

Recombinant hepatitis C virus-envelope protein 2 interactions with low-density lipoprotein/CD81 receptors

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Hepatitis C virus (HCV) envelope protein 2 (E2) is involved in viral binding to host cells. The aim of this work was to produce recombinant E2B and E2Y HCV proteins in Escherichia coli and Pichia pastoris, respectively, and to study their interactions with low-density lipoprotein receptor (LDLr) and CD81 in human umbilical vein endothelial cells (HUVEC) and the ECV304 bladder carcinoma cell line. To investigate the effects of human LDL and differences in protein structure (glycosylated or not) on binding efficiency, the recombinant proteins were either associated or not associated with lipoproteins before being assayed. The immunoreactivity of the recombinant proteins was analysed using pooled serum samples that were either positive or negative for hepatitis C. The cells were immunophenotyped by LDLr and CD81 using flow cytometry. Binding and binding inhibition assays were performed in the presence of LDL, foetal bovine serum (FCS) and specific antibodies. The results revealed that binding was reduced in the absence of FCS, but that the addition of human LDL rescued and increased binding capacity. In HUVEC cells, the use of antibodies to block LDLr led to a significant reduction in the binding of E2B and E2Y. CD81 antibodies did not affect E2B and E2Y binding. In ECV304 cells, blocking LDLr and CD81 produced similar effects, but they were not as marked as those that were observed in HUVEC cells. In conclusion, recombinant HCV E2 is dependent on LDL for its ability to bind to LDLr in HUVEC and ECV304 cells. These findings are relevant because E2 acts to anchor HCV to host cells; therefore, high blood levels of LDL could enhance viral infectivity in chronic hepatitis C patients.

Key words: HCV - E2 - LDLr - CD81

Hepatitis C virus (HCV) is a small, enveloped RNA virus. A single open reading frame encodes a precursor polyprotein that is cleaved *via* the signalling mechanisms of host and viral proteases, generating 10 different structural [core (C), envelope 1 (E1) and envelope 2 (E2)] and nonstructural 2-5 proteins, in addition to the protein P7 (Bartenschlager & Lohmann 2000, Penin et al. 2004).

The E2 protein is a 70-kDa viral envelope glycoprotein that includes an N-terminal ectodomain and a C-terminal transmembrane domain. After synthesis, the E2 protein undergoes a process of post-translational modification and presents nine to 11 potential glycosylation sites, which differ depending on virus genotype and subtype, but are nevertheless well conserved and are therefore good antigenic targets for developing antiviral molecules (Liu et al. 2001). E1 and E2 proteins perform essential functions during different stages of the HCV

replication cycle and are necessary for initiation of the infection process; their functions include receptor binding, fusion with the host cell membrane and invasion (Bartenschlager & Lohmann 2000, Dubuisson et al. 2008).

The mechanism through which HCV invades a cell is not well known, but there is strong evidence that viral proteins interact with cell surface receptors. For instance, the interactions of HCV with glycosaminoglycans (Germi et al. 2002), the liver/lymph node-specific intercellular adhesion molecules-3-grabbing non-integrin (SIGN) (Lozach et al. 2003) and the low-density lipoprotein receptor (LDLr) (Agnello et al. 1999) are thought to facilitate initial viral attachment to the cell. Subsequently, HCV can form interactions with tetraspanin CD81 (TAPA-1) (Zhang et al. 2004), scavenger receptor class B type 1 (SR-BI) (Scarselli et al. 2002), dendritic cell-SIGN (Lozach et al. 2003), the tight junction proteins claudin-1 (CLDN1) (Evans et al. 2007) and occludin (OCLN) (Ploss et al. 2009), signal transducer Harvey rat sarcoma viral oncogene homologs (Zona et al. 2014), the cholesterol uptake receptor Niemann-Pick C1-like 1 (Sainz et al. 2012) and transferrin receptor 1 (TfR1) (Martin & Uprichard 2013). Additionally, epidermal growth factor receptor and ephrin receptor A2 tyrosine kinases have been identified as HCV entry cofactors (Lupberger et al. 2011). Although data from several studies support the involvement of LDLr in HCV entry, several discrepancies remain. A study con-

doi: 10.1590/0074-02760140441

Financial support: FAPESP (2008/58957-0)

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Received 23 November 2014

Accepted 20 March 2015

ducted by Albecka et al. (2012) investigated the role of LDLr in the HCV life cycle by comparing virus entry to a process of lipoprotein uptake and showed that HCV particles can interact with LDLr. However, this interaction does not necessarily lead to a productive infection, but rather indicates a role for LDLr as a lipid-providing receptor, which modulates viral RNA replication.

