**Studying intracellular signaling pathways by quantitative immunoelectron microscopy during chronic human diseases**

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**ABSTRACT**

Most studies of signaling pathways analyze the levels of whole populations of modified/unmodified proteins in a group of cells. However, key signaling and cellular processes signal through different complexes and/or from various cellular compartments and differentially in individual cells. In addition, chronic diseases such as diabetic foot ulcers (DFUs) and hepatitis C virus (HCV) infection lead to cellular stress and extensive remodeling of intracellular membranes and organelles. To restore homeostasis under stress, cells adjust intracellular signaling and membrane contact sites (MCSs) allowing various processes such as organelle dynamics and autophagy. Heberprot-P (HPP) is an EGF-based therapeutic product (HeberBiotec S.A.,Havana, Cuba) that enhances healing of chronic wounds in patients with DFU. This study aimed to apply electron and immunoelectron microscopy (EM and IEM) approaches for ultrastructural study of cellular processes and to quantify distributions of relevant proteins across subcellular compartments under chronic diseases such as HCV and DFUs or after HPP therapy of DFUs. By using this methodology we have been able to link preferential subcellular localization and intracellular trafficking of key viral and host signaling molecules with remodeling of cellular membranes and processes. These effects were also associated with the therapeutic effect of HPP in DFUs. Notably, key MCSs like the mitochondria-associated endoplasmic reticulum membranes appeared as an important place for trafficking of these proteins and regulation of autophagy. Our approach revealed the value of quantifying subcellular localization of proteins at ultrastructural level to study intracellular dynamics of key signaling pathways in a relevant therapeutic setting.

**Introduction**

Under conditions of chronic diseases such as those related to diabetic foot ulcerations (DFUs) and hepatitis C virus (HCV) infection, affected cells develop various stress responses and processes to adapt to different pathological environments. Lower extremities ulcerations and the potential for amputation are major complications of diabetes (1). Thus, DFUs result in significant disability, morbidity and mortality (2). On the other hand, most of HCV-infected persons develop chronic hepatitis (70-80%) that can progress to liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC) and liver failure. In addition, chronic infection with HCV is the leading cause of end-stage liver disease, HCC and liver related death in the Western world (3, 4). Remarkably, it is predicted that the prevalence of HCV-related cirrhosis and HCC will considerably increase in the next decade with a significant burden on health care systems worldwide (4-6).

Interestingly, the pathogenesis of UPDs and HCV infection is associated with impaired wound healing responses (WHR). UPDs are chronic wounds where the normal healing process is stalled in the inflammatory phase leading to static wounds (7). In UPDs, the extracellular matrix (ECM) and growth factors degradation is increased while their synthesis is impaired (8). On the other hand, HCV-associated hepatic injury is related to fibrosis progression as a consequence of a sustained WHR caused by the chronic liver injury (9). In this case, unbalanced production and degradation of ECM components leads to an accumulation of connective tissue in the liver. Despite differences in the mechanisms of pathogenesis, microenviroments from both UPDs and HCV-related hepatic injury generate various common stresses such as cellular hypoxia, oxidative and endoplasmic reticulum stress (10-12). These cellular stresses are frequently associated with extensive remodeling of intracellular membranes and organelles. To restore homeostasis under stress, cells adjust intracellular signaling and membrane contact sites (MCSs) allowing various processes such as organelle dynamics and autophagy. However, the role of these cellular processes in the pathogenesis of UPDs and HCV in vivo are not completely understood. Importantly, Heberprot-P (HPP) (HeberBiotec S.A.,Havana, Cuba) is an epidermal growth factor (EGF)-based therapeutic product that enhances healing of chronic wounds in patients with DFUs safely and efficaciously (13). It provides a unique opportunity to study the mechanisms behind the therapeutic effect of HPP to restore the WHR in a clinical setting.

Transmission and immunoelectron microscopy (EM and IEM) have proved to be valuable tools for studying different cellular processes and intracellular dynamics localization of host proteins (14-16). Especially, a methodology to quantify intracellular immunogold localization using IEM has been previously shown to be a powerful approach for examining relative intracellular distributions of interesting molecules (17, 18). By comparing patterns of compartmental gold labeling within and between different groups of cells, preferential labeling of certain compartments within a cell or shifts in labeling patterns associated with experimental treatment can be evaluated. As spatial distributions of molecules can be mapped in the context of cellular ultrastructure, their intracellular trafficking may be efficiently monitored while showing details of cell fine structure. Therefore, we hypothesized that this approach may be particularly relevant to study key cellular processes and signaling pathways with therapeutics effects. This work aimed to apply EM and IEM approaches for ultrastructural studies of cellular processes and to quantify distributions of relevant proteins involved in EGF receptor (EGFR) signaling pathways across subcellular compartments under chronic diseases such as HCV and DFUs and after HPP therapy of DFUs.

