion, the lymphocyte stops rolling and penetrates through the blood vessel out to the lymphatic vessels. When inflammation occurs and T and B cells are activated with antigens and most of lymphocytes lose their original homing receptors and express special receptors to migrate to injury sites. The migration of activated lymphocytes and other white blood cells is mainly interceded by other selectins and integrin families.

Based on the fact that CAMs are important mediators of the inflammatory response, they are often a target for inhibition. For example, P-selectin is expressed on activated platelets and endothelium and mediates neutrophil binding to both cell types. Neutrophil infiltration at lesions on balloon angioplasty injured site is a major mediator in post-percutaneous transluminal coronary angioplasty restenosis. Substances which are capable of inhibiting P-selectin interactions may be able to reduce restenosis.

Restenosis and Percutaneous Transluminal Coronary Angioplasty

Percutaneous transluminal coronary angioplasty (PTCA) is a common procedure for providing relief of angina pectoris in patients with single and multivessel coronary artery disease. Angiographic renarrowing at the site of a previous PTCA is known as restenosis and occurs in approximately 40% of patients at 6 months (Lucas, Liu, Macen, Nash, Dai, Stewart, Graham, Etches, Boshkov, Nation, Bumen, Hobman & McFadden, 1996). Although the typical restenotic lesion is different from the atherosclerotic plaque in architecture and composition, both restenotic and atherosclerotic plaques contain smooth muscle cells and fibrous tissue. Restenosis after PTCA is the major problem limiting the long-term efficacy of PTCA. A critical step in restenosis is thought to be activation of inflammatory response, including platelet adhesion, platelet aggregation, fibrin deposition, and leukocyte infiltration (Hermans, Rensing, Bradley, Strauss & Serruys, 1991). Activation of the inflammatory response is followed by intimal smooth muscle cell proliferation and migration, which is initiated by both cellular components of the blood and damaged vascular endothelial cells (Liu, Roubin & King, 1989). Anticoagulants, antiplatelet agents, antiproliferative agents, calcium channel blockers, lipid-lowering agents, and inflammation inhibitors have all been unsuccessful in prevention of restenosis (Hermans, Rensing, Bradley, Strauss & Serruys, 1991).

Clinical Significance of Viral Polypeptides in Restenosis

The search for a so-called magic bullet, which prevents restenosis after PTCA, is on intense area of biological research. After the vascular injury caused by PTCA, inflammatory responses are major contributors to restenosis. The inflammatory responses involve many inflammatory enzymes and thrombotic enzyme cascades, which have serine proteinase activity and are regulated by serine proteinase inhibitors (Altieri, 1995). Coagulation and fibrinolytic enzymes are known to induce monocyte motility and chemotaxis, tissue infiltration, cytokine responses, cell proliferation, and apoptosis (Lucas, Liu, Macen, Nash, Dai, Stewart, Graham, Etches, Boshkov, Nation, Bumen, Hobman & McFadden, 1996). One of serine proteinase inhibitor, plasminogen inhibitor-1 (PAI-1), has been demonstrated to play a significant role in suppression of intimal hyperplasia. Transgenic mice that lack PAI-1 gene showed accelerated intimal hyperplasia as histologically (Carmeliet, 1995; Carmeliet, Seboojans, Kieckans, Ream, Degan, Bronson, DeVos, van den Oord, Collen & Mulligan, 1994). Likewise, inhibition of plasminogen with traexamic acid reduced smooth muscle cell migration in rats following arterial balloon injury (Jackson & Reidy, 1992). Lucas *et al.* (1996) obtained the purified serine proteinase inhibitor, (SERP-1), which was a myxoma virus encoded secreted glycoprotein, to observe effects on plaque growth after balloon angioplasty on rabbits when it is administered locally. Plaque development was reduced by inhibition of serine proteinase activities and was

characterized by a focal decrease in macrophage infiltration immediately after the balloon angioplasty.

Antiviral Therapy in the Treatment of Restenosis After Angioplasty

Coronary restenotic lesions have common features with benign tumors such as increased presence of proliferating vascular smooth muscle cells (McEwan, 1995). 50% of human benign tumors involve mutation of the p53 gene, which has a tumor suppressive activity. Spier *et al.* (1994) focused on effects of human Cytomegalovirus (HCMV) infection on atherosclerotic proliferation of vascular smooth muscle cells. HCMV is known for producing a protein, IE84, which elicits a repressor effect on p53 expression. Spier *et al.* (1994) found that vascular injury induced reactivation of HCMV infection at the angioplasty injured site which resulted in IE84 expression. Enhanced IE84 expression inhibited degradation and the repressor effect of p53 in the vascular smooth muscle cells. Therefore, the inhibition of p53 gene activity resulted in the proliferation of vascular smooth muscle cells, which is a common feature of the post-angioplasty restenosis. Though this finding had a great impact in restenosis research, it is still inconclusive. In addition, scientists who believe that restenosis is caused by viral infection investigated this further. Ohno *et al.* (1994) introduced the concept of applying antiviral therapy to the prevention of restenosis. The antiviral agent, gancyclovir, was locally injected to pigs after balloon catheter induced iliac artery injury. At the same time, a replication

deficient adenovirus, which possessed the viral enzyme thymidine kinase, was transfected in the injured vessels. The enzyme is known to transform gancyclovir to a cytotoxic metabolite that selectively kills actively proliferating cells. This experiment resulted in substantial inhibition on neointimal formation.

MATERIALS AND METHODS

Virus and Cell Lines

SPV was obtained from American Type Culture Collection, Rockville, Maryland, and cultivated in monolayers of Porcine Kidney 15 (PK-15) cells. PK-15 cells were grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10 % (v/v) newborn calf serum (NBCS), 200mM glutamine and antibiotics (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25μg/ml amphotericin B) (Growth Medium). Cells were cultured at 37°C in an incubator with 5% CO_2. Unless noted, cells were infected with 1~2 plaque forming unit (pfu) per cell followed by adsorption on a rocking platform at room temperature for one hour. Following the adsorption, infected cells were maintained in RPMI 1640 containing 2% (v/v) NBCS, 200mM glutamine, and antibiotics (Maintenance medium) at 37°C with 5% CO_2.

Expanded surface 1700 cm^2 plastic roller bottles (Corning, Rochester, NY) were also used with Earle's minimum essential medium (EMEM) containing 2% (v/v) NBCS, 200mM glutamine, and antibiotics to prepare bulk virus. Infected cells were harvested 7-8 days post-infection using a rubber policeman and cells were pelleted by centrifugation at 1500 ×g (IEC PR-6000 refrigerated superspeed centrifuge) for 20 minutes. The pellet was resuspended in serum free RPMI 1640 with antibiotics. In order to release intracellular viral particles, three cycles of freeze-

thawing was carried out. Then nuclei and large cytoplasmic debris were removed by centrifugation at 1500 ×g (IEC PR-6000 refrigerated superspeed centrifuge at 2000 rpm) for 15 minutes. Supernatants were then centrifuged at 85,000 ×g (Beckman L8-70M ultracentrifuge; Ti-70.I fixed angle rotor) for 30 minutes to pellet SPV. The SPV pellet was resuspended in RPMI 1640 containing 2% (v/v) NBCS, 200mM glutamine, and antibiotics to a final concentration of 100X. The concentrated SPV was stored at -20°C.

Titration of SPV

SPV was quantitated by serial dilution method. Monolayers of PK-15 cells in 6 well dishes were infected with 10^{-4}, 10^{-5}, and 10^{-6} dilution of 100X SPV followed by adsorption on a rocking platform at room temperature for one hour. Then monolayers were washed with serum-free RPMI 1640 with 200mM glutamine, and antibiotics three times. Monolayers were then incubated with overlay medium (maintenance medium containing 1 % (w/v) carboxymethylcellulose) for 14 days at 37°C with 5% CO_2. After incubation, infected monolayers were fixed and stained with 1% crystal violet in 10% formaldehyde to visualize plaques. 100X virus was titrated to be 3.25 X 10^8 pfu/cell on average.

Identification of SPV Secretory Proteins

Metabolic Labeling Studies of SPV Infected Cells

Fresh monolayers of PK-15 cells were grownin 60 mm dishes. Cells were infected with SPV at multiplicity of infection of 10 or 20 pfu/cell. After 1 hour adsorption at room temperature, maintenance medium was added to the dishes and incubated for 6 hours at 37°C with 5% CO_2. Then the supernatants were removed and monolayers were washed twice with serum-free RPMI 1640 with antibiotics, after which 2ml of methionine-free EMEM (Gibco Laboratories, Gland Island, NY) without serum was added to each dish. Dishes were then incubated at 37°C with 5% CO_2 for one hour. Following incubation, supernatants were removed and 2ml of methionine-free, serum-free EMEM supplemented with 100 μCi [S^{35}]methionine/ml at 1504Ci/mmol (ICN,Costa Mesa, CA) was added to monolayers. The supernatant was collected 10 to 18 hours later, depending on experiments. Cells in the supernatants were removed by centrifugation at 1500 ×g (IEC PR-6000 refrigerated superspeed centrifuge) for 20 minutes followed by ultra-centrifugation at 85,000 ×g (Beckman L8-70M ultracentrifuge; Ti-70.I fixed angle rotor) for 30 minutes to pellet cell debris and SPV. In order to recover SPV secretory polypeptides, the supernatants were then concentrated 5-fold by centrifugation in a Microcon 3 concentrator (Amicon, Beverly, MA) or in a Centriprep 3 concentrator (Amicon, Beverly, MA) for larger quantities. In an attempt to collect intracellular SPV proteins, monolayers were scraped off with a rubber policeman at the time of harvesting and centrifuged at 1500

×g (IEC PR-6000 refrigerated superspeed centrifuge) for 20 minutes. Then medium was decanted and NP-40 (BRL – Shell Oil Company, Rockville, MD) was added to the remaining pellet to avoid DNA contamination. Protein samples from concentrated supernatants were mixed with an equal volume of a protein loading solution and solubilized by boiling for 3 minutes. The solubilized proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10.5% gel. Radiolabeled proteins were visualized by autoradiography of the dried gel. The dried gel was exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) for a minimum of 48 hours. Where indicated, prior to autoradiography, presence of SPV secretory proteins were visualized with silver staining by using Bio-Rad Silver Stain Kit (Bio-Rad Laboratories, Richmond, CA) or Commasi blue staining. Low molecular marker (Bio-Rad Laboratories, Hercules, CA) was used to estimate molecular weights of proteins. All autoradiograms presented were scanned on a Microtek Laboratories Scanmaker E6PRO and organized with Adobe Photoshop 4.0. Autoradiograms were then printed on a 600 dpi × 600 dpi NEC Super Script 860 Laser Printer.

Early and Late Protein Experiment

Upon poxvirus infection, two major types of proteins are known to be produced. One is the early protein, which is a poxvirus gene product produced before poxvirus successfully shuts down host protein synthesis. The other is the late protein, which is a poxvirus gene product produced after the termination of host protein

synthesis. Protein labeling was conducted as described above. Cytosine β-D-Arobinofuranoside (AraC) (Sigma, St.Louis, MO), which blocks late but not early viral protein synthesis, was added to one of the samples (12.5 μl/ml) in addition to [S^{35}]methionine (100 μCi) so that only early proteins were detectable in samples. Amount of Ara C used in this experiment was chosen by results from a previous publication (Sharad & Boyle, 1990).

