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Sequential Ultrastructural Changes of WISH Cells Infected with Encephalomyocarditis Virus

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The objective of the present study is to evaluate the sequential ultrastructural changes due to encephalomyocarditis virus (EMCV) infection in human amnion derived WISH cells. The effects of recombinant human interferon-alpha (IFN-α) on viral multiplication and cell morphology were assessed in WISH cells. Supernatants and pellets obtained from EMCV- infected WISH cells, pre-treated or not with IFN-α, for 18, 24 and 36 h were used. Electron microscopy was employed to evaluate the fine cellular changes. EMCV infection within 24 h led to partial damage of the cell population with signs of organellar damage. Cellular alterations were clearly evident at late-phase of infection. Presence of the viral particles was much less evident in infected Wish cells pre-treated with IFN-α which did not pose any damage to WISH cells. Intracytoplasmic localization of EMCV in WISH cells may well reflect the sites of viral replication. The presence of virus particles in cell membrane protrusions and in vacuoles indicates that virus is possibly released not only by disintegration of the infected cells but also via exocytosis. It is also postulated that EMCV is released from infected WISH cells by budding in a low steady phase manner. It is concluded that IFN-α has an inhibitory effect on the EMCV replication activity.

Key words: EMCV, WISH cell lines, ultrastructure

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INTRODUCTION

Encephlomyocarditis virus (EMCV) belongs to the genus Cardiovirus of the family *Picornaviridae* (Matthews, 1979). EMCV has been recognized as a pathogen of pigs for many years (Murnane *et al*., 1960). Clinical signs of the induced disease vary considerably, while the virus causes sudden death of the infected piglets due to acute myocarditis (Gainer, 1967; Koenen and Vanderhallen, 1997), fetal death or abortion in pregnant sows have been reported (Brewer *et al*., 2004; Christianson *et al*., 1990; Nakayama *et al*., 2004; Papaioannou *et al*., 2003; Psychas *et al*., 2001; Su *et al*., 1998). Pig-to-pig transmission and transplacental transmission of EMCV contribute in dissemination of the infection among pigs (Foni *et al*., 1993). Small rodents are susceptible for EMCV infection (Su *et al*., 2003) and frequently have been considered reservoir hosts or carriers of EMCV, spreading the disease to a wide variety of animal species (Nowotny, 1996; Spyrou *et al*., 2004).

The susceptibility of different cell lines such as WISH, HEp-2, Vero, A549 and IT-26 VA4 for EMCV and the response to the inhibitory activity of interferon-alpha (IFN-α) on the viral cytopathic effects have been studied (Antalis *et al*., 1998). WISH cell line has been chosen for the present study since EMCV is able to replicate in WISH cells microcultures with the production of discrete plaques (Neumark *et al*., 1990). Morphological studies of the WISH cell lines have revealed the features of an epitheloid cell type with some characteristics of both the original human epithelium and the transformed state. Earlier work on viral infection of WISH cells line has shown inhibition of cell growth and a dose-and time-dependent decrease of cell viability (Detrick *et al*., 1996).

The mechanisms of interactions between virus and host cells that elicit cell-based antiviral responses and the resulting persistent infections are poorly understood. The intention of the present study is to describe the sequential ultrastructural changes in EMCV infected-WISH cells pre-treated or not with IFN-α. The hypothesis that pre-treatment of the EMCV-infected WISH cells with IFN-α could ameliorate the resultant ultrastructural changes are tested. The obtained data may contribute to reveal some aspects of the virus-cell interactions.

MATERIALS AND METHODS

IFN preparation: Human rINF-α2a (Roferon; Hoffman-La Roche, Basel, Switzerland) had a specific activity of 2×10^8 IU mg^{-1}, following manufacturer's instructions. IFN solution was calibrated with NIHGxa 01-901-535 IFN reference strain preparation; the final concentration was 10^9 IU mL^{-1}.