**Body of the paper**

***Materials and Methods***

*Biopsies samples and antibodies*

Liver needle biopsies samples of patients with chronic HCV infection and anti-core, anti-E1, anti-EGFR and anti-PCNA antibodies have been described before (16, 19). These patients have been previously illustrated and were recruited after informed consent in writing was obtained. Granulation tissue fragments of UPDs were obtained as previously described (16) from patients before and after (15 (T15), 45 (T45), and 60 (T60) minutes and 6 (T6) and 24 (T24) hours) HPP treatment.

##### *Transmission electron microscopy and immunoelectron microscopy*

Hepatic and granulation tissue samples were prepared for transmission electron microscopy and immunoelectron microscopy analyses as previously described (16, 19). Samples were examined with a JEOL/JEM 2000 EX transmission electron microscope (JEOL, Japan).

*Comparing distributions of gold particles between different compartments within a cell and at different times after treatment with EGF*

Comparing distributions of gold particles within a cell and at different times after treatment with HPP was done according to Mayhew et al. (18) using the ImageJ 1.38 software (Website: [rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)).

***Results and discussion***

To replicate its genome in cell culture in vitro systems HCV induces massive remodelling of primarily endoplasmic reticulum (ER)-derived membranes to create in the cytoplasm a microenvironment favourable for its replication (20). As shown in Figure 1, hepatocytes from HCV-infected samples showed ER dilatation which is characteristic of ER-induced stress (Figure 1A). Interestingly, these samples showed extensive MCSs in the mitochondria-associated ER membranes (MAMs). Notably, HCV core protein localized to both ER and mitochondria, including MAMs (Figure 1A). In addition, virus-like particles (VLPs) were observed in vesicles similar to autophagic vacuoles (AV) (Figure 1B). Similar VLPs were also seen in multivesicular bodies (MVBs) (Figure 1C). It is interestingly to note that AV showed several MCSs with the ER (Figure 1B). Another interesting finding was detection of extracellular vesicles or exosomes-like structures (ELS) immunostained with anti-HCV E1 protein suggesting the presence of HCV components in these vesicles (Figure 1D). These results indicate the involvement of various MCSs such as MAMs and contacts between ER and AV in the HCV life cycle and possibly, its pathogenesis. MAMs are central organizers of several metabolic processes, including calcium, apoptotic and innate immune signalling, synthesis of phospholipids and regulation mitochondrial morphology (21-23). Although MAMs have been suggested to contribute to HCV replication in vitro, findings from this work indicates the presence of HCV proteins in this location in hepatocytes of human patients. Moreover, MAMs may favour translocation of viral proteins to mitochondria affecting cell metabolism and survival. Interestingly, the presence of VLPs in both MVBs and AV, and HCV proteins in ELS suggest their involvement in HCV morphogenesis in vivo. Both autophagy and ELS have a key role in cell survival and communication and might also be involved in viral persistence and pathogenesis (15).

Scarce detection of both EGFR and PCNA in fibroblasts-like cells (FLCs) from DFUs was observed was observed before HPP treatment suggesting impairment of EGF/EGFR signaling pathway (not shown). Interestingly, strong immunostaining of the essential autophagy factor LC3B was detected in ER membranes, especially in MAMs of FLCs before HPP treatment (Figure 2A). This was related with increased detection of AV (Figure 2B) suggesting the induction of autophagy in FLCs from DFUs samples.

Importantly, intralesional EGF therapy induced the activation and nuclear translocation of EGFR and PCNA in FLCs early after EGF administration (Figure 3). In addition, EGFR and PCNA localized to MVBs and ELS (Figure 3C, D, E, F). Interestingly, HPP treatment inhibited the autophagy in FLCs (not shown). This may be related with the previously reported inhibitory effects of EFGR signaling on autophagy either directly or through activation of mTOR (24).