Pulse-Chase Experiment

A pulse-chase experiment was performed to exhibit kinetics of SPV protein expression. SPV secretory proteins and SPV polypeptides inside cells were chased for 20 hours postinfection (9 hours after radiolabeling) and pulsed for 11 hours postinfection (1 hour after radiolabeling) with 100 μCi [S^{35}]methionine/ml at 1504Ci/mmol (ICN, Costa Mesa, CA). Both supernatant and pellets were recovered and processed as described above.

Characterization of SPV Secretory Polypeptides

Interferon Gamma Assay

The biological activity of human IFN-γ (hIFN-γ) was assayed by its ability to inhibit cocal virus plaque formation. The protocol was basically duplicated from a previous publication was followed (Essani, Chalasani, Eversole, Beuving & Birmingham, 1994). hIFN-γ was assayed in cultures of PK-15 cells. Although,

human cells should be used due to the species specificity of the IFN-γ, PK-15 cells had to be used due to the fact that SPV only infects porcine cells. Cell monolayers in 6 well dishes (Gibco, Grand Island, NY) were pretreated with 2% RPMI 1640 containing 2% NBCS with antibiotics and indicated doses of IFN-γ and concentrated supernatants from SPV infected cultures. After a 10 hr incubation at 37°C with 5% CO_2, the cells were infected with 100 pfu/cell of SPV and incubated for a further 9 to 14 days at 37°C with 5% CO_2 in media containing 1 % (w/v) carboxymethylcellulose. After incubation, the supernatants were removed and monolayers were stained with crystal violet dye to count plaques. It has to be noted that virus-free supernatants from the infected cultures did not form plaques in the absence of SPV.

Human Recombinant Tumor Necrosis Factor Alpha and Human Recombinant Interleukin-1β Assay

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA). Cells were plated in a 75 cm^2 tissue culture flask (Corning, Indianapolis, IN) pretreated with 1% gelatin (Difco, Detroit, MI). Growth medium consisted of 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 2% fetal bovine calf serum (Clonetics, San Diego, CA), 50 μg/ml gentamicin, 50 ng/ml Amphotericin-B, 12 μg/ml bovine brain extract, and 10 μg/ml heparin. HUVEC were grown as monolayers in 96 well microtiter plates (Corning,

Rochester, NY) pretreated with 1% gelatin. The microtiter plates were incubated at 37°C with 5% CO_2, with daily changes of growth medium until confluent.

Treatment of HUVEC With Recombinant Human TNF α and SPV Protein Samples or Human Recombinant Interleukin-1β and SPV Protein Samples

Concentrated 5× supernatants from either SPV-infected or mock-infected cells were prepared as described above. Mixtures of indicated doses of recombinant human TNF-α (Genzyme, Cambridge, MA) or recombinant human IL-1β (R&D Systems, Minneapolis, MN) and the concentrated supernatants were introduced to the confluent HUVEC cells on microtiter plates. Microtiter plates were then incubated at 37°C for four hours. After incubation, the microtiter plates were washed with 1× PBS and fixed with 1% paraformaldehyde in PBS and stored at 4°C for 10 to 15 minutes. After fixation, the microtiter plates were washed with PBS and blocked with 2% bovine serum albumin (BSA) in PBS supplemented with NaN_3 (0.02 g/dl). The microtiter plates were incubated at 37°C for at least one hour. After removal of the blocking solution, the microtiter plates were washed with PBS and stained with primary antibody (1 μg/ml in PBS, 0.1 % BSA). Primary antibodies were mouse anti-ICAM-2 monoclonal antibody (Biosource International, Camarillo, CA), mouse anti-ICAM-1 monoclonal antibody (R & D Systems, Minneapolis, MN), mouse anti-VCAM-1 monoclonal antibody antibody (R & D Systems, Minneapolis, MN), mouse anti-E-Selectin monoclonal antibody (R & D Systems, Minneapolis, MN). After the addition of primary antibodies, the microtiter plates were incubated at 37°C for two

hours. Microtitier plates were rewashed with PBS containing 0.05% Tween 20 followed by a single wash with PBS. After the wash, 100 µl/ well alkaline phosphatase-conjugated goat anti-mouse IgG (1 µg/ml in PBS, 0.1% BSA) was added and incubated further at 37°C for two hours. The microtiter plates were washed with PBS containing 0.05% Tween 20 with PBS. Chromogenic substrate, p-nitrophenyl phosphate (1 mg/ml in PBS), was added and incubated at room temperature for 10 to 20 minutes depending upon the degree of reactions. Microtiter plates were read in a fluorescence microtiter plate reader at an optical density of 405 nm. Results were recorded with the aid of computer software.

Atherosclerosis Study

Pigs and Diet

Female and male domestic feeder pigs (Yorkshire-Hampshier-Landrace-Duroc crosses, 2-3 months old) were provided from Pharmacia & Upjohn Farms and fed on S-985 chow (18% crude protein, Pharmacia & Upjohn feed) until the percutaneous transluminal coronary angioplasty (PTCA) procedure. After PTCA, the animals were fed S-810-L chow (16% crude protein, including 200 gm/ton of the antibiotic lincomycin; Pharmacia & Upjohn feed) throughout the rest of the study.

SPV Production and Infection Procedures

A 100X SPV preparation was prepared as described above. The 100X virus was diluted to a final concentration of 1×10^8 pfu/ml RPMI 1640. Pigs selected for

this study were infected one week prior to a PTCA balloon over-inflation procedure. A 20 ml venous blood samples was also taken by a superior vena cava venipuncture. Blood samples were incubated in a 37°C water bath in order to separate sera. These blood samples were used as control serum samples and stored at 4°C until analysis. Pigs were subsequently injected subcutaneously in the right inguinal fold with either live SPV (1×10^8 pfu/ml RPMI 1640) or 1ml of RPMI 1640 vehicle of conscious pigs in separated rooms.

Percutaneous Transluminal Coronary Angioplasty (PTCA)

The procedure used to intentionally over-stretch a 2 cm segment of porcine left anterior descending (LAD) coronary artery has been described in detail (Humphrey, Simmons, Toombs & Shebuski, 1994). PTCA was performed on ketamine/midazolam anesthetized pigs via the right carotid artery to introduce a catheter to the LAD coronary artery. A GE-mobile fluoroscope connected to a video cassette recorder was used to locate the balloon visually and to record angiograms throughout the PTCA procedure. After each procedure, these angiograms were replayed on a 12 inch monitor to calculate over-inflation ratio (OI ratio), which denoted the diameter ratio of the inflated balloon catheter (3.0 or 3.5 mm PTCA catheter inflated with contrast dye) to the diameter ratio of the original coronary arterial site subjected to PTCA. Throughout all PTCA procedures, attempts were made to overstretch a 2 cm segment of LAD coronary artery between 30% and 50% (OI ratio = 1.3 – 1.5). This level of overstretch had previously been shown to

effectively cause a neointimal proliferative response in the artery without causing collapse of the vessel (Humphrey, Simmons, Toombs & Shebuski, 1994). This degree of overstretch was applied twice in 60 second intervals with each inflation lasting 20 seconds at 8 atmospheres. After all surgical devices were removed and incision points surgically repaired, effectiveness of the PTCA was confirmed by a repeat angiogram. In order to avoid fibrillation which is usually associated with this procedure, pigs were treated with a coronary vasodilator (nitroglycerin), antiarrhythmic (bretylin), and antithrombotic agent (Aspirin) (Humphrey, Simmons, Toombs & Shebuski, 1994). At the end of the procedure, each pig was administered penicillin G intramusculary (1.5 million units) and recovered in their own cages over 2 to 3 hours post-procedure. While pigs were maintained for the next two weeks, daily oral doses of aspirin (80 mg/day) were given as a post-PTCA treatment.

Histopathology and Morphometric Analysis of Porcine LAD Coronary Artery

Two weeks following the PTCA procedure, pigs were anesthetized with ketamine/midazolam which was administered intramuscularly. The external jugular vein was surgically exposed to gain access for a catheter. Heparin (200 U/kg) was administered systemically followed by Evan's blue dye (1% v/v in physiological saline) to stain the lesion site at the LAD coronary artery. Evan's blue dye is excluded by intact endothelium, thus cites of dye uptake indicate balloon-induced endothelial injury. After sacrificing, the hearts were harvested and retrogradely perfused at 100 mmHg with physiological saline for 15 minutes. The hearts were

then perfused with Omni-fix II fixative (An-Con Genetics, Inc., Melville, NY) for 30 minutes. Three consecutive 1 cm LAD coronary artery segments were taken from each balloon injured site then fixed in Omni-Fix II fixative for more than 24 hours for subsequent paraffin embedding. Ten cross-sections were obtained throughout the entire length of each of the three sample segments. The sections were stained with aldehyde fuschin and counter stained with Van Giesson's solution to differentiate the internal elastic lamina (IEL). On up to 30 cross-sectioned coronary arteries (Evan's blue stained 20 sections from the 2 cm segment; 10 sections from 1 cm segment with no Evan's blue stain), morphometric measurements were performed with a computer aided image analysis system (The Microscope CO., Medina, OH). The degree of vascular injury caused by PTCA balloon overinflation and the degree of neointimal hyperplasia was determined by previously established methods (Humphrey, Simmons, Toombs & Shebuski, 1994).

Indirect Immunofluorescence

Currently, a widely accepted method for anti-SPV antibody titer in serum is not available, though an attempt was made to measure anti-SPV antibody titer in porcine serum samples taken at the time of infection, the time prior to PTCA, and at 2 weeks post-PTCA. Immediately after the collection of the serum samples, samples were incubated at 37°C for 30 minutes in an attempt to separate serum. Serum samples were then stored at 4°C until assay.

Confluent monolayers of PK-15 (Porcine Kidney-15) cells were grown in eight well chamber slides in RPMI 1640 with 10 % NBCS (Nunc Inc, Naperville, IL). Cells were then infected with SPV (10 pfu/cell) and incubated at 37°C with 5% CO_2 for 36 hours. Microscopic examination at this time was carried out to confirm CPE. Each well on the slides was then washed 3 times with PBS containing 1 % (w/w) gelatin (Bio-Rad Laboratories, Richmond, CA) and cells were fixed for 30 seconds using ethanol precooled to –20°C. Wells were rewashed 3 times with PBS/gelatin immediately prior to sample addition. Porcine serum samples were diluted 1:10,000 with PBS/gelatin and 75µl aliquots of this dilution were added to the fixed PK-15 cells and incubated for 2 hours at 37 C° in a humidified incubator. After the incubation, the wells were washed 5 times with PBS/gelatin and exposed to 75 µl aliquots of a 1:64 dilution (in PBS) of fluorescein isothiocyanate (FITC) labeled rabbit anti-pig IgG (Sigma, St. Louis, MO) and incubated for 1.5 hours at 37 C° in a humidified incubator. The wells were then washed 5 times with PBS/gelatin, mounted in Vectar Shield (Vectar Laboratory, Burlingame, CA), and observed under UV microscope (Nikon, Tokyo, Japan). Intensity of fluorescence was measured by recording exposure time required to activate camera. Therefore, in theory, serum levels of anti-SPV antibody should be inversely proportional to the recorded exposure time. In short, the higher the exposure time was, the lower the antibody titer in serum sample was.