Virus and cell line: The Encephalomyocarditis virus (EMCV), ATCC VR 995, was propagated in human epithelial amnion WISH (He La marker) cell lines. Cells were grown in Minimal Essential Medium (MEM) (GIBCO BRL, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Ultra-low endotoxin) and 1% (v/v) antibiotics (GIBCO BRL, Grand Island, NY). WISH cells were pre-treated or not with IFN-α (1000 IU mL^{-1}) (WISH/IFN-α^+ or IFN-α^-) for 20 h and then infected with EMCV virus at multiplicity of infection (MOI) 0.1 for 18, 24, 36 h, respectively. Cells mock infected with virus and not pre-treated with IFN-α were used and harvested at similar time interval (WISH/IFN-α^-/EMCV$^-$).

Electron microscopy: For electron microscopic study, cell lysates were obtained by trypsinization and pellets or supernatants, containing both intracellular and extracellular viral particles, were collected after centrifugation at 300 x g at 4°C. The resultant pellets and supernatants were mixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C, then post-fixed with 1% osmium tetroxide (OsO_4) in the same buffer. Subsequently, dehydration was done using ascending series of ethanol and specimens were cleared in propylene oxide and finally embedded in Epon: Aroldite mixture. Ultra-thin sections (70-80 nm) were prepared using a diamond knife on an ultramicrotome (Ultracut UCT, Austria) and double contrasted with uranyl acetate and lead citrate. The sections were observed with a transmission electron microscope (TEM) (JEOL 100 CX).

RESULTS

The uninfected cells manifested regular well-defined outline (mostly rounded) and possessed variable number of microvilli, of normal size and length, evenly distributed on the cell surface. The cells had abundant cytoplasmic mass containing the standard mammalian cell set of organelles including mitochondria and rough and smooth endoplasmic reticulum (SER and RER) (Fig. 1 and 2). The mitochondria were spherical or oval with distinct linear cristae and the abundant RER appeared as parallel stalks having narrow cisternae and attached surface dense ribosomes (Polyribosomes). There were numerous free ribosomes (monosomes) and scattered vesicles and vacuoles of pinocytotic nature. Nuclei were mostly

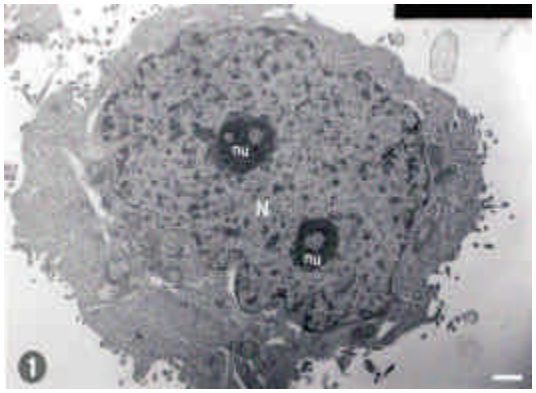

Fig. 1: Transmission electron micrograph showing non-infected WISH cell with regular outline and possesses normal surface microvilli which are evenly distributed on the cell surface. Note the high nuclear cytoplasmic ratio (large nucleus, N), the set of cytoplasmic organelles and the prominent nucleoli (nu). Note the regular distribution of nuclear chromatin. Scale bar = 1 μm

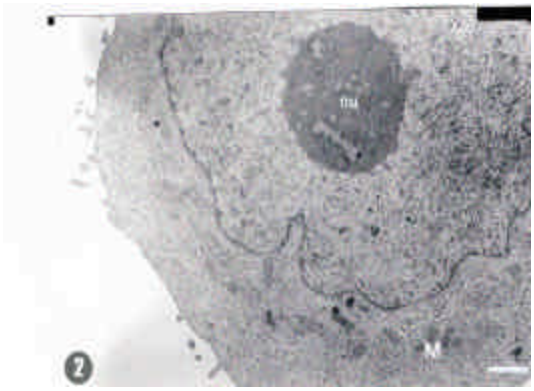

Fig. 2: Non-infected WISH cell showing the standard mammalian set of organelles including mitochondria (M) and RER. There are also numerous free ribosomes. Nuclear chromatin is not clumped and nucleolus (nu) has distinct pars fibrosa and graulosa. Scale bar = 1 μm

rounded and occupied large proportion of cytoplasm (high nuclear : cytoplasmic ratio). Nuclear chromatin was abundant but of normal density and distribution and no chromatin clumps were noticed along the inner nuclear membrane, i.e., scarce peripheral heterochromatin. One or two prominent nucleoli with distinct pars fibrosa and granulosa were recognized.