A more detailed characterization of distributions of immunogold labeling for EGFR at later times after EGF treatment showed an increase in labelling of EGFR in the ER and golgi complex at T6-T24 (Figure 4A). As illustrated at T6-T24, a shift in labelling showed augmented golds in mitochondria and decreased EGFR immunolabelling in nucleus. Similarly, a shift in PCNA labelling was observed from T6 to T24 with increased immunolabelling detected in mitochondria and reduced gold particles in nucleus (Figure 4B). These findings suggest that nuclear functions of EGFR and PCNA prevail early after HPP treatment. Nonetheless, the therapeutic effect of HPP might also be related with major mitochondrial functions of EGFR and PCNA at later times following EGF infiltration.

EGFR is a multifunctional regulator in various subcellular organelles including cell membrane, cytoplasm, mitochondria and nucleus (25). Different roles of EGFR may depend on its subcellular localization and specific cellular microenviroment. This is the first report showing a relationship between EGFR and PCNA accumulation in nucleus and mitochondria of FLCs from DFU and the therapeutic effect of EFG in treated patients. EGFR phosphorylate and promotes PCNA activity in nucleus which is related to cellular proliferation and may contributes to FLCs activation and the WHR. Identification of EGFR and PCNA in mitochondria of FLCs is also a valuable finding of this work. Several reports have indicated that mitochondrial functions are affected in diabetis (26). On the other hand, previous *in vitro* studies have shown that EGFR localization in mitochondria is related with cell survival (27). EGFR can regulate mitochondrial dynamics through interaction with mitofusin 1 (Mfn1) (28). It was shown that inhibition of Mfn1 induced mitochondrial fission, altered mitochondrial subcellular distribution, improved ATP production and cell motility. Moreover, mitochondrial PCNA has been involved in the regulation of the energy generating system in the mitochondria (29). Interestingly, the relative mitochondrial area represented 9% of the total FLCs area at T6-T24 that was higher to the 3,7% observed at T15-T60 (P<0.001, z test of proportions). This finding suggests increased biogenesis of mitochondria at later times after HPP therapy. Therefore, possible therapeutic effects of mitochondrial EGFR and PCNA in FLCs of DFU may involve the EGFR-mediated modulation of mitochondrial dynamics, increased energy production and fibroblast migration thus contributing to cellular activation and wound healing-related responses observed in EGF-treated patients. Localization of both EGFR and PCNA to MAMs suggest its possible role in intracellular signaling and trafficking of these molecules.

Several biological important functions have been described for extracellular vesicles and exosomes in both, normal and pathological conditions (30). Particularly, exosomes have been suggested as mediators in intercellular signal communication by delivering a variety of molecules, including proteins, lipids, mRNAs, microRNAs, and DNAs, from donor cells to recipient cells via membrane fusion (30). Interestingly, exosomes derived from mesenchymal stem cells have been shown to facilitate cutaneous wound healing by promoting collagen synthesis and angiogenesis, as well as inducing proliferation and migration of fibroblasts derived from normal donors and chronic wound patients (31, 32). Therefore, accumulation of EGFR and PCNA containing exosomes-like vesicles in the ECM might be relevant. Further studies will be necessary to elucidate their role in HPP-induced WHR.

***Conclusions***

This study revealed MCSs and cellular processes such as autophagy and mitochondrial dynamics involved in HCV life cycle in vivo and HCV-related and DFUs pathogenesis. Our approach revealed the value of quantifying subcellular localization of proteins at ultrastructural level to study intracellular dynamics of key signaling pathways in a relevant therapeutic setting.