ELISA

In addition to the immunofluorescent procedure, in order to titer anti-SPV antibody in serum samples, enzyme linked immunosobent assay (ELISA) was carried out as described in published literature (Hyman & Perombelon, 1990). Confluent monolayers of PK-15 cells were infected at multiplicity of 10 pfu/cell or mock-infected and incubated at 37°C with 5% CO_2 for 36 hours. After the incubation, supernatant and cells were recovered and centrifuged at 1500 ×g (IEC PR-6000 refrigerated superspeed centrifuge) for 5 minutes. Then supernatants were decanted and deionized water was added to pellet. The pellet was frozen and thawed three times and centrifuged again at 1500 ×g (IEC PR-6000 refrigerated superspeed centrifuge at 1000 rpm) for 15 minutes. Supernatants were saved and mixed with the same amount of 2× coating buffer. Then 50 µl aliquots of the mixtures were dispensed to each well of Immulon II 96 well plates (Flow Laboratories, Horsham, PA) and incubated for overnight at 37 C° in a humidified incubator. After the incubation, the plates were washed three times with PBS with 0.05% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20) (Sigma, St. Louis, MO) followed by addition of 100µl aliquots of blocking buffer (PBS- 0.1% gelatin) to each well. The plates were left at room temperature for one hour, then the plate was washed two times with PBS-Tween 20. Final wash was decanted and 100 µl aliquots of each 1:10,000 diluted pig serum sample (diluted in blocking buffer) were introduced to designated wells. Hyperimmune serum kindly provided by Dr. Richard Moyer

(University of Florida, Gainesville, FL) was diluted 1:100 in blocking buffer. After the addition, the plates were incubated at room temperature for 90 minutes. The plates were then washed five times with PBS-Tween 20 (0.05%). 50µl aliquots of goat anti-pig IgG peroxidase conjugated antibody, diluted 1:10,000 in blocking buffer, was added to each well and incubated at room temperature for 60 minutes, followed by five washes with PBS-Tween 20 and a wash with deionized water. Substrate made with OPD (4mg/10ml), DMSO (50 - 100 µl), and Phosphate-Citrate buffer (0.05M, pH 5.0) was mixed with H_2O_2 (100µl/10ml) and 50µl aliquots of the mixture was added immediately to the plates. The plates were incubated at room temperature for 30min in a dark place and reaction was stopped by addition of 2M H_2SO_4 (50µl/well). The plate was then observed with a microplate reader (Bio-Rad, Richmond, CA) at an absorbance of 450 nm.

Production of Monoclonal Antibody (mAb)

Production of Hybridomas

Methods used for production and selection of hybridomas have been described in detail elsewhere (Ouchi, Fujiwara, Hatano, Yamada & Nii, 1992). SPV was produced by following a protocol described above. At week intervals for four weeks, female Bulb/c mice (Charles River, Kalamazoo, MI) were immunized with PK-15 cells infected with SPV (10 pfu/cell). After the four weeks, the mice received a booster injection intraperitoneally with a mixture of PK-15 cells infected with SPV. Five days after the booster injection, the mice were sacrificed and the spleen cells

were harvested. The spleen cells were fused with the mouse myeloma cell line P63Ag8 (American Type Culture Collection, Rockville, MD) pretreated with 8 azaguanine using polyethylene glycol (Gibco Laboratories, Grand Island, NY). Fused cells were seeded on 96 well dishes and hybridomas were selected with a medium containing hypoxantine / aminopterin / thymidine (HAT medium). Hybridomas were screened for the presence of anti-SPV antibodies by indirect immunofluorescent tests.

Indirect Immunofluorescence

This test was done with a slight modification of the indirect immunofluorescent procedure described above. PK-15 cells cultured to confluence on coverslips were infected with SPV at a multiplicity of infection of 10 pfu. At 48 hours postinfection, infected cells on coverslips were washed with serum free RPMI 1640 with antibiotics and fixed for 30 seconds using 200 proof ethanol precooled to –20°C. The coverslips were then washed with PBS-gelatin (0.01%) several times. The supernatants of hybridoma cultures (50 µl/coverslip) were used as the primary antibody and incubated for 2 hours at 37 C° in a humidified incubator. 50 µl aliquots of a 1:64 dilution (in PBS) of fluorescein isothiocyanate (FITC) labeled rabbit anti-pig IgG (Sigma, St. Louis, MO) was used as the secondary antibody and incubated for 2 hours at 37 C° in a humidified incubator. The coverslips were then washed 5 times with PBS/gelatin, mounted in Vectar Shield (Vectar Laboratory, Burlingame, CA), and observed under UV microscope (Nikon, Tokyo, Japan).

RESULTS

Viral Polypeptides

SPV Replication in Tissue Culture

Only four published studies have described SPV infection in tissue culture systems (Kasza, Bohl & Jones, 1960; Massung & Moyer, 1991a; Massung & Moyer, 1991b; Massung, Jayarama & Moyer, 1993). According to these studies, SPV is a relatively slow growing virus compared to other poxviruses.

In order to assess the cytopathology of SPV infection, PK-15 cells were infected with SPV. The cytopathic effect (CPE), which denotes changes in the microscopic appearances of cultured cells following virus infection, was examined. Plaque formation was also observed. Plaques, in a confluent PK-15 cell monolayer, were clear circular areas, approximately 2 mm in diameter, and resulted from cell rounding and lysis of cells 10 days postinfection.

A confluent monolayer of PK-15 cells was infected with SPV at 0.1 pfu/cell followed by adsorption on a rocker table for one hour. These infected cells were incubated at 37°C with 5% CO_2. CPE was evident 7 days post-infection and an important distinctive feature of the CPE was the presence of intracytoplasmic inclusions and vacuoles in the cytoplasm of infected cells. In parallel experiments, CPE was evident 2 days post-infection when monolayers were infected with 10

pfu/cell. Infection with SPV at a multiplicity of 20 pfu/cell also produced a CPE 2 days post-infection.

Synthesis of Swinepox Virus Protein

Most of the studies for SPV protein production have been limited to DNA sequence analyses for prediction on what proteins would be expressed upon SPV infection (Massung & Moyer, 1991a; Massung, Jayarama & Moyer, 1993). Proteins secreted from SPV infected cells have never been analyzed or characterized in the published literature.

Experiment designed to determine early and late SPV proteins were conducted. Monolayers of PK-15 cells in 60 mm dishes were either mock-infected or SPV-infected. Multiplicity of infection was 20 pfu/cell. After the initial infection, monolayers were incubated in maintenance medium for 2 hours followed by washes with serum-free RPMI 1640. The monolayers were then starved in methionine-free medium for one hour prior to labeling with [^{35}S] methionine. In addition to [^{35}S] methionine, Cytosine β-D-Arobinofuranoside (AraC), which blocks DNA synthesis, was also added to monolayers to monitor the synthesis of early viral proteins. Both pellet (total protein) and supernatant samples (secretory proteins) were collected as described in Materials and Methods. Proteins were then separated on 11.5% SDS-PAGE. Samples were analyzed under reducing (with 2-mercaptoethanol) and non-reducing conditions (without 2-mercaptoethanol).

After the gel electrophoresis, autoradiography was performed to visualize proteins. Cytoplasmic extracts from SPV-infected PK-15 cells, 22 hours post-infection, revealed no visible viral proteins (Figure 1, Lanes H and I), as compared to mock-infected cells (Figure 1, Lane G). A major protein (35 kDa) is secreted from infected cells 22 hours post-infection (Figure 1, Lanes B, C, E, and F). The protein appeared in supernatants from SPV-infected cells treated with AraC (Figure 1, Lanes C and F) and the protein continued to appear in supernatants from SPV-infected cells not treated with AraC (Figure 1, Lanes B and E). Therefore, the protein is classified as an early protein. Also the protein did not appear in supernatants from mock-infected cells (Figure 1, Lanes A and D).

Post-Translational Cleavage of Viral Polypeptides

It has been previously shown (Katz & Moss, 1970) that during vaccinia virus maturation at least three precursor polypeptides are proteolytically cleaved into product polypeptides which become structural components of the virus. Almost all poxviruses examined thus far demonstrate this activity (Moss, 1996). Since no data is available on SPV maturation, we were interested in determining whether post-translational cleavage plays any role in SPV maturation. To determine this, a pulse-chase experiment was designed. PK-15 cells were infected with 20 pfu/cell with SPV. At 8hours post-infection, cells were washed and starved in methionine-free medium for one hour and pulse labeled with [^{35}S] methionine (50μCi/ml) for one hour. The cell pellet was removed at the end of the pulse. The other pulse labeled sample was

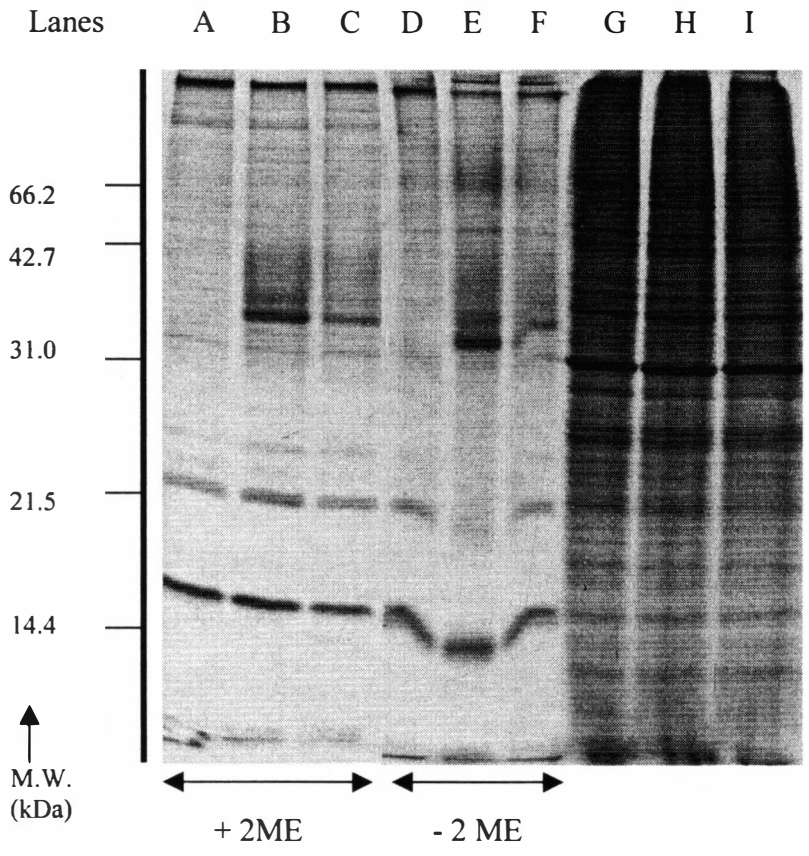

Figure 1. Synthesis of Viral Polypeptides From SPV Infected Cells.

Autoradiogram of 11.5 % Polyacrylamide gel electrophoresis. Samples are supernatant from mock-infected (A,D), supernatant from SPV infected cells 22 hours post-infection (B,E), supernatant from SPV infected cells with a presence of AraC (C,F), cytoplasmic extract from mock-infected cells (G), cytoplasmic extract from SPV infected cells 22 hours post-infection (H), cytoplasmic extract from SPV infected cells with a presence of AraC (I). +2ME indicates a presence of 2- mercaptoethanol and -2ME indicates an absence of 2-mercaptoethanol.

chased for 12 additional hours. Polypeptides present in the total cytoplasm (pellet) were solubilized with SDS and 2-mercaptoethanol and separated by polyacrylamide gel electrophoresis. An equal amount of TCA precipitable protein was loaded into each lane. Radioactive bands were detected by autoradiography.