Vascular changes in the cirrhotic livers of patients with chronic hepatitis C have attracted increasing interest because little is known about the relationship of HCV with endothelial cells. As this pathology is associated with major complications and prognostic implications, the necessity of acquiring a more detailed characterisation of the interactions between HCV and cells during infection is required. Previous studies using hepatoma cell lines, lymphocyte cell lines and nonhuman cell lines have already demonstrated that the E2 protein can bind to these cells. In the current study, we tested E2 binding to human umbilical vein endothelial cells (HUVEC) and ECV304 cells, both of which are endothelial cell models. Therefore, we aimed to study the binding interactions between recombinant E2 proteins and LDLr and CD81, two well known binding partners of E2, in two different endothelial cell lines: HUVEC, which have a high concentration of LDLr but no CD81 receptors, and ECV304 cells, which are a derivative of the human urinary bladder carcinoma T24 cell line and possess a high concentration of LDLr and a low concentration of CD81. To investigate how human LDL and differences in protein structure (glycosylated or not) affected binding efficiency, we examined recombinant proteins that were either associated or not associated with lipoproteins.

MATERIALS AND METHODS

Strains, cell lines and media - The DH5a *Escherichia coli* strain (Invitrogen, USA) was used for general propagation of plasmids and the *E. coli* Rosetta (DE3) strain was used to express the E2 protein. The KM71H^(Mut^S) *Pichia pastoris* strain (Invitrogen) was used as an expression host. HUVEC (ATCC[®] CRL-2873TM) and ECV304 cells (ATCC[®] CRL-1998TM) were used to analyse viral binding to host receptors. All cell lines were grown according to Urbaczek et al. (2014).

Cloning, expression and purification of E2 protein - HCV cDNA was obtained from viral RNA that was extracted with a QIAamp Viral RNA Mini Kit (QIAGEN, USA) using sera that was collected and pooled from individuals with HCV genotype 1a. *E. coli* and *P. pastoris* were used to express E2 proteins. The E2B protein was expressed in *E. coli* and the E2Y protein was expressed in *P. pastoris*. Cloning, expression and purification were performed according to Urbaczek et al. (2014).

Immunoreactivity of recombinant proteins - Recombinant E2 proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Pooled sera from HCV positive patients was diluted 1:100 and added to the membrane for 1 h. As a negative control, pooled sera that tested negative for HCV and select other infectious diseases (Chagas, syphilis, hepatitis B, human immunodeficiency virus-1/2 and human T-lymphotropic virus) was

used at a dilution of 1:10. The reaction was visualised using biotinylated human immunoglobulin G (IgG) diluted 1:1000, avidin-peroxidase and chromogenic substrate 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide.

Immunophenotyping - HUVEC and ECV304 [cultivated with and without foetal bovine serum (FCS)] cells were suspended at a concentration of 1×10^6 cells/mL and immunophenotyped for CD81 (TAPA-1) and LDLr cell surface receptors following the addition of anti-CD81 mouse monoclonal antibody (MAb), IgG1-fluorescein isothiocyanate (FITC) conjugate and anti-LDLr mouse MAb IgG1-phycoerythrin (PE)-conjugated; cells were incubated with the indicated immunoglobulins for 30 min at 4°C. The cells were analysed by flow cytometry (FACS Canto BD Biosciences and FACSDiva software v.6.1.3). In total, 30,000 cells were analysed. All experiments were repeated on at least three different occasions and their results were combined. The results are expressed as average percentage fluorescence.

Binding assay - HUVEC and ECV304 cells were grown in media with and without the addition of FCS and were then washed twice in phosphate buffered saline, pH 7.2. Following this, the cultures were suspended at a concentration of 1×10^6 cells/mL and incubated at 37°C for 45 min with 5 µg/mL untreated E2 protein (E2B or E2Y) or 5 µg/mL E2 protein pre-incubated with 10 µg/mL human LDL. For these assays, human LDL was obtained from pooled sera taken from individuals who were HCV negative and who exhibited high concentrations of LDL (values above the normal reference). The cells were incubated with an anti-His mouse MAb IgG1-Allophycocyanin (APC) conjugate for 30 min at 4°C and were then analysed by flow cytometry. A total of 30,000 cells were analysed for each experiment and all experiments were repeated a minimum of three times. The proportions of cells bound to the proteins were measured and pooled and the mean was calculated. A total of 10 experiments were performed to calculate the means and standard deviation (SD).