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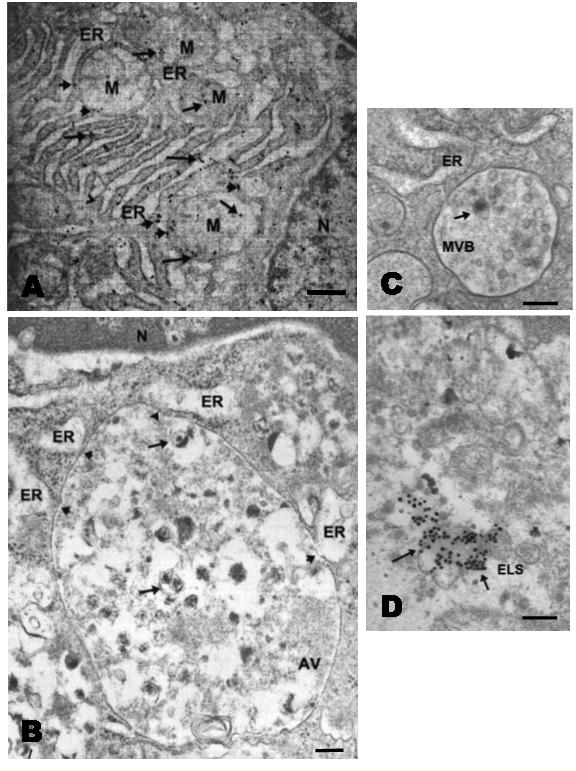
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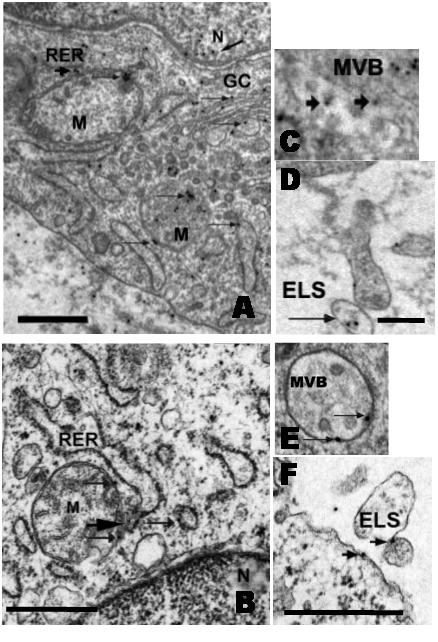
**Figure 1**: Electron micrographs of liver biopsies from HCV-infected patients. **A**) Ultrastructural changes in part of an hepatocyte with dilatation of ER. Immunolabeling of HCV core protein in ER and mitochondria (arrows) and MAMs (arrowheads) (Bar=500 nm). **B**) Virus-like particles were observed in vesicles similar to autophagic vacuoles (AV) (arrows) (Figure 1B). Note MCSs linking the AV with the ER (arrowheads) (Bar=200 nm). **C**) A VLPs inside a multivesicular body (MVB) (Bar=200 nm). **D**) Immunostaining of HCV E1 in exosomes-like structures (ELS) (Bar=200 nm).



**Figure 2**: Evidences of autophagy in FLCs of DFUs before HPP treatment. Immunostaining of LC3B (LC3B) in MAMs (Bar=500 nm) (**A**) and early (Vat) and late (Vad) autophagy vacuoles (Bar=250 nm) (**B**).



**Figure 3**: Electron micrographs of UDFs after HPP treatment. **A**) Immunolabeling of EGFR in part of a FLC from a DFU obtained after HPP treatment. EGFR (arrows) localized to nucleus (N), rough ER (RER), golgi complex (GC), mitochondria (M), extracellular matrix and MAMs (arrowhead) (Bar=500 nm). **C,D**) Immunostaining of EGFR in MVBs and ELS (Bar=100 nm). **B**) Immunolabeling of PCNA in part of a FLC from a DFU obtained after HPP treatment. PCNA (arrows) localized to nucleus (N), rough ER (RER), mitochondria (M), extracellular matrix and MAMs (arrowhead) (Bar=500 nm). **E,F**) Immunostaining of PCNA in MVBs and ELS (Bar=500 nm).



**Figure 4**. Ratio of observed (No) and expected distributions (Ne) of gold particles for EGFR (**A**) and PCNA (**B**) in organelle compartments of Fibroblast-like cells (FLCs) contributing to the difference observed at different times (T15-T60; T6-T24) after treatment with EGF. Ratios of No/Ne are shown for those cellular compartments contributing to differences between labeling distributions obtained at different time points that were analyzed by contingency table analysis. Negative values represent No/Ne ratios below 1. **A**) Note that FLCs at T15-T60 have fewer-than-expected gold particles at mitochondria (No/Ne ratio=0.5) and more-than-expected gold particles in nucleus (No/Ne ratio=1.27) while FLCs analyzed at T6-T24 have more-than-expected gold particles at mitochondria (No/Ne ratio=1.48) and fewer-than-expected gold particles in nucleus (No/Ne ratio=0.67). **B**) Note that FLCs at T15-T45 have more-than-expected gold particles in nucleus (No/Ne ratio=1.37) and fewer-than-expected gold particles in mitochondria (No/Ne ratio=0.6); while FLCs analyzed at T6-T24 have more-than-expected gold particles at mitochondria (No/Ne ratio=1.48) and fewer-than-expected gold particles in nucleus (No/Ne ratio=0.72).

**A**



**B**