Result from the autoradiogram (Figure 2), an evidence of post-translational cleavage was found. A polypeptide band (8.6 kDa) appeared in the SPV pulse sample (Figure 2, SPV pulse indicated with →) and appeared as faded polypeptide bands (35 kDa) in the SPV chase sample (Figure 2, SPV chase indicated with ←*). The polypeptide band was not observed in the mock pulse sample (Figure 2, mock pulse). Also, a polypeptide band appeared in the SPV chase sample (Figure 2, indicated with ←*), which was not detected in the mock pulse sample and the SPV pulse sample. These results suggest that the polypeptide was cleaved from precursor polypeptides.

The SPV Secretory Polypeptide Differed From TPV 38 kDa Polypeptide

An earlier report from our laboratory (Essani, Chalasani, Eversole, Beuving & Birmingham, 1994) describes a 38 kDa secretory protein from TPV infected cells. In order to determine if a major secretory protein (35 kDa) found in SPV-infected samples differs from the 38 kDa polypeptide found in TPV, owl monkey kidney cells (OMK) were infected with TPV and PK-15 cells were infected with SPV. Both concentrated supernatant from TPV-infected OMK cells and SPV-infected PK-15 cells were solubilized and proteins were resolved by electrophoresis in a 11.5 % polyacrylamide gel. As shown in Figure 3, the tanapox virus 38 kDa protein was

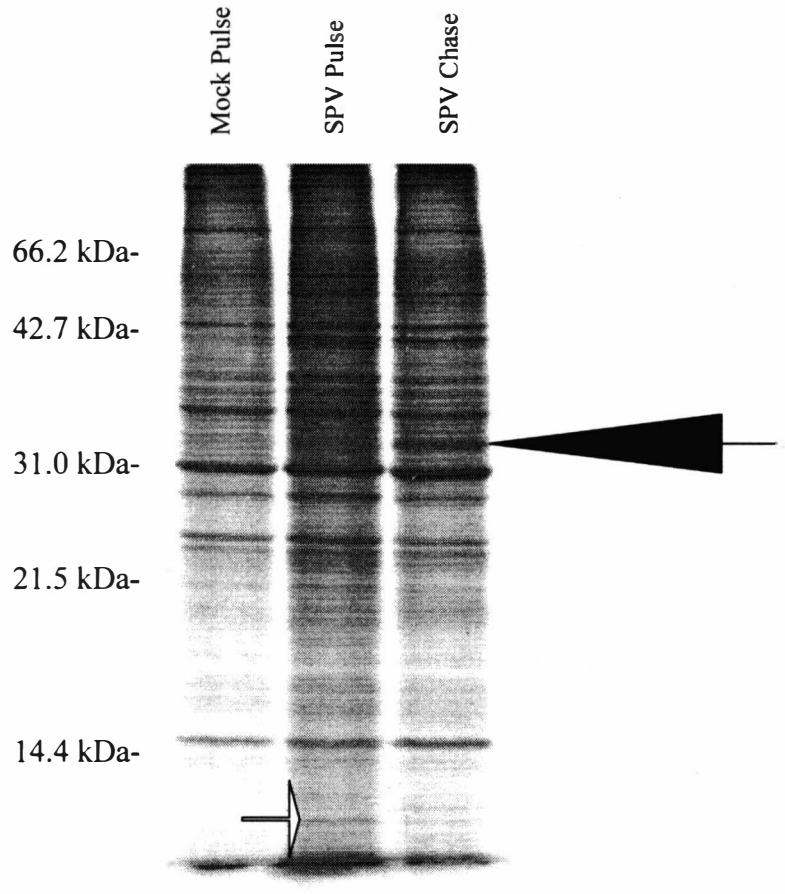

Figure 2. Post-Translational Cleavage of Viral Proteins.

Equal amount of TCA precipitable counts were loaded to each lane on 11.5% Polyacrylamide gel. Radioactive bands were demonstrated by autoradiography. An original gel was exposed for 120 hours. Post-translational cleavage of a polypeptide was detected. A polypeptide indicated with a solid arrow appeared in the SPV pulse sample and appeared as a faded band in the SPV chase sample, suggesting post-translational cleavage. Also an open arrow indicates a polypeptide appeared in the SPV chase sample, which did not appear in the SPV pulse sample.

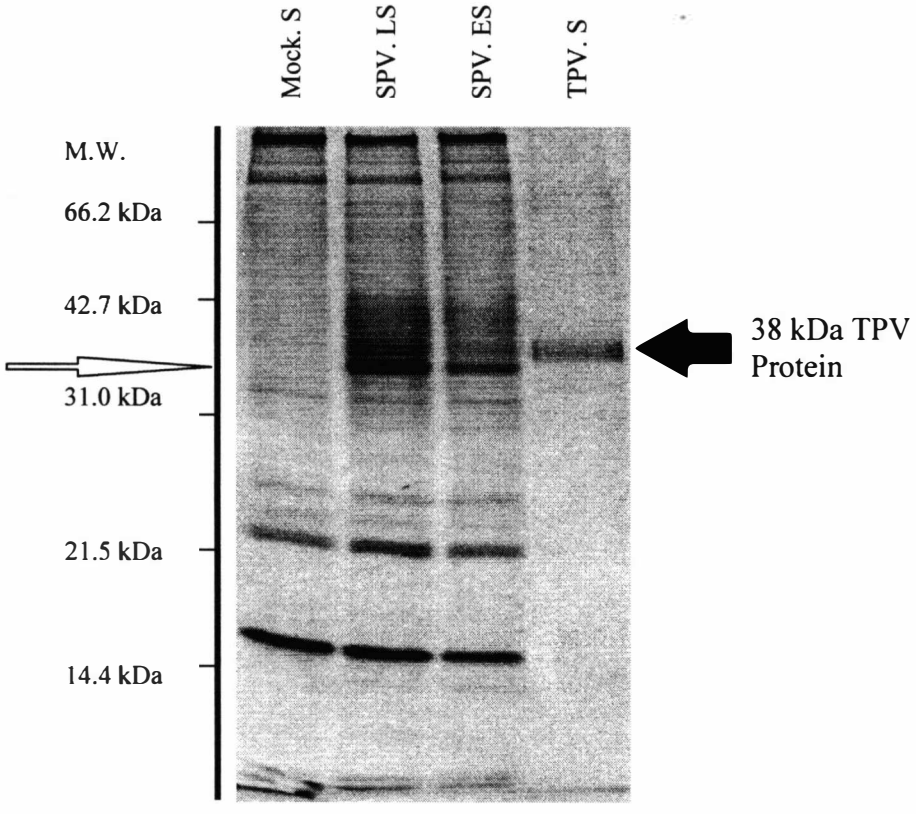

Figure 3. Comparison Between a TPV 38 kDa Secretory Protein and a Major Secretory Protein Found in Supernatant From SPV-Infected PK-15 Cells.

An open arrow (points to bands on SPV LS and SPV ES) indicates a major secretory protein from SPV-infected PK-15 cells (35 kDa). A 38 kDa secretory protein in supernatant from TPV infected OMK cells is indicated on TPV lane with a solid arrow. The SPV secretory protein (35 kDa) clearly differs form the TPV 38 kDa protein. Mock. S indicates supernatant from mock-infected Pk-15 cells. SPV. LS indicates supernatant from SPV-infected cells and the sample was harvested at 22 hours post-infection. SPV. ES indicates that supernatant from SPV-infected PK-15 cells and the sample was treated with AraC. PK-15 cells were infected with SPV and OMK cells were infected with TPV under same conditions. Supernatants were collected and concentrated. Samples were resolved by SDS-PAGE. A autoradiography was performed by exposing a X-OMAT film on dried gel for 115 hours. (M.W. = Molecular Weight.)

different from a major polypeptide (35 kDa) discovered in the supernatant obtained from SPV-infected PK-15 cells. As described below, the unavailability of swine cytokines prevented us from demonstrating any anti-cytokine activity associated with the 35 kDa SPV secretory protein.

Anti-Interferon-γ Activity in the Supernatants From SPV-Infected Cells

Many viruses in the family *Poxviridae* are known to secrete polypeptides which bind to interferon gamma (INF-γ). Myxoma virus (Upton, Mossman & McFadden, 1992) and vaccinia virus (Symons, Alcami & Smith, 1995) are known poxviruses which encode soluble INF-γ receptors. In myxoma virus, the IFN-γ receptor homologue is a major secretory polypeptide from infected cells and binds to IFN-γ and competitively inhibits the binding of INF-γ to original receptor (Upton, Mossman & McFadden, 1992). In order to determine whether SPV encoded soluble receptors of human recombinant interferon gamma (hIFN-γ), virus free supernatants from mock-infected or SPV infected cells were incubated on PK-15 cells with 100 units of hIFN-γ and these cells were subsequently challenged with SPV at 100 pfu/well. If SPV secretory proteins bind to hIFN-γ, antiviral activity of hIFN-γ would be inhibited and 100 plaques should be observed after a subsequent challenge with SPV 100 pfu/cell.

As shown in Table 1, several attempts were made to clarify the ability of putative SPV encoded secretory polypeptides to neutralize hIFN-γ antiviral activity.

Table 1

Results of Interferon Gamma Assay

Well	1	2	3	4	5	6
IFNγ	0 units	100 units	50 units	50 units	0 units	0 units
Protein Samples	Mock 5× Super	None	None	SPV5× Super	SPV 5× Super	None
SPV Infection	100 pfu	100 pfu	100 pfu	100 pfu	0 pfu	0 pfu
Experiment 1	35 pfu	16.5 pfu	17.5 pfu	37.5 pfu	0 pfu	0 pfu
Experiment 2	78 pfu	68.5 pfu	82 pfu	86.5 pfu	0 pfu	0 pfu
Experiment 3	92.5 pfu	77 pfu	79.5 pfu	76 pfu	0 pfu	0 pfu

Confluent monolayers of PK-15 cells in 6 well dishes were treated with mixtures of IFN-γ and protein samples as indicated. Subsequent infection with SPV or mock took place as indicated. Following incubation for 14 days in overlay medium, monolayers were fixed with formaldehyde and stained with 1 % crystal violet. Plaques were counted under a microscope.

The negative results may suggest that anti-IFN-γ activity is species specific. Unavailability of swine IFN-γ prevented additional studies.

Anti-Tumor Necrosis Factor-α Activity in the Supernatants From SPV Infected Cells

Myxoma virus is known to encode T2 protein which is capable of inhibiting rabbit TNF-α (Schreiber & McFadden, 1994). TNF-α is an important component of the immune system which induces mitogenic effects on vascular endothelial cells and produce acute inflammation with neutrophil infiltration (Stites & Terr, 1991). In an

attempt to verify whether SPV encodes soluble TNF-α receptor homologues, a TNF-α assay was carried out using hTNF-α. Because swine TNF-α was not available, anti-TNF-α activity of SPV encoded secretory proteins was estimated by assaying for expression of cell adhesion molecules (CAMs) on human umbilical vein endothelial cells (HUVEC). Human TNF-α (hTNF-α) is known to up-regulate the expression of ICAM-1, VCAM-1, and E-Selectin in HUVEC upon binding of hTNF-α to cellular TNF-α receptors (Williams & Hellewell, 1992). Also, ICAM-2 is known to be expressed constitutively (Williams & Hellewell, 1992). If SPV encodes secretory proteins that bind to hTNF-α as shown in myxoma virus, up-regulation of ICAM-1, VCAM-1, and E-Selectin on HUVEC would be hindered. By measuring expression of CAMs after addition of a mixture of hTNF-α and SPV encoded secretory proteins, it should be possible to establish whether these proteins are capable of inhibiting hTNF-α.

