Evaluation of ultrastructural changes associated with encephalomyocarditis virus in the myocardium of experimentally infected piglets

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Objective—To evaluate the ultrastructural changes and localization of encephalomyocarditis virus (EMCV) and viral pathogenesis in the myocardium of experimentally infected piglets.

Animals—Eight 20-day-old piglets.

Procedure—Six piglets were inoculated oronasally with 5 ml (10^6 median tissue culture infective dose/ml) of EMCV suspension, and 2 were used as uninfected controls. Piglets were euthanatized or died between postinoculation days 1 and 3. Samples of heart tissue from all piglets were evaluated histologically, by virus isolation, and by use of immunohistochemistry and electron microscopy.

Results—All infected piglets had gross or microscopically detectable lesions of interstitial myocarditis. Immunohistochemically, EMCV antigen was detected in the cytoplasm of cardiac muscle cells, Purkinje fibers, and endothelial cells of capillaries and intranuclei in cardiac muscle cells. The cell membranes of the Purkinje fibers and endothelial cells had distinct protrusions that contained virus particles. In control piglets, no lesions were found, and no EMCV antigen was detected.

Conclusions—Localization of EMCV intracytoplasmically or intranuclei in various myocardial cells may reflect the sites of viral proliferation. The presence of virus particles in cell membrane protrusions and in vacuoles within the lumen of capillaries indicates that virus is released not only by disintegration of the host cell but also via exocytosis. (Am J Vet Res 2001;62:1653–1657)

Encephalomyocarditis virus (EMCV) is a member of the genus Cardiovirus of the family Picornaviridae and has a worldwide distribution. The EMCV genome consists of a single-stranded RNA enclosed in an icosahedral capsid. The virus particle is roughly spherical, nonenveloped, and 24 to 30 nm in diameter. In swine, EMCV is recognized as a cause of mortality resulting from acute myocarditis in young pigs or reproductive failure in sows. In Europe, the myocardial form of the disease has been reported in Greece, Italy, and Belgium. The reproductive form, in which there is no mortality, has been reported only in Belgium. In the United States, the virus was initially identified as a cause of mortality; it was later proposed that the virus was associated with reproductive failure. Infection with EMCV can be demonstrated by virus isolation in cell culture and by use of reverse transcription-polymerase chain reaction. Another diagnostic method is identification of the virus by immunohistochemical analysis, using the indirect immunoperoxidase technique to analyze myocardial tissue from affected animals.

It has also been reported that EMCV can be detected by use of transmission electron microscopy (TEM). The most important findings included the presence of 1 or more aggregates of virus particles and crystallloid viral structures in the cytoplasm of cardiac muscle cells and endothelial cells.

In the study reported here, lesions of the heart caused by EMCV in experimentally infected piglets and localization of the virus within myocardial cells were evaluated immunohistochemically and by use of TEM.

Materials and Methods

Virus—The EMCV strain 424/90 (isolated from a pig that died unexpectedly) was passaged serially 4 times on baby hamster kidney (BHK-21) cell culture and tested for extraneous viruses. Virus infectivity of the inoculum was 10^6 median tissue culture infective dose (TCID50)/ml.

Animals and experimental design—Eight 20-day-old Large White/PIEtain crossbred piglets were obtained from an EMCV antibody-free pig farm. They were housed in the Laboratory of Microbiology and Infectious Diseases, Aristotle University, Thessaloniki, Greece, and were identified by ear tags. All piglets were fed a commercially available sterile milk substitute, and water was provided ad libitum. The piglets were divided into 2 groups. Six (group A) were inoculated oronasally with 5 ml (10^6 TCID50/ml) EMCV suspension, using 3 ml orally and 2 ml intranasally (1 ml was instilled deeply into each nostril). The 2 other piglets (group B) were similarly inoculated with sterile cell culture medium and used as uninfected controls. Each group was housed in an isolation box on straw bedding at room temperature (between 20 and 24°C). After infection, the piglets were euthanatized using 3 ml orally and 2 ml intranasally (1 ml was instilled deeply into each nostril) of a 1 ml was instilled deeply into each nostril).
Virus isolation—Virus isolation was performed on heart tissue samples from all piglets, as described.8 Briefly, tissue supernatants were incubated on BHK-21 cell monolayers. On samples with evidence of cytopathic effects, a neutralization test was performed, using specific EMCV-antiserum to identify the isolate. Samples with negative results were subjected to 3 blind passages consisting of 3 days each.9