Binding inhibition - To inhibit the binding of E2 recombinant proteins to HUVEC and ECV304 cell surface receptors, cell cultures were grown to 1×10^6 cells/mL (grown in medium with and without FCS) and LDLr and CD81 receptors on the cell surface were blocked through the addition of anti-LDLr and anti-CD81. The blocked cells were then incubated at 37°C for 45 min with 5 µg/mL E2 protein (E2B or E2Y) or 5 µg/mL E2 protein pre-incubated with 10 µg/mL human LDL (obtained from pooled sera taken from HCV negative patients that exhibited high concentrations of LDL) at 37°C for 1 h. After washing, an IgG1-APC anti-His mouse MAb conjugate was added and the cells were incubated at 4°C for 30 min. The cells were analysed by flow cytometry. A total of 30,000 cells were analysed for each experiment and all experiments were repeated a minimum of three times. A total of 10 experiments were performed to calculate the means and SD.

Statistical analysis - All statistical analyses were performed using Prism 5.0 GraphPad Software Inc (USA). All sample comparisons were made using ANOVA and Tukey tests to compare percentage fluorescence. Values of $p < 0.05$ were considered significant.

RESULTS

Sequence amplification and correspondence to E2 recombinant proteins - A sequence was amplified that corresponded to an E2 protein without a transmembrane domain (GenBank accession AF009606); when including restriction sites, this resulted in an 852-bp-long sequence. A recombinant pET-42a vector was sequenced and analysed using BLAST by alignment with the HCV subtype-1a genome sequence (GenBank accession M67463). The recombinant E2 proteins corresponded to the native form of E2 but without the transmembrane domain (amino acid residues from 384-661), which increased their solubility.

Expression analysis and immunoreactivity of E2 recombinant proteins - Protein expression levels were confirmed by their corresponding electrophoretic bands. The protein that was produced in *E. coli* and fused to a glutathione-S-transferase protein (GST) and 6x His tag was found to be 63.5 kDa (E2B) (Fig. 1). The protein that was produced in *P. pastoris* and fused to a 6x His tag was found to be 50 kDa (E2Y) (Fig. 2) because of N-glycosylation. Molecular weight differences were related to the different fusion proteins that were employed and to the varying post-translational modifications that were imparted by different organisms. These results are

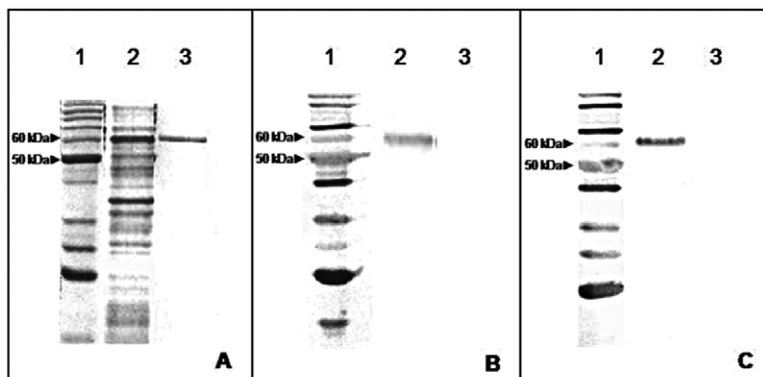


Fig. 1A-C: protein size markers. Expression and reactivity of the envelope 2 (E2) protein produced in *Escherichia coli* (E2B). A: sodium dodecyl sulfate polyacrylamide gel electrophoresis stained with Coomassie blue [2 in A: supernatant of the induced bacterial culture; 3 in A: recombinant E2 protein fused to glutathione-S-transferase protein (GST) and 6x His tag (63.5 kDa) after purification in the GST column]; B: polyvinylidene difluoride (PVDF) membrane transferred with the E2 recombinant protein [2 in B: recombinant E2 protein localised by anti-His tag primary antibody and alkaline phosphatase anti-immunoglobulin G (IgG) secondary antibody; 3 in B: negative control and alkaline phosphatase anti-IgG secondary antibody]; C: PVDF membrane transferred with the E2 recombinant protein for test of reactivity with human serum [2 in C: reactivity of the E2 recombinant protein with a pool of hepatitis C virus (HCV) (+) human serum (diluted 1:100); 3 in C: no reactivity of protein with a pool of HCV (-) human serum (diluted 1:10)].