The infected WISH cells were incubated with EMCV for 18 h revealed marked budding activity, there were

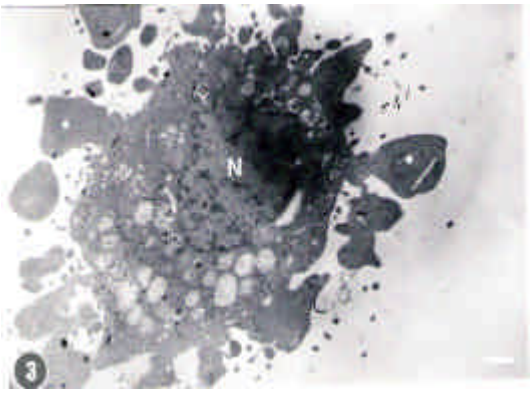

Fig. 3: EMCV-infected WISH cell (18 h) revealing marked membrane blebbing (*). There is large number of membrane-bound structures adjacent to cell surface. Nucleus (N) is obviously irregular. Scale bar = 1 μm

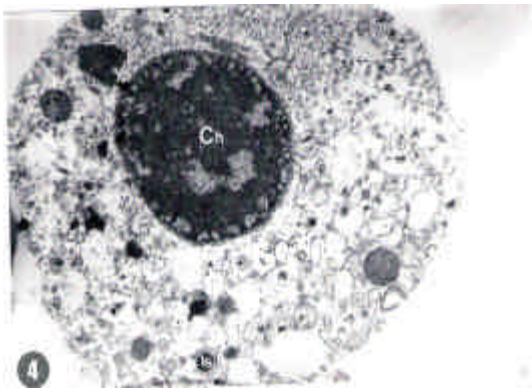

Fig. 4: Infected WISH cell (24 h) showing marked condensation of the nuclear chromatin (Ch). Heterochromatin is abundant. There are lysosomal structures (ls) and autophagic vacuoles (V) in the infected cell. Scale bar = 0.5 μm

numerous irregular membrane-bound structures adjacent to cell surfaces (Fig. 3). The membrane-bound structures contained fine granular material.

Supernatants obtained from WISH cells infected with EMCV for 24 h manifested deteriorated cytoplasmic organelles associated with increased number of primary and secondary lysosomes. However, the cell outlines were still preserved. The outstanding ultrastructural feature was the nuclear shrinkage accompanied with marked condensation of the nuclear chromatin (Fig. 4). In the shrunken nuclei, heterochromatin was abundant and euchromatin was of much less contribution. Some cells showed obvious blebbing of their cytoplasmic

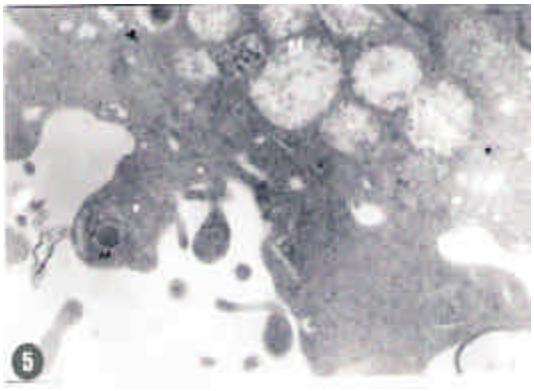

Fig. 5: Infected WISH cell (24 h) showing obvious blebbing of the cell membrane (*). Note the deteriorated mitochondria (M) and the vesicles containing immature viral particles (V). Scale bar = 0.3 μm