Five fold concentrated supernatants from mock-infected and SPV-infected samples were mixed with fixed units of hTNF-α prior to its application to HUVEC cells. The mixture was added to HUVEC cells followed by incubation and fixation of treated HUVEC. ELISA was performed to measure the expression of CAMs. Mouse anti-CAMs IgG antibody was used as primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG was added as secondary antibody followed by incubation and washing. Chromogenic substrate, p-nitrophenyl phosphate, was added and absorbance at 405 nm (in the microtiter plates) was read using a fluorescence microtiter plate reader. Results were recorded with the aid of peripheral computer

software. The results in this case were also negative, demonstrating that none of the secretory proteins from SPV infected cells can bind to hTNF-α.

Expression of ICAM-1 was not inhibited as the amount of concentrated samples of SPV encoded secretory proteins increased (Figure 4. C). Initially it appeared that SPV secretory proteins reduced ICAM-1 expression in a dose dependant manner. Increased concentration of mock-infected samples also reduced expression of ICAM-1 in a similar manner. Similarly, expression of VCAM-1 was not reduced by the inhibition of hTNF-α by SPV secretory proteins (Figure 4. A). As observed with ICAM-1, it again appeared that increasing doses of SPV encoded secretory protein inhibited TNF-α. This may have resulted reduced expression of VCAM-1 but the reduction may also have been due to the use of mock-infected samples. With only hTNF- α addition, E-selectin was stimulated to show more than 0.3 in 405 nm absorbance (Figure 4. B). When absorbances from zero to100 µl SPV secretory proteins with hTNF-α were compared, no drastic reduction of E-selectin expression was observed. It appeared that addition of 200 µl SPV secretory protein with hTNF-α reduced the expression of E–selectin, but this reduction was also evident with mock-infected samples. Therefore, it is not clear if SPV encoded secretory proteins were capable of inhibiting hTNF-α.

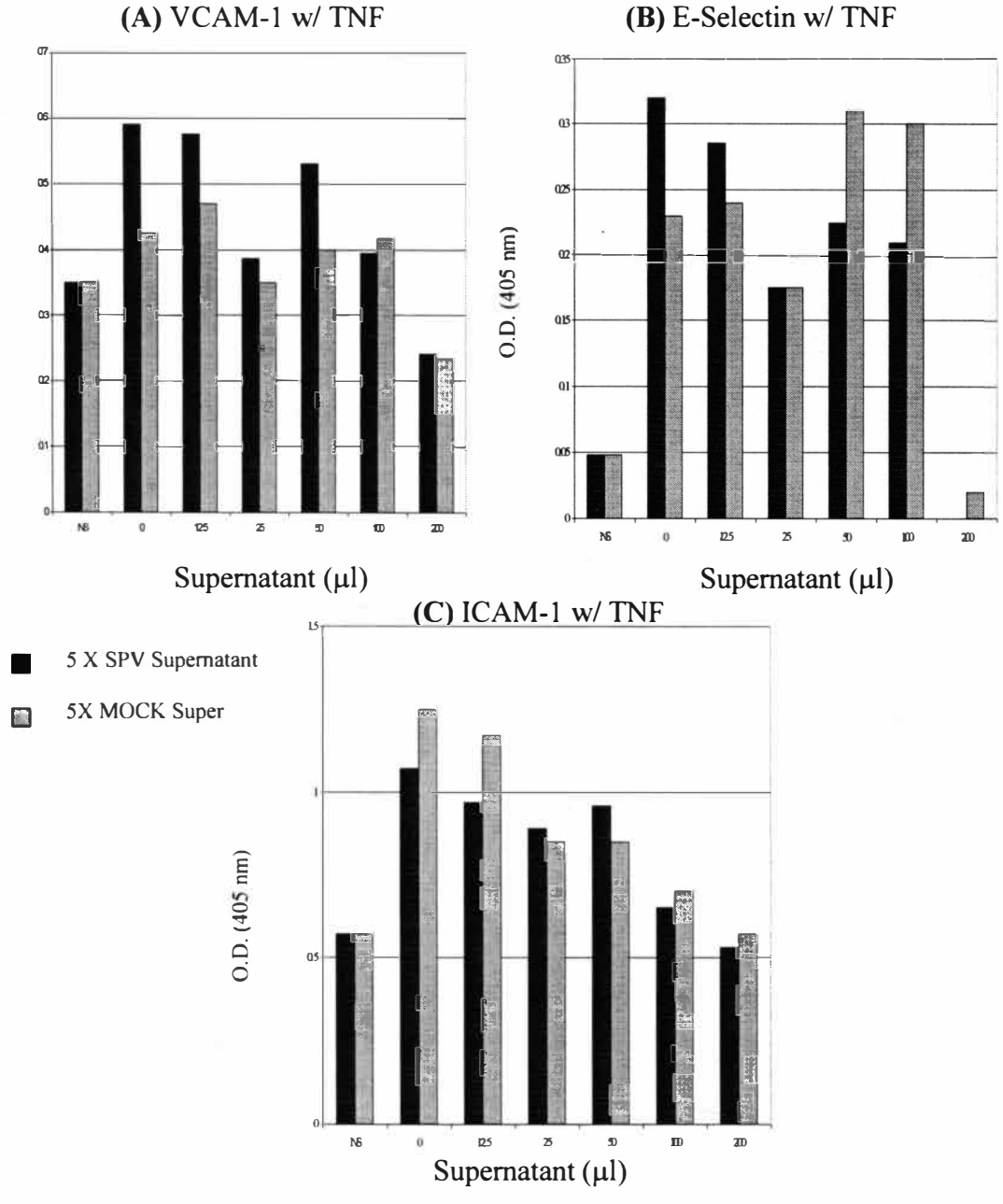

Figure 4. Inhibition of Expression of CAMs With SPV Encoded Secretory Proteins (TNF-α Stimulated).

(A) VCAM-1 (B) E-Selectin (C) ICAM-1 expressions. HUVEC cells were stimulated with TNF–α with concentrated supernatant from SPV – infected cells or mock-infected cells.

Anti-Interleukin-1β Activity in the Supernatants From SPV Infected Cells

A soluble receptor for IL1-β is encoded by vaccinia virus (Alcami & Smith, 1992) and cowpox virus (Spriggs, Hruby, Maliszewski, Pickup, Sims, Buller, and Vanslyke, 1992). IL-1β plays an important role in the production of antibodies by B-cells (Stites & Terr, 1991). IL-1β is also known to up-regulate expression of VCAM-1, ICAM-1, and E-Selectin. Therefore, the same methods used for TNF-α assays were used to determine whether SPV encodes a similar soluble receptor for IL1-β. It was not possible to verify the existence of a SPV encoded soluble inhibitor of human recombinant IL-1β.

Five fold concentrated supernatant from mock-infected and SPV-infected samples were mixed with fixed units of IL-1β. The mixture was added to HUVEC cells and expression of CAMs were assayed. Although several attempts were made to stimulate HUVEC cells with Interleukin 1β (IL-1β), it was not successful to up-regulate expression of all CAMs to satisfactory levels. Only E-Selectin and ICAM-1 showed satisfactory up-regulation with the addition of IL-1β.

It was clearly shown that 0 to 100 μl addition of SPV secretory protein with IL-1β did not down-regulate the expression of ICAM-1 (Figure 5. A). When only SPV secretory protein was added to HUVEC cells, the expression of ICAM-1 returned to its base expression levels. This phenomenon was also observed with concentrated supernatants from mock-infected cells. It was also shown that 0 to 100

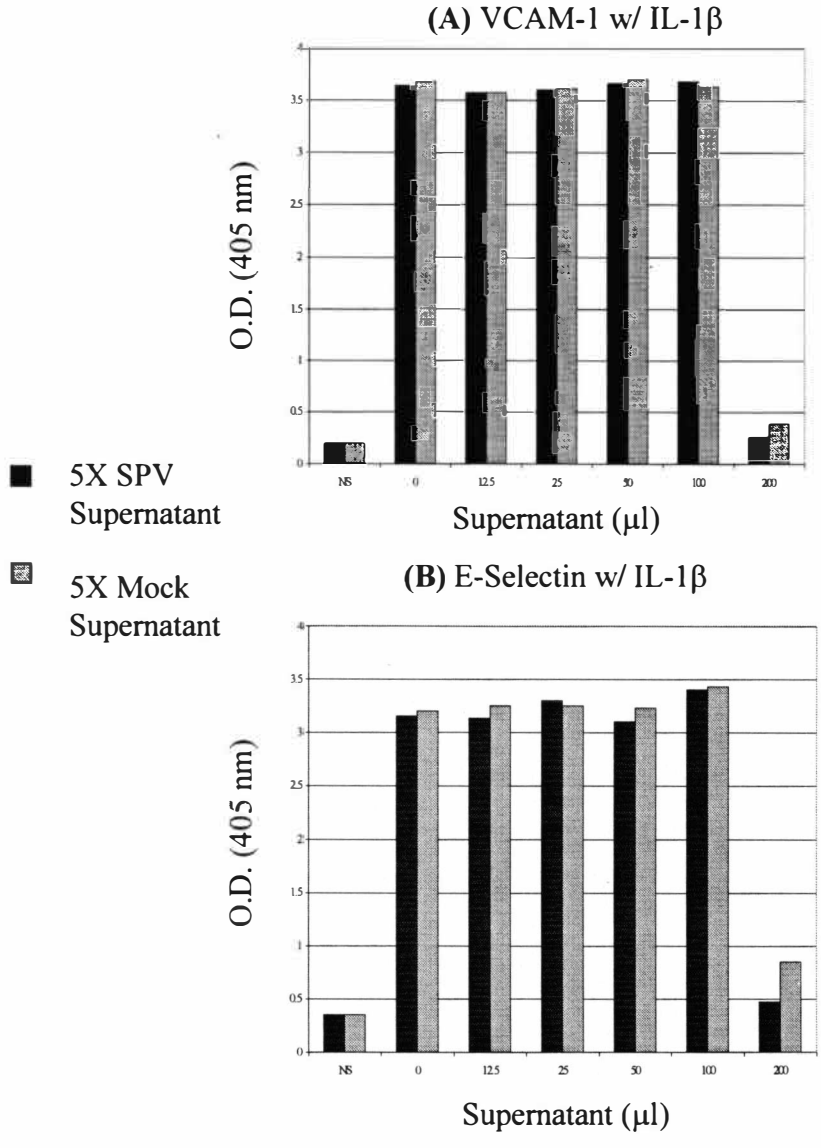

Figure 5. Inhibition of Expression of CAMs With SPV Encoded Secretory Proteins (IL-1β stimulated).