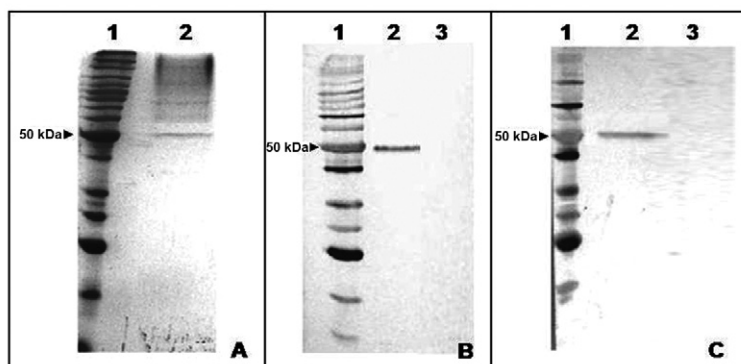


Fig. 2A-C: protein size markers. Expression and reactivity of the envelope 2 (E2) protein produced in *Pichia pastoris* (E2Y). A: sodium dodecyl sulfate polyacrylamide gel electrophoresis stained with Coomassie blue [2 in A: recombinant E2 protein fused to glutathione-S-transferase protein (GST) and 6x His tag (50 kDa) after purification in the GST column]; B: polyvinylidene difluoride (PVDF) membrane transferred with the E2 recombinant protein [2 in B: recombinant E2 protein localised by anti-His tag primary antibody and alkaline phosphatase anti-immunoglobulin G (IgG) secondary antibody; 3 in B: no reactivity of the E2 recombinant protein with primary antibody (anti-core) - negative control and alkaline phosphatase anti-IgG secondary antibody]; C: PVDF membrane transferred with the E2 recombinant protein for test of reactivity with human serum [2 in C: reactivity of the E2 recombinant protein with a pool of hepatitis C virus (HCV) (+) human serum (diluted 1:100); 3 in C: no reactivity of protein with a pool of HCV (-) human serum (diluted 1:10)].

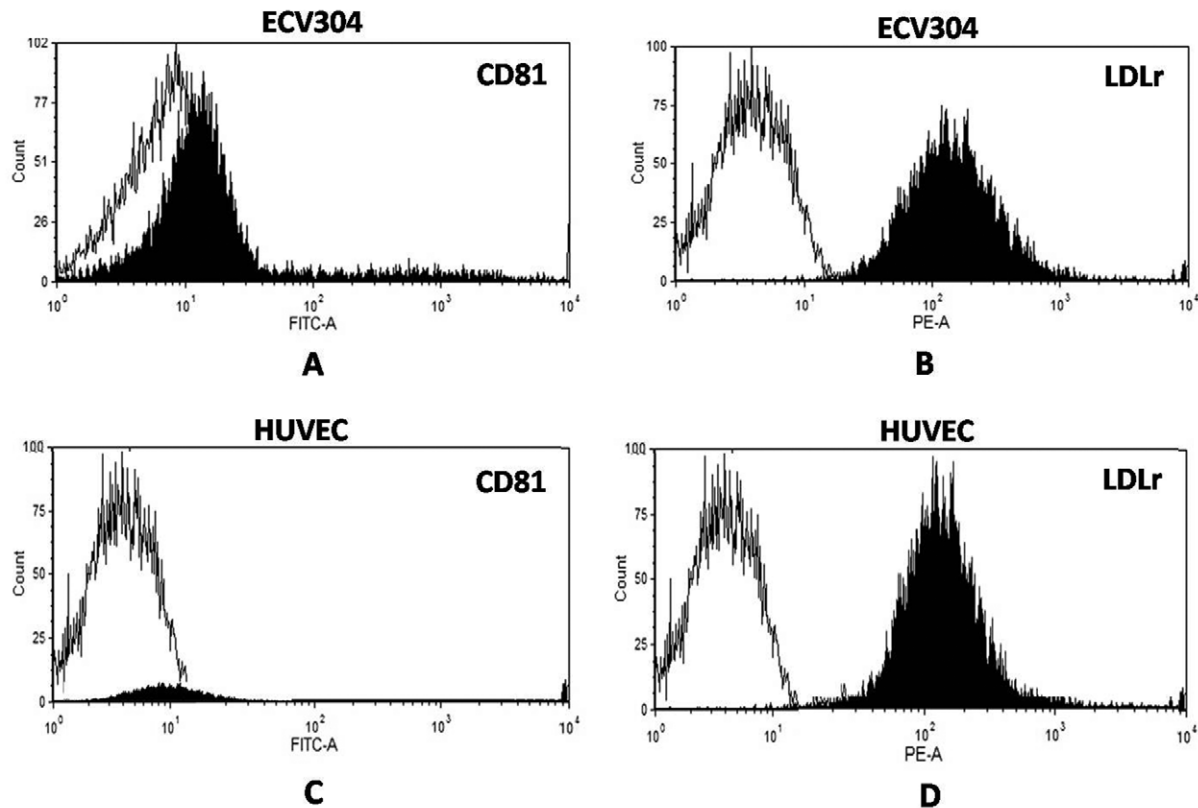


Fig. 3: immunophenotyping assay of the low-density lipoprotein receptor (LDLr) and CD81 surface receptors in ECV304 and human umbilical vein endothelial cells (HUVEC). Blank peak indicates isotype control used for calibration of the flow cytometer. Black peak indicates the fluorescence. FITC: fluorescein isothiocyanate; PE: phycoerythrin.

in agreement with previous publications that evaluated the same organisms and gene sequences (Yurkova et al. 2004, Martinez-Donato et al. 2006).