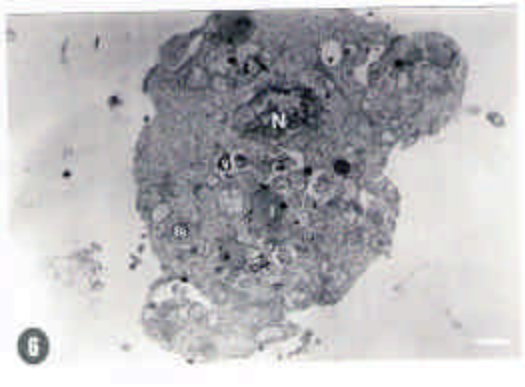

Fig. 6: Infected WISH cell (24 h) showing a nuclear fragment, arised from a cleaved nucleus (N), which has condensed chromatin. There are large cytoplasmic lipid droplets (l), lysosomal structures (ls) and autophagic vacuoles (V). Scale bar = 1 μm

membranes, the membrane blebs were of varying size and shape (Fig. 5). Mitochondria of these cells were severely affected, as revealed by their swelling and destruction of their cristae. Considerable number of cells showed fragmented nuclei, the nuclear fragments had condensed chromatin (Fig. 6). Large lipid droplets, lysosomal structures and autophagic vacuoles in association with deteriorated organelles were seen in those cells.

Supernatants obtained from EMCV-infected WISH cells (24 h) and pre-treated with IFN-α revealed different ultrastructural characteristics. The infected cells of that infected lane showed much less deterioration of cytoplasmic organelles with increased number of free monoribosomes and cytoplasmic vacuoles (Fig. 7). Some

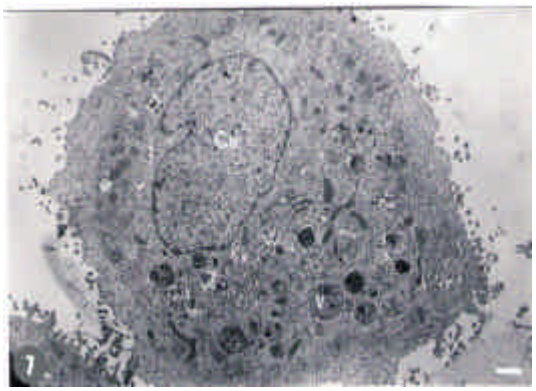

Fig. 7: EMCV-infected (24 h) WISH cell pretreated with IFN-α showing less deterioration of the cytoplasmic organelles with increased number of the autophagic vacuoles (V). Nuclear chromatin (Ch) is not condensed but the nuclear shape is irregular. Scale bar = 1 μm

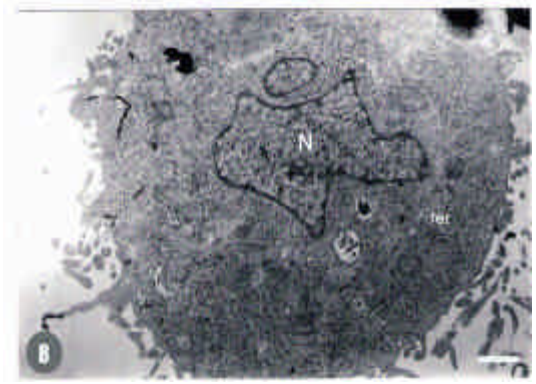

Fig. 8: Infected (24 h) pretreated WISH cell showing irregular shaped nucleus (N) which is separated into two parts probably due to deep indentation of the nuclear envelope. Rough endoplasmic reticulum (rer) is proliferated and there is increased number of monoribosomes. Scale bar = 1 μm

of the pre-treated infected cells had increased number of secondary lysosomes and autophagic vacuoles. RER was moderately proliferated. Nuclear chromatin was not condensed, but the nuclear shape was still irregular probably due to indentation of the nuclear envelope, which was deep enough to separate parts of the altered nuclei (Fig. 8). Less number of these infected cells possessed lobulated nuclei, however even in these nuclei the nuclear chromatin was not condensed (Fig. 9). Other pre-treated infected cells revealed irregular nuclei having nucleoli of normal appearance. In general, there was

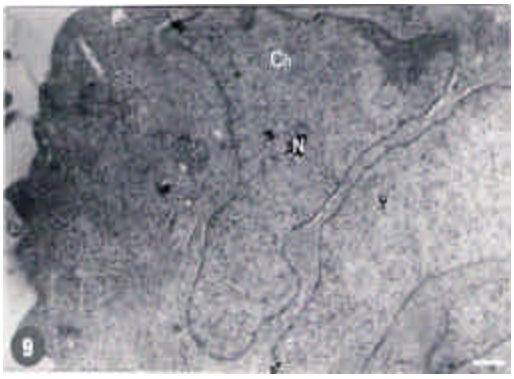

Fig. 9: Infected (24 h) pretreated WISH cell showing cleaved nucleus (N). The nuclear chromatin (Ch) is not condensed. Scale bar = 0.4 μm