Histologic and immunohistochemical examination—Samples of heart tissue were fixed in neutral-buffered 10% formalin, embedded in paraffin wax, sectioned at 4 to 6 µm, and stained with H&E. Unstained sections from the same samples were used for immunohistochemical examination. Viral antigens were detected by use of an indirect immunoperoxidase technique, using the monoclonal antibody 3E5.10

Transmission electron microscopy—Myocardial samples from the walls of the right and left ventricles were fixed in 4% glutaraldehyde solution in a sodium phosphate buffer (pH 7.3) solution, and were then placed in 2% osmium tetroxide solution with a veronal acetate buffer (pH 7.2). Subsequently, the tissue specimens were dehydrated and embedded in epoxy resin. Thin sections (70–90 nm) of selected areas were cut with an ultramicrotome.12 Sections were then mounted on copper grids, stained with uranyl acetate and lead citrate, and examined by use of an electron microscope at an accelerating voltage of 80 kV.

Results

Macroscopic findings—Gross lesions were observed in the myocardium of infected piglets that died or were euthanatized on PID 2 and 3. The lesions varied in severity, consisted of multiple white-gray linear or spherical areas, and were visible in the wall of the ventricles, especially of the right ventricle, whereas the remaining myocardium was discolored. Macroscopic lesions were not detected in the myocardium of control piglets.

Virus isolation—Encephalomyocarditis virus was isolated from heart tissue samples of inoculated piglets that died or were euthanatized on PID 2 and 3. Virus was not isolated from heart tissue of piglets euthanatized on PID 1 but was isolated from other tissues (spleen). Virus was not isolated in any tissue from control piglets.

Histologic and immunohistochemical findings—Histologically, multifocal or diffuse interstitial myocarditis with foci of myocardial degeneration and necrosis of cardiac muscle cells and Purkinje fibers were detected in all infected piglets. The cellular infiltrate consisted of mononuclear cells, whereas degenerated myocardial cells were observed in foci of myocarditis or in adjacent sites. The intensity of inflammation was greater on PID 3. Immunohistochemically, EMCV antigen was detected in the cytoplasm of cardiac muscle cells, Purkinje fibers, and endothelial cells and in the nucleus of cardiac muscle cells (Fig 1) and Purkinje fibers in all infected piglets. No lesions were found in control piglets, nor was EMCV antigen detected.

Ultrastructural findings—Lesions in the myocardium were similar in all infected piglets. Alterations were detected in cardiac muscle cells, Purkinje fibers, and endothelial cells of capillaries. Virus particles were found intracytoplasmically and intranuclearly in cardiac muscle cells and only intracytoplasmically in Purkinje fibers and endothelial cells. These virus particles were identified as 3 types: granular, crystalline, and vermicular.11 Virus particles were either electron-dense or empty, measuring 23 to 27 nm in diameter with an almost round shape.

Alterations of cardiac muscle cells were characterized by intracellular edema, necrosis, and myofibrillary or glycogenic degeneration. Necrosis was evident from the characteristic shape, size, and chromatin morphologic characteristics of the nuclei. Nuclei had a wavy outline and were smaller in size, compared with normal nuclei, and chromatin was condensed in patches. Karyorrhexis was seldom evident. Another common feature was the presence of chromatin near the inner part of the nuclear envelope (margination). The perinuclear space was often enlarged and electron-lucent. Myofibrillar degeneration referred to the myofilaments; Z-lines had a wavy appearance and were thickened, and disruption of the actin filaments in the Z-line area was frequently detected. Mitochondria were edematous and had disintegration of cristae. Cardiac muscle cells with karyorrhexis had further mitochondrial changes, such as swelling and lysis of the cristae, lysis of the outer membrane, and dilation between the inner and the outer membrane. Myelinic figures and giant and ring-form mitochondria were also observed. Cisternae of smooth endoplasmic reticulum and Golgi apparatus were mildly dilated.

In cardiac muscle cells, intranuclear virus particles were observed only in cells with pyknosis of the nucleus or margination of the chromatin. The virus particles formed aggregates of electron-dense or empty virus particles and were vermicular in appearance (Fig 2 and 3). Granular- or crystalline-type viral particles were...
also evident in the sarcoplasm of cardiac muscle cells. Furthermore, virus aggregations arranged in parallel arrays and located close to the nuclear pole were detected in the sarcoplasm. These aggregations consisted of virus particles connected together in sequence, with thin and light electron-dense filaments (Fig 4). Moreover, membranous vacuoles were evident in proximity to the nuclear envelope, and some virus particles were found inside or around the outer surface of their membrane.