The antigenicity of each recombinant protein was tested using serum samples taken from chronic hepatitis C carriers. We found that human anti-IgG demonstrated a specific recognition of E2 proteins [Figs 1 (2 in C), 2 (2 in C)]. As a control, a PVDF membrane that had been incubated with E2 proteins was tested against a pool of HCV negative serum; no interactions were found [Figs 1 (3 in C), 2 (3 in C)]. No immunoreactivity was observed with respect to 6x His-tag and GST fusion proteins in HCV-negative and positive serum pools.

Cellular immunophenotyping - LDLr and CD81 (TAPA-1) surface receptors were characterised in HUVEC and ECV304 cells by flow cytometry and representative histograms that were obtained using FITC-conjugated anti-CD81 and PE-conjugated anti-LDLr are shown in Fig. 3. In HUVEC, 93% were positive for LDLr, whereas no significant fluorescence was found with respect to CD81 (approximately 1%). In ECV304 cells, 15% were positive for CD81 and 98% were positive for LDLr. The presence or absence of FCS in culture medium did not significantly alter the expression of receptors.

Binding of E2 recombinant proteins to LDLr and CD81 receptors - Assessing the binding of the recombi-

nant E2 proteins to HUVEC and ECV304 cells revealed that E2 proteins could bind to receptors without having to form a heterodimer with E1. In HUVEC, binding capacity was found to be totally dependent on the presence of FCS, as the percentage of binding was reduced for both E2 proteins when FCS was removed from culture media (Fig. 4). However, the addition of human LDL to the media both rescued and increased the binding capacity of both E2 for all combinations studied and regardless of the presence or absence of FCS (Fig. 4). It is notable that in HUVEC, these recombinant proteins were only found to bind to LDLr because this cell line did not present significant amounts of CD81 receptors under the experimental conditions that were used in this work.

With respect to ECV304 cells, the presence of FCS was important for the binding efficiency of both recombinant E2 proteins. However, these results differed from those obtained with HUVECs, which, in the absence of LDL, were totally dependent on the presence of FCS in culture media. Similar to the results obtained using HUVEC, in ECV304 cells, the addition of human LDL led to a significant increase in binding in all of the experimental conditions that were tested (Fig. 5).

Furthermore, we also observed more significant binding ($p < 0.01$) of E2Y vs. E2B in both cell types in the presence of LDL or FCS.

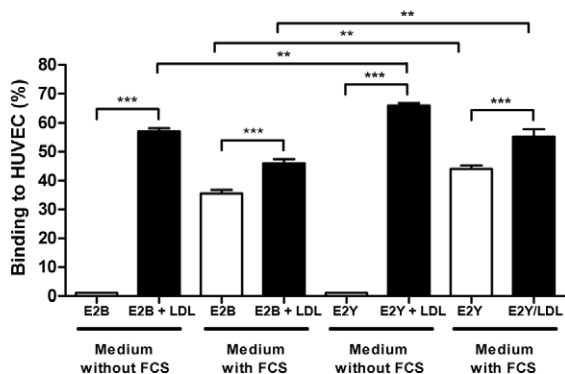


Fig. 4: binding of recombinant E2B and E2Y to human umbilical vein endothelial cells (HUVEC) - effect of the addition of low-density lipoprotein (LDL) and foetal bovine serum (FCS). The results are mean and standard deviation. **: $p < 0.01$; ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

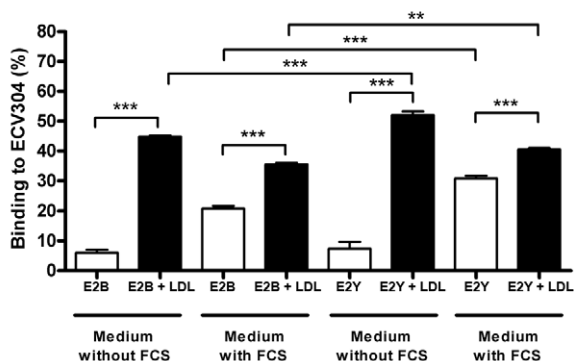


Fig. 5: binding of recombinant E2B and E2Y to ECV304 - effect of the addition of low-density lipoprotein (LDL) and foetal bovine serum (FCS). The results are mean and standard deviation. ** $p < 0.01$; ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