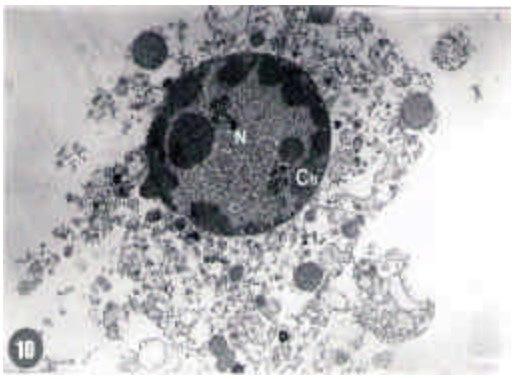

Fig. 10: WISH cell incubated with EMCV for 36 h and pretreated with IFN-α, revealing ruptured cell membrane and deteriorated organelles. Nucleus (N) is shrunken and has condensed chromatin clumps (Ch) along the inner nuclear membrane. Scale bar = 1 μm

relatively less number of cytoplasmic vesicles containing viral particles and less accentuation of the cell membrane blebbing in the infected cells pre-treated with INF-α.

Regarding the supernatant of WISH cell line infected for 36 h with EMCV and pre-treated with IFN-α, it contained cells with indistinct cell membrane which was partially ruptured. Most of the cytoplasmic organelles were either deteriorated and or degenerated (Fig. 10). The remaining mitochondria were swollen and had destructed cristae and RER cisternae were dilated and contained fine granular material. Nuclei were shrunken and had condensed chromatin clumps along the inner nuclear membrane; the central nuclear portion was still occupied by euchromatin. Few of these infected cells possessed distinct cell membrane which revealed obvious blebs. However, these cells also contained deteriorated

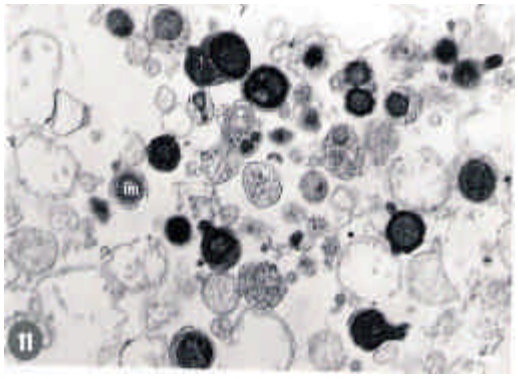

Fig. 11: Deteriorated and degenerated organelles released from EMCV-infected (36 h) WISH cell. There are lamellate membrane-bound dense bodies (l m), autophagic vacuoles (*) and vesicular structures (V) containing immature viral particles. Scale bar = 0.4 μm

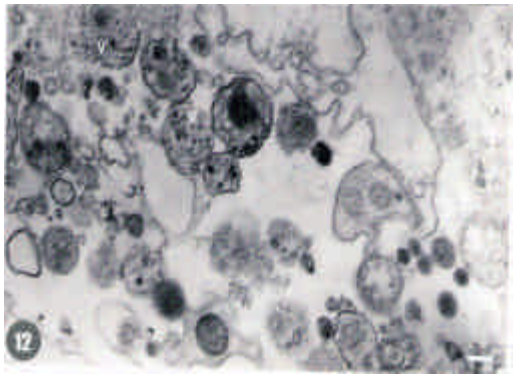

Fig. 12: Vesicular structures (V) containing a large number of immature viral particles. These structures were released from EMCV-infected (36 h) WISH cell. Scale bar = 0.2 μm

organelles, especially mitochondria, numerous autophagic vacuoles and lysosomal structures. Although nuclear chromatin was not condensed in such cells, but the nuclear membrane was markedly irregular. Pellets prepared from WISH cells incubated with EMCV for 24 h and pre-treated with IFN-α manifested ultrastructural features similar to that observed in supernatant of the infected cell line (36 h) and pre-treated with IFN-α. On the other hand, pellets prepared from WISH cell lines incubated for 36 h with EMCV contained numerous totally destructed cells with the only remaining poorly identified organelles. Large number of released lamellate membrane-bound dense bodies and autophagic vacuoles were present (Fig. 11 and 12). Vesicular structures containing immature viral particles were also recognized.