(A) Expression of VCAM-1 (B) Expression of E-Selectin. HUVEC cells were stimulated with IL-1β with concentrated supernatant from SPV-infected cells or mock-infected cells. NS = no stimulation (no IL-1β stimulation). 0 = No supernatant, IL-1β only. 200 = Supernatant only, no IL-1β.

μl addition of SPV secretory protein with IL-1β did not down-regulate the expression of E-Selectin with dose dependant manner (Figure 5. B).

Anti-Restenosis Activity of Viral Polypeptides

Poxvirus Secretory Polypeptides as an Anti-Restenosis Agent

Percutaneous transluminal coronary angioplasty (PTCA) is a common treatment for coronary artery diseases caused by artherosclerotic plaque deposition. After the vascular injury caused by PTCA, inflammatory responses are known to be major contributors for restenosis, which result in neointimal formation due primarily to proliferation and migration of smooth muscle cells. The inflammatory responses involve many inflammatory enzymes and thrombotic enzyme cascades, which have serine proteinase activity and are regulated by serine proteinase inhibitors. The serine proteinase inhibitor, SERP-1, was verified as a potent inhibitor of restenosis (Altieri, 1995). Lucas *et al.* (1996) obtained the purified serine proteinase inhibitor (SERP-1), which was a myxoma virus encoded secreted glycoprotein, to observe effects on neointimal formation and plaque growth following balloon injury in rabbits. Plaque development was reduced by inhibition of serine proteinase activity and was characterized by a focal decrease in macrophage infiltration immediately after the balloon angioplasty (Lucas, Liu, Macen, Nash, Dai, Stewart, Graham, Etches, Boshkov, Nation, Bumen, Hobman & McFadden, 1996).

In order to determine if SPV encodes secretory proteins which exhibit similar effects on restenosis, pigs were infected either with SPV or vehicle a week before PTCA. Rather than local delivery of a specific SPV secretory protein to the site of PTCA, systemic SPV infection was chosen, since purified SPV encoded secretory proteins were not available.

Numbers of Pigs Included in This Study

Within a group of 22 pigs, 19 pigs survived the entire two weeks recovery after PTCA balloon over-inflation. Two of the SPV infected pigs died from lethal arrhythmia due to acute balloon failure. One of control pig died from respiratory failure at the time of post PTCA recovery. From the 19 surviving pigs, a total of 2 pigs (1 control and 1 SPV infected) were excluded from analysis, because they developed abnormal double-lumen morphorogy at the balloon-injured site. Also, one SPV infected pig was excluded from analysis due to an unusually large injury index. In total, data from 8 control and 8 infected pigs were utilized to examine effects of SPV infection on restenosis.

Symptomology in Swine

Since poxviruses cause lesion formation on their hosts, symptoms on pigs were carefully examined as a part of verification of SPV infection. As shown in Figure 6, all pigs infected with live SPV developed a lesion at the site of subcutaneous injection site within one week post-infection. However, by three weeks

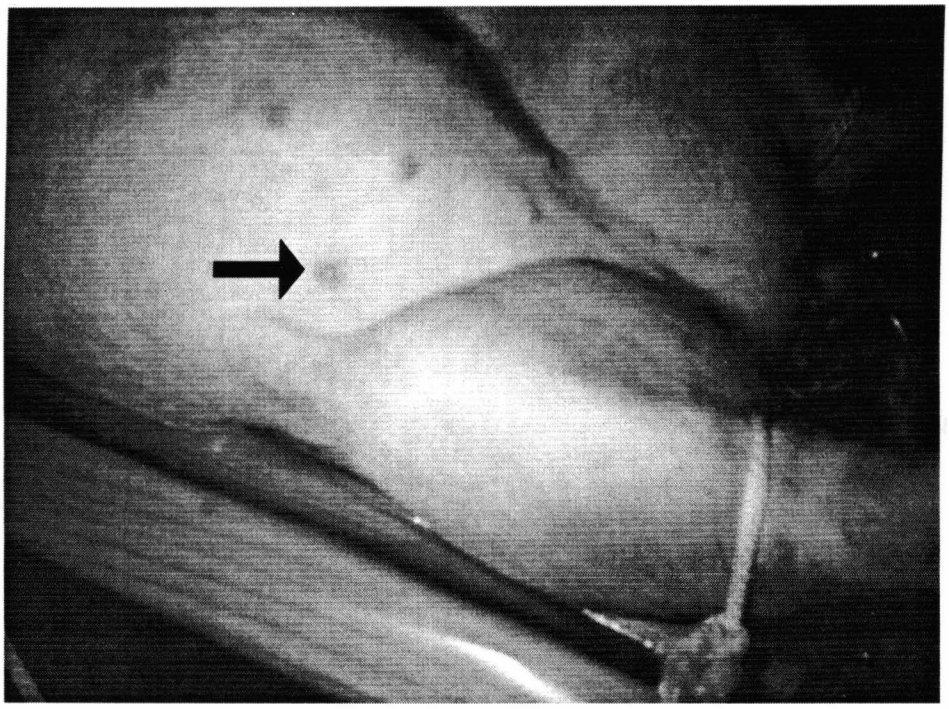

Figure 6. A Photograph Taken on a SPV-Infected Female pig a Week Post-Infection (at the time of PTCA).

An arrow indicates the injection site, which is characterized by papule formation. This papule formation at injection site was the most common symptom for SPV infected pigs. Also note that the papule formation is limited to the injection site. Papule like structures appear above the injection site are nipples.

post-infection, only 2 out of 8 SPV-infected pigs sustained escalated skin lesions throughout the groin area and the axillary regions. On the other hand, none of the mock-infected pigs developed characteristic pox lesions at the injection site. It has to be noted that 2 out of 8 mock-infected pigs showed unusual rashes throughout their inguinal areas.

Morphometric Analysis of Swine LAD Coronary Arteries

In order to assess the effects of SPV infection on restenosis, cross-sections of balloon-injured porcine LAD coronary arteries were morphologically compared between mock-infected group and SPV-infected group. Table 2 shows the magnitude of arterial over-stretch (Over-inflation or OI ratio), the severity of internal elastic lamina (IEL) rupture (=injury index), neointimal and medial areas, total areas, demarcated by the external elastic lamina (EEL), and the extent of neointimal hyperplasia (proliferation index or NI/M) measured in balloon-injured porcine coronaly arterial segments two weeks after PTCA over-inflation. The mean OI ratios were similar between the mock-infected (1.43) and SPV-infected (1.41) groups. Alternatively, the degree of vascular injury induced by this degree of over-stretch was also similar between groups, as mean injury indexes of the mock-infected and SPV-infected pigs were 25.9 ± 2.3 % and 25.7 ± 3.7 %, respectively. The magnitude of neointimal proliferation in terms of the NI/M ratio was 18 % lower in the SPV infected group (0.85 ± 0.09) compared to the NI/M ratio observed in the mock-infected group (1.04 ± 0.16), but this difference was not statistically significant.

Table 2

Morphological Analysis of Balloon-Injured Porcine Coronary Artery Segments Harvested 2 Weeks After Intentional PTCA Over-inflation.

Group	Over Infration Ratio	Injury Index (IEL*/IEL × 100)	Neointimal Area (mm^2)	Medial Area (mm^2)	EEL** Area (mm^2)	Proliferation Index (NI/M)
Control (Mock Infected) Group Number = 8	1.43 ± 0.01	25.9 ± 2.3	0.49 ± 0.07	0.50 ± 0.03	2.82 ± 0.14	1.04 ± 0.16
SPV Infected Group Number = 8	1.41 ± 0.02	25.7 ± 3.7	0.40 ± 0.04	0.48 ± 0.02	2.80 ± 0.12	0.85 ± 0.09

*Internal Elastic Lamina
**External Elastic Lamina

Figure 7 represents the linear regression analysis that was performed to compare the severity of injury to the subsequent degree of neointimal proliferation in the two groups. Although the slope of linear regression line of the mock-infected group (3.65 ± 1.91, $r = 0.52$) was 2.5 fold larger than the slope of linear regression of the SPV-infected group (1.45 ± 1.22, $r = 0.71$), the overall relationship between the extent of injury and consequent proliferation was not statistically significant. ($p=0.35$ according to the general linear analysis).

A typical cross-section from SPV-infected pig LAD coronary artery is shown in Figure 8. Also, a typical cross-section of mock-infected pig LAD coronary artery is shown in Figure 9. When those two figures are compared, the degree of neointimal smooth muscle cell proliferation at rupture sites do not appear to differ from each other.

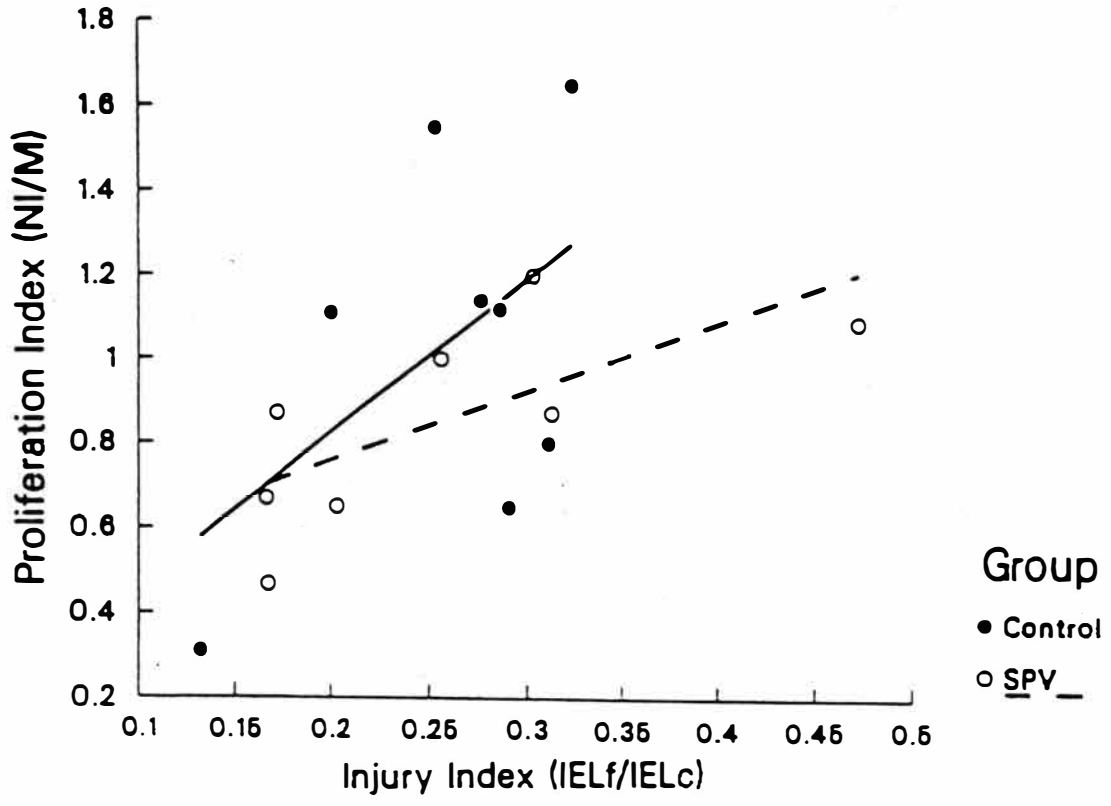

Figure 7. Linear regression analysis illustrating the positive correlation between the severity of vascular injury (Injury index) and the extent of neointimal proliferation (Proliferation Index) in balloon-injured coronary arteries obtained of mock- and SPV- infected domestic feeder pigs. Differences in the slopes of the regression lines were not statistically significant between groups ($p=0.35$ via the general linear models procedure).