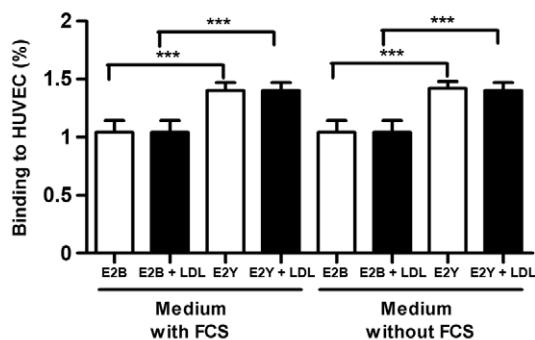


Fig. 6: binding of recombinant E2B and E2Y to human umbilical vein endothelial cells (HUVEC) with blocked low-density lipoprotein receptor (LDLr) - effect of the addition of LDL and foetal bovine serum (FCS). The results are mean and standard deviation. ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

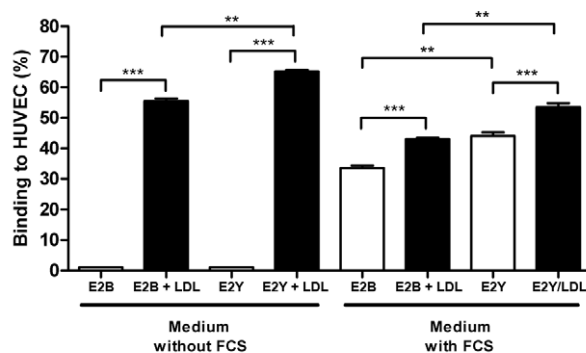


Fig. 7: binding of recombinant E2B and E2Y to human umbilical vein endothelial cells (HUVEC) with blocked CD81 - effect of the addition of low-density lipoprotein (LDL) and foetal bovine serum (FCS). The results are mean and standard deviation. **: $p < 0.01$; ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

Inhibiting recombinant E2 proteins from binding to cell receptors - In HUVEC, blocking LDLr led to a significant reduction in E2B and E29 binding both in the presence and absence of FCS and/or LDL in the media (Fig. 6). Blocking the CD81 receptor produced a markedly different result, as both the binding and the effects produced by FCS and/or LDL were similar to the results that were obtained in experiments that did not include the CD81 inhibitory antibody (Fig. 7). In another set of experiments, both LDLr and CD81 receptors were blocked and only a small amount of fluorescence was detected (Fig. 8), suggesting that these proteins might be binding to other receptors that are also able to bind E2 proteins, such as SR-BI, CLDN1, OCLN and TfR1. We also cannot rule out the possibility that LDL might modify E2 (e.g., conformation) and alter its interactions with CD81 or other cellular receptors in the absence of LDLr. Nevertheless, these results suggest that E2 proteins primarily bound to LDLr and not to another cell receptor. In ECV304 cells, blocking LDLr also led to a decrease in the binding of both recombinant proteins under all of the experimental conditions that were tested. However, this effect was not as intense as that which was observed in HUVEC. In Fig. 9, it can be observed that blocking LDLr could not completely eliminate binding. This may indicate that both CD81 and other E2 receptors that are present in this cell line, such as SR-BI, CLDN1, OCLN and TfR1, represent alternative binding sites, which may be more relevant in the context of E2Y than E2B. When blocking CD81, a small but significant decrease in binding capacity was also observed under all of the experimental conditions that were tested in ECV304 cells (Fig. 10). These results confirm that CD81 is also relevant to the binding of E2 recombinant proteins to ECV304 cells and they differ from what was observed in HUVEC, which were practically insensitive to blockage of this receptor. Finally, the simultaneous blockage of both receptors (Fig. 11) reduced the binding of E2 proteins to ECV304 cells, similarly to what was observed in HUVECs.

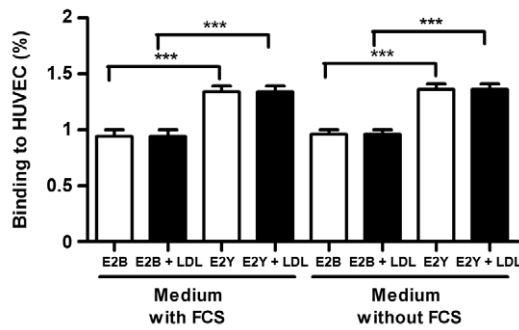


Fig. 8: binding of recombinant E2B and E2Y to human umbilical vein endothelial cells (HUVEC) with blocked low-density lipoprotein receptor (LDLr) and CD81 - effect of the addition of LDL and foetal bovine serum (FCS). The results are mean and standard deviation. ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