In the organelles of Purkinje fibers and endothelial cells, lesions similar with those found in cardiac muscle cells were detected. Furthermore, margination of the chromatin and myofibrillar degeneration such as that seen in cardiac muscle cells was observed in Purkinje fibers.

In endothelial cells, granular- and crystalline-type virus particles were evident in the cytoplasm. Crystalline-type was the most common and primarily consisted of empty particles. Granular-type particles were rarely detected and consisted of electron-dense and empty virus particles. These particles were found free in the cytoplasm or were surrounded by membrane. Oftentimes the cell membrane protruded into the lumen of capillaries and contained free virus particles. Virus particles in membrane vacuoles were often identified in capillary lumens as well (Fig 3A and B).
In Purkinje fibers, virus particles were of the granular type and were evident in membranous vesicles located in the inner area and periphery of the sarcoplasm. Virus particles were also found free just under the sarcolemma and in sarcoplasmic processes, which were prone to bud (Fig 5C). Viral particles consisted of electron-dense and empty particles.

Discussion

Results of the present study confirmed the ultrastructural findings previously reported by Tsangaris et al., who detected viral particles in the cytoplasm of endothelial cells and in the sarcoplasm of cardiac muscle cells of naturally infected piglets, and by Marcato et al., who detected viral particles in the cytoplasm of endothelial cells of experimentally infected piglets.

A remarkable finding was the detection of virus particles in the nucleus of cardiac muscle cells. Particles were electron-dense or empty and were observed in the nucleus after pyknosis of the nucleus or chromatin margination had occurred. Particles were not detected intranuclearily and intracytoplasmically in the same cell.

These findings are not in agreement with the established view that multiplication of picornaviruses occurs entirely in the cytoplasm. Viral proteins are synthesized in polyribosomes and cleaved to 15S polyribosomes that are assembled to form 80S pentamers. Infected cells often contain empty 80S protein shells. Viral RNA is synthesized on smooth endoplasmic reticulum. Provirions are assembled by packaging RNA within capsid pentamers to form infectious 150 to 160S particles. Completed virus particles, which often are forming crystals in infected cells, are ultimately released by disintegration of the host cell. However, intranuclear crystalloids, which consist of immature virus particles, have been observed in cell cultures infected with poliovirus or echovirus and incubated in a suboptimal temperature. Anzai and Ozaki hypothesized that this may be attributable to migration across the damaged nuclear membrane, but Kawanishi presumed that the presence of intranuclear crystalloids of echovirus may have some relevance to the composition of the virus constituents, and that may be a feature common to the picornaviruses. In addition, poliovirus proteins VP0, VP1, and VP2 were detected in higher amounts in the nucleus than in the cytoplasm by use of electron microscopy autoradiography, whereas VP3 was extruded from the nucleus.

Our observation of virus particles in the nucleus by electron microscopy is confirmed by immunohistochemistry, concerning the intranuclear detection of EMCV antigen by use of a monoclonal antibody (3E5) that strongly reacts with VP1 and in a lesser degree also with VP2 and VP3 in this study as well as in a previous study of ours.

To the authors’ knowledge, the peculiar type of viral aggregations in the sarcoplasm of cardiac muscle cells has not been described in association with EMCV. In the aggregations we observed, the particles were connected together with thin small electron-dense fibrils. This finding does concur with the observations of Godman and Minor, who infected cell cultures with echovirus and poliovirus, respectively.

The cell membrane of the Purkinje fibers and endothelial cells protruded into the interstitial connective tissue and into the lumen of capillaries. These processes contained electron-dense and empty virus particles. Additionally, virus particles in membrane vacuoles were often identified in the capillary lumen. These observations indicate that the virus is released not only by disintegration of the host cell (as is described for picornaviruses) but also via exocytosis.

The main alterations associated with EMCV included edema, degeneration, and necrosis of cardiac muscle cells and Purkinje fibers. Glycogenic and myofibrillar degeneration of cardiac muscle cells has been reported by other investigators. However, to our knowledge, glycogenic degeneration of Purkinje fibers has not been reported. The necrotic lesions of cardiac muscle cells and Purkinje fibers were characterized by pyknosis, karyorrhexis, and chromatin margination, along with the simultaneous presence of virus particles in these cells. These alterations could result from lesions in the endothelial cells of capillaries or from direct virus action.

Another important finding was the presence of viral particles within Purkinje fibers. This finding, combined with damage of these fibers and of cardiac muscle cells, may offer an explanation for the sudden death that occurs in piglets infected with EMCV.

References