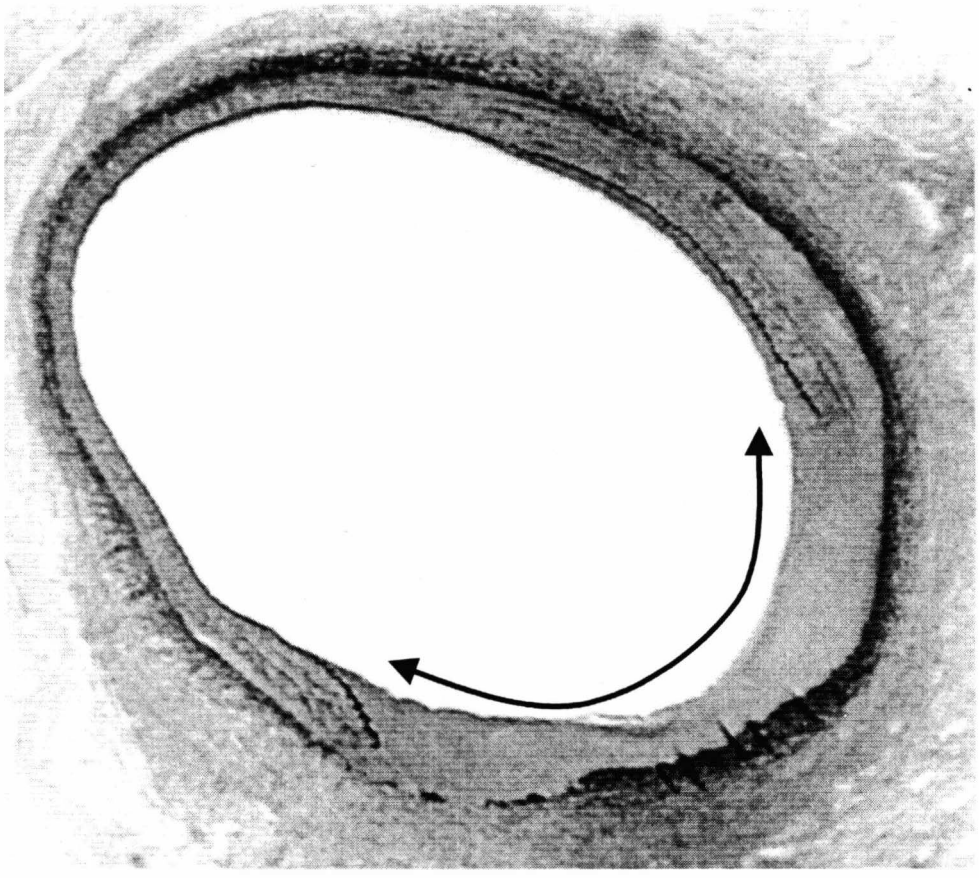

Figure 8. A Typical Cross Section of a LAD Coronary Artery From a Pig in a SPV Infected Group (n=8).

An area indicated with an arrow is a rupture site made by the balloon over-inflation. The area also shows neointimal cell proliferation, which is a main characteristic of the restenosis. Notice that degree of the neointimal cell proliferation does not differ from that of mock-infected pig.

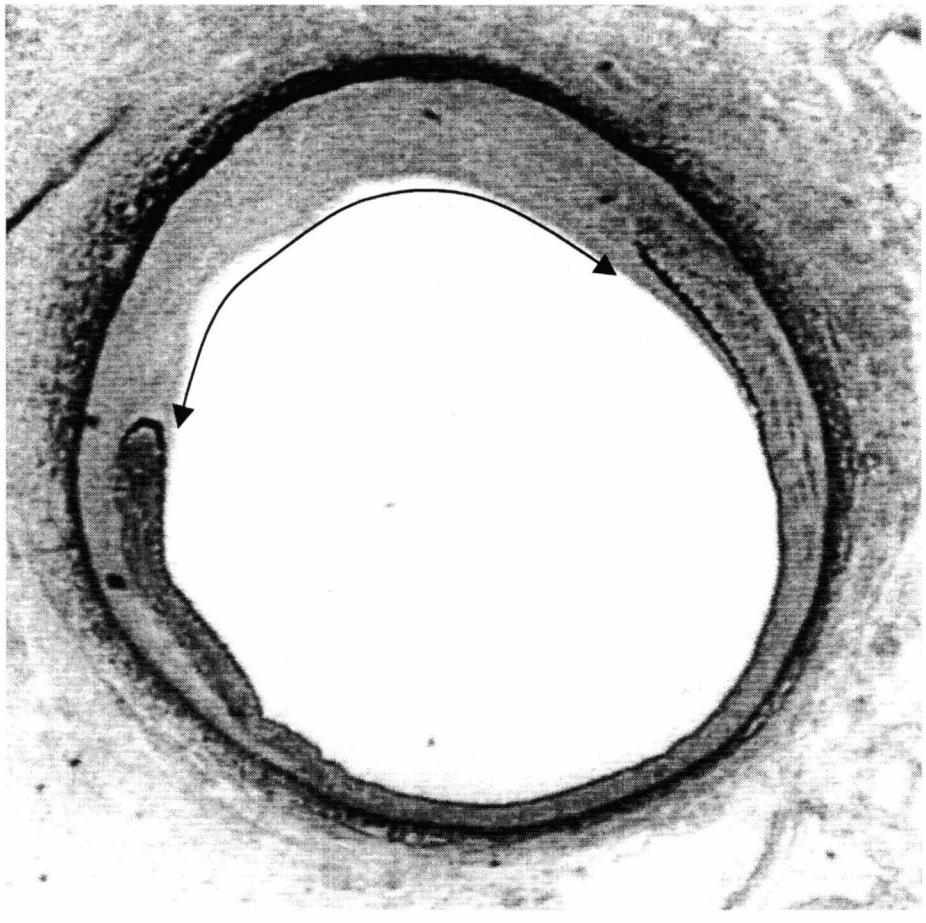

Figure 9. A Typical Cross Section of a LAD Coronary Artery From a Pig in a Mock Infected Group (n=8).

An area indicated with an arrow is a rupture site made by the balloon over-inflation. The area also shows neointimal cell proliferation, which is a main characteristic of the restenosis. Notice that degree of the neointimal cell proliferation does not differ from that of SPV-infected pig.

DISCUSSION

SPV Polypeptides

Swinepox Virus

Although SPV was first appeared in the early 19th century as a common disease in swine population, very little was known about the virus. Besides previous DNA sequence analyses and clinical studies, no additional studies were conducted on SPV encoded secretory proteins. This is the first attempt to demonstrate SPV encoded secretory polypeptides. No other studies have ever clarified biological activities of SPV encoded secretory proteins. The major limitation in this study was that almost no swine cytokines are commercially available.

It is evident that SPV is a relatively slow growing virus as compared to vaccinia virus (Massung & Moyer, 1991a). The cytopathology of SPV infection in PK-15 cell monolayers is characterized by CPE and plaque formation. Intracytoplasmic inclusions and vacuoles in cytoplasm characterize the CPE. Plaques are clear circular areas with a diameter of 2 mm in a confluent PK-15 cell monolayer.

A major 35 kDa SPV encoded secretory protein was found exclusively in supernatants from SPV-infected PK-15 cells (Figure 3. SPV.LS & SPV.ES). This protein was not detected in mock-infected cells or TPV infected cells. The protein is virologically classified as an early polypeptide as it is synthesized in the presence of

cytosine β-D-arobinofuranoside (AraC), an inhibitor of DNA synthesis (Figure 1, Lane C).

An earlier study showed that SPV exhibits highly attenuated phenotype and mild infections in swine population as described above. Analysis on the SPV DNA sequences of the highly variable near-terminal regions suggested that approximately 20,000 base pairs of DNA near the left terminus including host range genes C7L and K1L of vaccinia virus is clearly absent in SPV (Massung, Jayarama & Moyer, 1993). This finding suggests that SPV exhibits limited host range and ineffectiveness to infect mammals other than swine.

Likewise, homologues of genes encoding complement C4b binding protein, secreted protein N1L, and vaccinia virus growth factor found in the left terminal region of vaccinia virus were not found in SPV DNA (Massung, Jayarama & Moyer, 1993). Therefore, it is reasonable to believe that SPV has lost a part of left terminal region responsible for its pathogenesis during a course of evolution.

Anti-Cytokine Activities Secreted From Poxvirus Infected Cells

In order to suppress the multiplication of viruses inside animal body, immune system of vertebrates has developed non-specific immune factors such as complement, interferons and natural killer cells to halt virus replication. Cytotoxic T cells and antibodies further eradicate viruses. Because of selective pressures, certain proteins that are secreted from poxvirus-infected cells enhance the ability of the virus to replicate and deter host immune responses (Barinaga, 1992). Poxviruses are

reported to encode secretory proteins which inhibit cytokines important for onset of inflammatory responses (Moss, 1996).

In order to characterize biological activities of the SPV encoded secretory polypeptides, studies of anti-cytokine activities are important. It has been demonstrated that tanapox virus (TPV) encoded 38 kDa polypeptide, which is an early gene product, binds to hIFN-γ, hIL-5, and hIL2 (Essani, Charasani, Eversole, Beuving & Birmingham, 1994). Also the polypeptide was recently reported to bind to hTNF-α (Paulose & Essani, Unpublished data). Since a major 38 kDa secretory polypeptide found exclusively in supernatant of TPV-infected owl monkey kidney cells exhibited multiple anti-cytokine activities, there was a great possibility that SPV encodes similar polypeptides upon infection of PK-15 cells.

Throughout our study, a major problem was the unavailability of swine cytokines, therefore we used human cytokine products. As described below, SPV secretory polypeptides show no anti- human cytokine activities. When we consider the fact that SPV is capable of causing disease in swine, SPV must encode secretory polypeptides against swine cytokines. The negative results in this study suggest that SPV encoded secretory polypeptides may be species specific. Hence, we were unable to demonstrate anti-cytokine activities using a variety of human cytokines.

Evidence for this notion comes from studies demonstrating an ORF C6L in the left terminus of SPV genome that is known to encode a polypeptide with 274 ammino acids with a molecular weight of 32.3 kDa (Massung, Jayarama & Moyer, 1993). The C6L ORF shows only 25% homology to the hIFN-γ receptor (Massung,

Jayarama & Moyer, 1993). C6L was also reported to have a putative hydrophobic leader sequence and likely to be secreted from infected cells.

Interestingly enough, approximate molecular weight of 35 kDa polypeptide that we found exclusively in SPV-infected cell supernatant, is close to the suggested molecular weight of ORF C6L encoded polypeptide, hence careful comparisons and examinations of both polypeptides are required to truly understand anti-hIFN-γ activities of SPV encoded secretory polypeptides. Considering the species specificity, we expect that the SPV C6L encoded polypeptide will exhibit high homology to swine IFN-γ. It would be interesting to purify the 32.3 kDa polypeptide and carry out experiments to see if the polypeptide is able to bind to either human recombinant IFN-γ or swine IFN-γ.