Table 1: Comparison of the ultrastructural features manifested by infected and non-infected WISH cells

	Wish cells							
		Infected						
Feature	Non-Infected	1*	2	3	4	5	6	7
Cell membrane	Distinct	Distinct	Distinct	Distinct	Distinct	Distinct	Indistinct	Distinct
Membrane blebbing	Absent	Obvious	Obvious	Only processes	Not obvious	Present	Absent	Not-obvious
Cytoplasmic organelles	Intact	Deteriorated	Deteriorated	Less deteriorated	Deteriorated	Deteriorated	Deteriorated	Deteriorated
Autophagic vacuoles	Absent	Present	Present	Present	Present	Present	Present	Present
Nuclear shrinkage	Absent	Absent	Present	Absent	Present	Absent	Absent	Present
Chromatin condensation	Absent	Absent	Marked	Absent	Present (marginal)	Absent	Absent	Present (marginal)
Viral particles	Absent	Present	Present	Not identified	Not identified	Present	Present	Not identified

1*, Supernatant of EMCV-infected WISH cell line (18 h); 2, Supernatant of EMCV-infected WISH cell line (24 h); 3, Supernatant of infected cell line (36 h) and pre-treated with IFN-α.; 4, Supernatant of infected cell line (36 h) and pre-treated; 5, Pellets from infected cell line (24 h); 6, Pellets from infected cell line (36 h); 7, Pellets from infected cell line (24 h) and pre-treated with IFN-α

Table 1 shows the comparison of the ultrastructural features manifested by the infected and non-infected WISH cells.

DISCUSSION

In the present study, we followed up the sequential ultrastructural changes manifested by WISH cells infected with EMCV and pre-treated or not with IFN-α. It was noticeable that in the various lanes of the infected WISH cells, except for the pellets prepared from WISH cells incubated 36 h with EMCV, cell membranes were intact despite the observed organellar changes. Membrane blebbing was obvious only in WISH cells incubated with EMCV for 24 h and either pre-treated or not with IFN-α. Changes of the cytoplasmic organelles were a feature of all lanes of the infected WISH cells. Similarly, cytoplasmic autophagic vacuoles were a constant feature in all infected lanes. Nuclear shrinkage and chromatin condensation were most noticeable in WISH cells incubated with EMCV for 24 h and not pre-treated with IFN-α. Viral particles were recognized in pellets prepared from infected (24 and 36 h) and not pre-treated WISH cells and in supernatant of WISH cells 24 h post-infection. Regarding, the cell membrane integrity of the infected cells, it was only disrupted in WISH cells 36 h post-infection. This finding may indicate that long incubation with the virus could provoke marked structural membrane alterations that eventually caused marked swelling, probably due to disturbed osmotic gradient balance, beyond the capability of the cell membrane to withstand the increased intracellular pressure.

The described cell membrane blebbing in WISH cells 24 h post-infection without IFN-α treatment was probably related to active membrane changes relevant to virus budding from the infected cells. Blebbing of cell membrane reflects disrupted membrane integrity which has been approved to be a feature of viral infections (Mattana *et al*., 1994; Meek and Davis, 1986). Absence of membrane blebbing in other infected lanes may be attributed to lesser virus activity related to longer incubation period and possible development of persistent infection. In cell lanes pre-treated with IFN-α, absence of obvious membrane blebbing was most probably ascribed to the inhibitory effect of IFN-α on the virus replication activity. Alterations of the cytoplasmic organelles noticed in all infected cell lanes are undoubtedly related to the virus damaging effect. In this respect, viruses are known to use cellular organelles to reproduce (Cheville, 1970) with development of organelle changes such as mitochondrial swelling. Finally, organelles of the infected cells are disintegrated and replaced by viral structures.