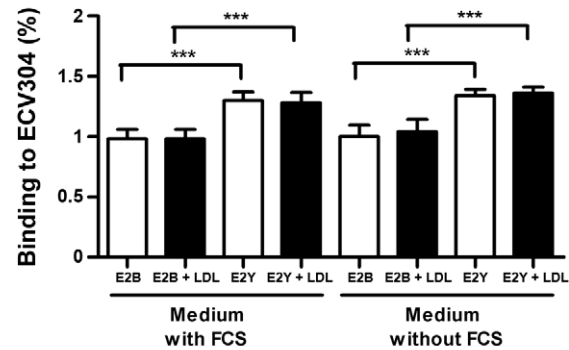


Fig. 11: binding of recombinant E2B and E2Y to ECV304 with blocked low-density lipoprotein receptor (LDLr) and CD81 - effect of the addition of LDL and foetal bovine serum (FCS). The results are mean and standard deviation. ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

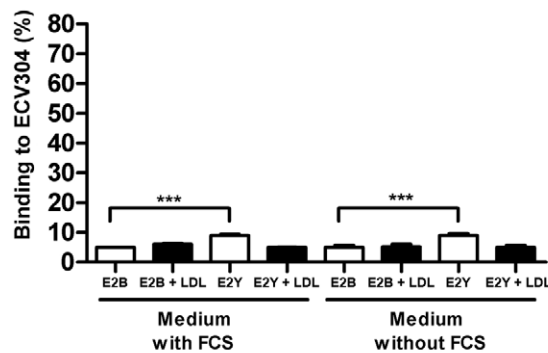


Fig. 9: binding of recombinant E2B and E2Y to ECV304 with blocked low-density lipoprotein receptor (LDLr) - effect of the addition of LDL and foetal bovine serum (FCS). The results are mean and standard deviation. ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

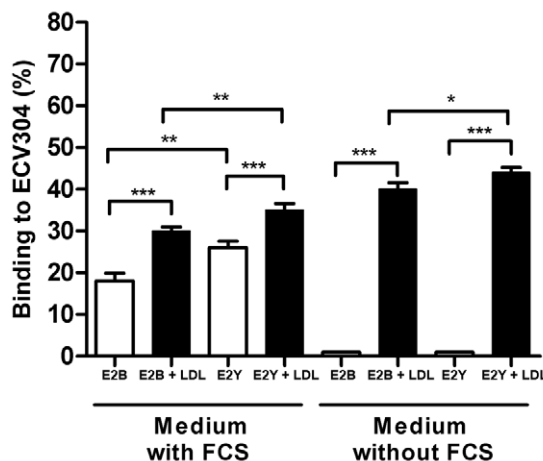


Fig. 10: binding of recombinant E2B and E2Y to ECV304 with blocked CD81 - effect of the addition of low-density lipoprotein (LDL) and foetal bovine serum (FCS). The results are mean and standard deviation. **: $p < 0.01$, ***: $p < 0.001$ (ANOVA and Tukey's multiple comparison test).

DISCUSSION

Many details of HCV receptors in host cells are not completely understood; however, there is evidence that E2 is the main protein responsible for the initial events that occur during the coupling of the virus to host cells (Zibert et al. 1995, Farci et al. 1996). Moreover, LDL and CD81 receptors are believed to mediate this process (Scarselli et al. 2002, Voisset & Dubuisson 2004). The absence of significant fluorescence that was noted with respect to CD81 is an indication that this receptor is not constitutively expressed in HUVEC under physiological conditions. Consistent with this, the expression of CD81 has previously been found to be reduced in endothelial cells in the absence of inflammation (Rohlén et al. 2009). In ECV304 cells, fluorescence was low but significant, which is an indication that the CD81 receptor was expressed. This result can be explained by the fact that ECV304 is a hybrid cell line that was created from endothelial cells that were contaminated with epithelial cells from bladder carcinoma (T24/83) (Brown et al. 2000). We believe that this might explain the very low but constitutive expression of CD81 in this cell line. However, we cannot rule out that the low percentage of ECV304 cells expressing CD81 may be related to a particular cell cycle phase.

The binding capacity of the recombinant E2 proteins to surface receptors in ECV304 cells was dependent on the presence of FCS and/or LDL in the culture media. These results suggest that E2 proteins are binding to CD81, as this receptor was present in a lower percentage of cells than was LDLr. Moreover, an absence of LDL in the culture media impeded the binding of E2 to LDLr, which was found to be present in a higher percentage of cells.