Similarly no DNA sequence homologues for hTNF-α receptors were found in either the left terminus or the right terminus of the SPV DNA. The question, whether the swine TNF-α receptor is totally different from that of humans, remains an open question. SPV K1R ORF showed only 32.1% homology to cowpox virus 38 kDa protein (Massung, Jayarama & Moyer, 1993), which was shown to inhibit hIL-1β converting enzyme to inhibit IL-1β activity (Ray, Black, Kronheim, Greenstreet, Sleath, Salvesen & Pickup, 1992).

Anti-Atherosclerotic Activity of SPV Encoded Polypeptides

Atherosclerosis Study

It has been thought that initiation of restenosis involves activation of inflammatory and thrombotic cascades, which are commonly regulated by serine proteinase enzymes and inhibitors (Lucas, Liu, Macen, Nash, Dai, Stewart, Graham, Etches, Boshkov, Nation, Bumen, Hobman & McFadden, 1996). Inflammatory cascades also include adhesion of leukocytes after PTCA balloon over-inflation, which is induced by cytokine-leukocyte signaling. Many poxviruses encode proteins which inhibit or bind to host cytokines. Those proteins are known as immunomodulatory proteins. In a case of myxoma virus, the virus secretes a serine proteinase inhibitor (SERP-1) upon infection, which inhibits serine proteinase like plasminogen (Macen, Upton, Nation & McFadden, 1993; Upton, Macen, Wishart & McFadden, 1990). Local introduction of purified immunomodulatory proteins encoded by vaccinia virus has been reported to inhibit neointimal hyperplasia induced by PTCA ballon over-inflation (Lucas, Liu, Macen, Nash, Dai, Stewart, Graham, Etches, Boshkov, Nation, Bumen, Hobman & McFadden, 1996). In our study, based on the fact that all poxviruses secrete some sort of anti-cytokine polypeptides upon infection, SPV was assumed to secrete similar polypeptides. Since SPV encoded polypeptides had never been characterized, systemic SPV infection on domestic feeder pigs was used to see if SPV induced immunosupression could cause an anti-

proliferative effect in a porcine model of neointimal proliferation which mimics restenosis in humans.

Morphological Tests to Reveal no Effects of SPV Infection on Restenosis

In order to measure the degree of neointimal proliferation, at three weeks post-infection with either vehicle or SPV, pig LAD coronary artery were harvested and morphological examination was performed. Subcutaneous administration of SPV to domestic feeder pigs a week prior to PTCA balloon over-inflation did not reduce neointimal hyperplasia compared to mock-infected pigs. When NI/M ratio was measured, the magnitude of neointimal proliferation was 18 % lower in the SPV-infected group compared to the mock-infected group (Table 2). This difference was not considered statistically significant. Also linear regression analysis was conducted to compare the severity of balloon injury to the resultant degree of neointimal proliferation in the two groups (Figure 7). Though the analysis revealed that the regression was larger in mock-infected group compared to SPV-infected group, the difference was not statistically significant.

Serological Examination

Unavailability of a reproducible serological technique to demonstrate anti-SPV antibodies restricted our ability to confirm the experimental SPV infections in pigs. It also restricted our ability to demonstrate that the pigs employed in these experiments were free from SPV.

Unless immunomodulatory polypeptides encoded by SPV are purified and SPV-free pig populations are available, real effects of the polypeptides on restenosis in pig will remain unknown. It has been reported that ORF K1R in the right terminus of SPV DNA shows homology to members of serine proteinase inhibitor protein family (Massung, Jayarama & Moyer, 1993). The K1R homology was highest with vaccinia virus spi-2, which was also found in cowpox virus as a 38 kDa protein (crmA). The crmA protein was a serine proteinase inhibitor and was shown to inhibit the cellular IL-1β converting enzyme (Ray, Black, Kronheim, Greenstreet, Sleath, Salvesen & Pickup, 1992). If serine proteinase inhibitor is an only factor to reduce restenosis as reported in a case of the myxoma virus SERP-1 protein, it would be interesting to purify K1R protein to conduct further restenosis study.

Appendix A

An Approved Animal Use Protocol From the
Corporate Animal Care and Use Committee,
Pharmacia and UpJohn Company

1ST RENEWAL JAN 19 RECT

INTERNAL USE ONLY
Destroy Upon Disposal

RENEWAL OF ANIMAL USE PROTOCOL (AUP) FORM

Corporate Animal Welfare Committee (CAWC) 70-1860-2 2
The Upjohn Company G:\FORMS\AUPRENEW.

1. Please note an AUP form can be renewed a maximum of 2 times before it must be rewritten. NOT TO BE USED FOR CATEGORY V'S.
2. Return the renewal form and a copy of your original authorized AUP form and any amendments to: AS&LS Main Offi 7273-25-7.
3. For assistance with an AUP form call 5-7614. Animal orders will not be processed without an authorization code. New codes are needed every 12 months.
4. If the form is not completed adequately and legibly, it will be returned to the responsible scientist and may cause a delay in approval.
5. Please use the current version of the form. To access: on a blank WordPerfect screen, press Shift F10, type G:\FORMS\AUPRENEW.FO and press enter.

NAME OF SCIENTIST RESPONSIBLE FOR STUDY (PRINT OR TYPE)	PHONE	UNIT	BLDG.	FLR.	AUTHORIZATION CODE*
Ronald J. Shebuski	5-7243	7243	209	3	284-01-5N320
* Assigned by AS & LS. If there is a significant change to the protocol after approval, the scientist should contact the attending veterinarian or AS & LS.		LOG NO.	SIGNATURE OF CAWC REPRESENTATIVE		DATE OF CAWC APPROVAL
		CC16			1-25-95

Previous AUP Authorization Code (10 digits) and Log # (3 digits): AUP# 284-01-4-N320; Log# 547

Category of procedure on authorized AUP: III
Note: Enter II/U(unknown) if most animals are expected to have a Category II experience and an unknown number may have a Category V experience.

Animal Species: Porcine
Stock or Strain Name: Domestic Feeder Pigs and/or Yucatan Minipigs

Approximate number of animals to be used per year:
150

Anticipated location of research or testing procedure: Bldg(s): 209 Flr: 3 Rm(s): 325A,325,324 324.1

For reporting purposes to AAALAC, for company accreditation, please provide a short (four words or less description of the procedure (e.g., cardiac reperfusion, antibody production - Freunds adjuvant, infectious disease):
Angioplasty-induced arterial restenosis

List any changes/additions to anesthetics, analgesics, or other treatments.

None

List any other changes in the protocol.

The procedure described for the "Balloon angioplasty procedure" and the "Chronic intravenous [illegible] procedures" described in 1994's AUP form will remain the same.

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The following procedural addendum may be performed as time permits in 1995:

Addendum: Effect of Swine Pox Virus Infection on Neointimal Hyperplasia in Pigs

Background: Dr. Karim Essani (Western Michigan University) has been investigating the production of anti-inflammatory proteins by pox viruses, some of which were found to inhibit intimal hyperplasia induced by balloon angioplasty of rabbit femoral arteries (G. McFadden et al., 10th International Conference on Poxviruses and Iridoviruses, April 30-May 5, 1994). Under Dr. Essani's guidance, we will investigate the potential anti-restenotic effect of swine pox virus infection in our porcine balloon overinflation model of coronary artery restenosis.

Infection protocol:

1. Swine pox virus (10^8 pox forming units or pfu) will be injected intravenously in chronically cannulated domestic feeder pigs.

2. Seven days later, antibody titers in serum will be measured to confirm swine-pox infection.

3. Once infected, pigs will undergo the standard balloon angioplasty procedure, with resultant vascular injury responses quantitated as previously described.

4. Stringent methods will be used to keep the swine pox infection localize the infected pigs to one recovery room; i.e. use of boots and coveralls that stay in the infected area; use of disinfectant shoe-trays.

Describe the alternatives considered during the past year to the procedures that may cause more than momentary or slight pain or distress to the animals, including methods and sources used to determine that alternatives were not available (e.g., The Animal Welfare Information Center).
As described in the 1994 AUP form, we feel that all necessary procedures that cause more than momentary pain or distress are accompanied by adequate use of anesthesia /analgesia. No alternative methods were uncovered in reviewing current literature on pig models of restenosis (Corporate Technical Library Medlars II Literature Review of Restenosis Articles).

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RENEWAL OF ANIMAL USE PROTOCOL (AUP) FORM

Please indicate below information on post-operative analgesics and restraint, if appropriate. Please check the long form for required information (G:\FORMS\AUP.FO).

1. For our Category III AUP, here is a summary of the sedatives, analgesic or anesthetic of choice:

Drug	Ketamine (Anesthetic)	Midazolam (Induction Agent)	Torbugesic (Analgesic)
Dose (e.g., mg/kg body wt.)	25 mg/kg + 20-30 mg/kg/hr supplemental	0.6 mg/kg + 0.3-0.4 mg/kg/hr supplemental	0.05-0.1 mg/kg
Route and method of administration	intra-muscular, bolus injection	intra-muscular, bolus injection	intra-muscular, bolus injection

2. Unanesthetized animals will not be physically restrained under this AUP.

3. Pain will be monitored during the surgical procedures by monitoring reflex responses in the animals (tail twitch upon needle prick to hindquarters/ limb withdrawal or movement upon hoof squeeze) every 30-40 minutes. Upon recovery such abnormal behavior as lethargy, failure to eat, or extreme agitation will be used as an indication of moderate to severe pain. Animal behavior will thus be monitored daily during the recovery period. Pigs experiencing post-operative pain will be administered Torbugesic as outlined above.

4. Euthanasia is a planned event at the end of our study and will be achieved via i.v. injections of sodium pentobarbital (Socumb, dosage ≥ 50 mg/kg). If coronary balloon angioplasty complications occur that would require major thoracic surgery to repair, the animal will be euthanized rather than introduce more complicated and extensive procedures to the protocol. Additionally, any recovery animals experiencing behavioral signs of pain that prevent it from eating and drinking and which cannot be abated with either analgesic or antibiotic therapy, will also be euthanized promptly.

In signing this renewal you acknowledge that the concerns listed under "Responsible Scientist's and Management's Verification" (see G:\FORMS\AUP.FO) have been appropriately considered.

SIGNATURE OF SCIENTIST RESPONSIBLE FOR STUDY	DATE
	1/19/95
DIRECTOR-LEVEL APPROVAL	DATE
	1/19/95

If another person should be contacted regarding questions on the completion of the AUP or the procedure in general, or should be notified of its approval, please include their name and extension (PRINT OR TYPE).

William R. Humphrey extension 5-7577 or 5-7238

Appendix B

Protocol Clearance From the Institutional Animal Care and Use Committee (IACUC), Western Michigan University

College of Arts and Sciences
Department of Biological Sciences
Laboratory of Virology

Kalamazoo, Michigan 49008-3899
Telephone: 616 387-5636
FAX: 616 387-2849
E-mail: BIOL_RES@gw.wmich.edu

WESTERN MICHIGAN UNIVERSITY

January 27, 1998

TO WHOM IT MAY CONCERN

This is to certify that the animal protocols used in this study were approved by the IACUC in this university (95-08-01 for monoclonal antibodies) and the animal use committee at the Pharmacia and Upjohn Laboratories (AUP # 284-01-5-N320: log # 1995-0016 for swine studies).

Sincerely,

Karim Essani, Ph.D.
Associate Professor

Bruce Bejcek, Ph.D.
Chair IACUC

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