The consistency of the autophagic vacuoles in all infected cells is most likely related to the existence of damaged organelles. Autophagy, the process of uptake and degradation of damaged cellular organelles into autophagic vacuoles, serves to sequester the damaged cytoplasmic structures resulting from acute cell injury (Dunn, 1990a; Dunn, 1990b).

Nuclear shrinkage and chromatin condensation were recognized most obviously in WISH cells 24 h post-infection. The demonstrated nuclear morphology may represent apoptotic nuclei. Nuclear alterations such as chromatin margination and condensation are among the characteristics features of nuclear apoptosis described *in vivo* as well *in vitro* studies (Karpman *et al*., 1998; Oberhammer *et al*., 1993; Romero *et al*., 2003; Searle *et al*., 1982; Walker *et al*., 1988, 1989). Chromatin condensation has been found the initial event in the apoptosis sequence (Cohen and Duke, 1984; English *et al*., 1989) and considered the most reliable morphological feature for recognition of apoptosis (Karpman *et al*., 1998; Oberhammer *et al*., 1993). The demonstrated cell membrane blebbing in the infected WISH cells is also considered one of the morphological apoptotic changes (Yeung *et al*., 1996). Apoptosis has been defined as an

important process for many of the pathological consequences of diseases (Kwon *et al*., 2003). The presently recognized apoptotic process is an induced one initiated by EMCV virus infection.

A dose and time dependent increase of cells in the G_2/M phase with the appearance of apoptotic cells in EMCV-infected cell line has been recognized by flow cytometric analysis (Detrick *et al*., 1996). It is postulated that DNA damage was a contributing factor in the development of the currently described apopototic nuclear change. This may conform to the action of some toxins which induce apoptotic mode of cell death (Corcoran *et al*., 1994; Corcoran and Ray, 1992; Qu *et al*., 2005). DNA damage is thought to be the essential event for initiating apoptotic cell death (Corcoran *et al*., 1994). It has been shown that EMCV infection of HEp-2 cells induces lower nuclear ploidy, reduced nuclear area and fewer nucleoli which may point to the occurrence of DNA damage (Karalyan *et al*., 2005). The mechanisms and signaling mediators that regulate the virus-induced apoptosis are not well understood. Moreover, viral infection can serve as an apoptotic signal and apoptosis has been considered a cellular response for many viral infections (Yeung *et al*., 1999). According to the latter authors, there are some apoptotic pathways which are important for viral clearance and these pathways are induced by a number of factors such as tumor necrosis factor-α (TNF-α). The currently demonstrated apoptotic changes of the infected WISH cells may represent a form of resistance.

Viral particles were detected in WISH cells 24 and 36 h post-infection. All cell lanes pre-treated with IFN-α did not manifest identifiable viral particles which points to the effective antiviral activity of the used IFN-α. Interferons (IFNs) are a family of related cytokines and type I IFNs regulate different biological processes including the antiviral activities, cellular growth and differentiation and modulation of immune functions (Balachandran *et al*., 1998; Der and Lau, 1995). Induction of antiviral action of IFNs differ quantitatively and depend on virus strain and the type of the infected host cell. PKR, protein Mx and RNase are among the best characterized contributors to the IFN-α induced antiviral state displayed at the level of single virus-infected cell (Antalis *et al*., 1998).

The effectiveness of the antiviral response of IFNs has led to many viruses developing mechanisms that antagonize the production or actions of IFNs. Evident cell lysis was obvious in the pellets prepared from WISH cells 36 h post-infection without IFN-α pre-treatment. This finding is supposed to be an evidence of the virus-induced cytolysis in absence of the factors which promote the virus-induced apoptosis. It was found that PKR is an apoptogenic factor and *in vitro* cells deficient in PKR show a delay in EMCV-induced apoptosis (Yeung *et al*., 1999). Presently absence of IFN-α pre-treatment and longer incubation period with the virus (36 h) clearly promoted the virus cytolytic activity.

In the present study, an *in vitro* system was employed to avoid the effects of the host immune defenses and thus make it possible to reveal the direct cell-virus interactions. The present results, demonstrated as sequential ultrastructural changes, clearly indicate that IFN-α treatment inhibits viral replication and the virus-induced cytolysis. Future work will focus on the cellular factors which may contribute to establishment of a state of persistent viral infection.

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