The presence of human LDL was also critical to the binding of both proteins to HUVEC when FCS was absent from the medium. This is an indication that the virus primarily bound to LDLr. Coupled with the absence of CD81 in HUVEC, this finding suggests that, in both of the cell types that were used in this study, the interaction of E2 with LDLr seems to be at least partially mediated by LDL. Consistent with our findings, experiments that

were performed to assess the binding properties of E2 proteins that were expressed in Chinese Hamster Ovarian cells revealed that binding was increased in a human acute lymphoblastic leukaemia cell line (MOLT-4) when using media that contained both LDL and FCS (Wünschmann et al. 2006). It is notable that several studies have used HCV particles that were produced in vitro (Bartosch et al. 2003, Zhong et al. 2005), which are structurally different from each other and from particles produced in vivo (André et al. 2002). In this study, we tested the recombinant proteins in two different organisms and found effects that were similar to previously reported findings.

When HUVEC were blocked against CD81 and cultured in media that lacked FCS, neither of the recombinant proteins was able to significantly bind to the cells until after the addition of LDL. Additionally, when cells were incubated with anti-LDLr, the binding of E2 proteins was not significant, suggesting that the binding of E2 proteins to cells occurs completely *via* LDLr. Altogether, these results suggest that LDLr is the primary target of both proteins under conditions in which they are associated with LDL. In agreement with these findings, CD81-deficient cells have previously been demonstrated to internalise HCV after it binds to LDL and uses LDLr (Agnello et al. 1999). Additionally, LDLr-deficient cells are less susceptible to HCV infection (Hishiki et al. 2010). Indeed, LDLr has long been considered HCV's route of entry into host cells (Wünschmann et al. 2000, Seipp et al. 1997). LDLr has also been shown to be relevant for the cell entry of various members of the Flavivirus genus (Wünschmann et al. 2000).

Another important finding was the verification that E2Y always exhibited a greater binding capacity than E2B. The primary difference between these two proteins is the glycosylation of E2Y. These results are in agreement with previous reports in which glycosylation was found to be indispensable for the global folding and conformation of this viral envelope protein (Goffard & Dubuisson 2003, Voisset & Dubuisson 2004, Bian et al. 2009, Lin et al. 2009). Moreover, glycosylation is also important for the ability of E2Y to bind to LDL and to other receptors of host cells, thereby enabling the coupling and entry of HCV into cells (Dubuisson & Rice 1996, Goffard et al. 2005, Tello et al. 2010). A previous report by Yurkova et al. (2004) found that glycosylation was not important for the binding of E2 to CD81. This finding was not corroborated by our experiments, as we found that E2Y more efficiently bound to both CD81 and LDLr than did E2B.

In vivo assays have shown the presence of HCV RNA-containing particles in low-density fractions of plasma, a finding that has been associated with high infectivity. However, the nature of circulating HCV particles and their association with immunoglobulins or lipoproteins, as well as the details of their cell entry mechanisms, have all been subject to conflicting reports (André et al. 2002). Nevertheless, the importance of HCV's association with LDL during viral entry through LDLr has been observed both in vivo and in vitro.

In conclusion, we have observed that the recombinant E2 HCV protein is dependent on LDL for its ability to bind to LDLr in HUVEC and ECV304 cell lines. The as-

sociation between E2 and LDL during the coupling of E2 to host cells has been demonstrated elsewhere (Thomssen et al. 1992, Nahmias et al. 2006); hence, the major contribution of this work is the demonstration that endothelial cells are also a potential target for this association. This may be related to the initial inflammation that is caused by HCV in endothelial cells of the hepatic portal vein, which may contribute to HCV-mediated cirrhosis.

This finding is very relevant because E2 serves as an anchor protein during the binding of HCV to host cells and high blood levels of LDL might enhance viral infectivity in chronic hepatitis C patients. In fact, LDL has been shown to promote an increase in HCV infectivity in several previous studies (Lavillette et al. 2005, Meunier et al. 2005, Nahmias et al. 2006, Siagris et al. 2006, Hishiki et al. 2010). The glycosylation of E2 is of critical importance for enabling the virus to anchor to LDLr. Additionally, although many studies have indicated that the E1E2 heterodimer is necessary for viral association with host cell receptors, we demonstrated here that the E2 protein is able to independently bind to these receptors. Finally, the putative mechanism of cellular infection involving E2, LDL, CD81 and LDLr could offer potential new targets for the development of novel antiviral therapies. Moreover, controlling serum LDL levels might also be helpful for the treatment of chronic hepatitis C.

ACKNOWLEDGEMENTS

To Prof Iracilda Zeppone Carlos (School of Pharmaceutical Sciences/UNESP), Prof Dulcinéia Saes Parra Abdalla (School of Pharmaceutical Sciences/USP), Prof Marcelo Dias Baruffi (School of Pharmaceutical Sciences of Ribeirão Preto/USP), Marisa Polezi Placeres, Felipe Fortino Verdã da Silva, Livia Carolina de Abreu Ribeiro, Thalita Athiê Nêo and Thaís Ferreira Isabel (School of Pharmaceutical Sciences of Araraquara/UNESP).

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