Short Communication

Surface expression of squamous cell carcinoma antigen (SCCA) can be increased by the preS1(21–47) sequence of hepatitis B virus

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A variant of the serpin squamous cell carcinoma antigen (SCCA) has been identified as a hepatitis B virus binding protein and high expression of SCCA has recently been found in hepatocarcinoma. Since HBV is involved in liver carcinogenesis, experiments were carried out to examine the effect of HBV preS1 envelope protein on SCCA expression. Surface and intracellular staining for SCCA was assessed by FACS analysis. Preincubation of HepG2 cells and primary human hepatocytes with preS1 protein or with preS1(21–47) tetrameric peptide significantly increased the surface expression of SCCA, without modification of its overall cellular burden, suggesting a surface redistribution of the serpin. An increase in HBV binding and internalization was observed after pre-incubation of the cells with preS1 preparations, compared to cells preincubated with medium alone. Pretreatment of cells with DMSO, while not influencing SCCA basal expression, was responsible for an increase in the efficiency of HBV internalization and this effect was additive to that obtained after incubation with preS1 preparations. In conclusion, the HBV preS1(21–47) sequence is able to induce overexpression of SCCA at the cell surface facilitating virus internalization, while the increased efficiency of HBV entry following DMSO addition is not mediated by SCCA.

A variant of squamous cell carcinoma antigen (SCCA) has been recently isolated by affinity chromatography from solubilized HepG2 plasma membranes (De Falco *et al.*, 2001b) and identified as a hepatitis B virus binding protein. This protein belongs to the serpin ovalbumin family, a large superfamily of serine protease inhibitors with pleiotropic biological activities. Previous studies have shown a cytosolic localization for SCCA, not associated intimately with membrane-bound organelles or cytoskeletal structures (Uemura *et al.*, 2000). More recent data indicate an additional surface localization for this serpin (De Falco *et al.*, 2001b; Mills *et al.*, 2002), suggesting an expanded role as a functional surface receptor or as an active proteinase inhibitor. Several studies indicate that SCCA is physiologically involved in the regulation of differentiation in normal squamous epithelium and is overexpressed in neoplastic tissue of epithelial origin, where it might be involved in the apoptotic pathway as a protease inhibitor (Suminami *et al.*, 1998). Recent findings have shown that SCCA variants are also highly expressed in hepatocarcinoma (Pontisso *et al.*, 2003), a hepatic malignancy and here the role of HBV infection is supported by epidemiological and molecular evidence (Beasley *et al.*, 1981; Bueinda, 1998). Experiments

carried out with serum-derived HBV particles have demonstrated that isolated SCCA protein is responsible for HBV binding to and internalization into human liver cells, underlying its potential biological role in HBV infection (De Falco *et al.*, 2001b). Many cellular receptors, including the retinoic acid receptor (de Thé *et al.*, 1989) and interleukin-2 receptor α chain (Imbert *et al.*, 2002), upregulate their expression upon ligand binding. To explore whether this possibility also occurs for SCCA, we have examined what effect HBV binding has on cellular expression of the serpin. In addition, since DMSO has been shown to improve the efficiency of HBV infection in cultured cells (Rumin *et al.*, 1996), we have evaluated whether DMSO treatment can induce modifications of SCCA expression at the cellular membrane level.

Surface expression of SCCA was assessed using HepG2 cells, maintained in culture in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}), supplemented with 10 % bovine calf serum (Sigma). Samples obtained 24 h after seeding and containing 10^6 cells were mechanically detached and incubated with 10 µg of a monospecific rabbit

anti-SCCA variants antibody (Xeptagen, S.p.A, Italy) or with pre-immune rabbit serum for 20 min at 4 °C, washed with phosphate buffered saline (PBS) containing 10 % bovine calf serum (PBS–10 % FBS) and further incubated with FITC-conjugated goat anti-rabbit antiserum (Santa Cruz Biotechnolgy Inc.) at a ratio of 1:10 for 20 min at 4 °C. To assess the overall cellular burden of SCCA, parallel immunostaining experiments were carried out in cells permeabilized with a commercially available kit (Fix & Perm, Caltag), following manufacturer's instructions.

Surface SCCA expression was also assessed in HepG2 cells pretreated with 2 % DMSO for 18 h and washed twice with PBS–10 % FBS.

Cellular fluorescence was evaluated using a FACScan analyser (Becton and Dickinson) and data were processed using the CELLQuest software program (Becton and Dickinson). The difference between the mean log fluorescence intensity (MFI) of different samples was calculated using the Kolmogorov–Smirnov test for analysis of histograms.

Since the preS1(21–47) amino acid region of HBV was previously identified as the envelope virus sequence involved in the interaction with surface SCCA, we assessed the effect of different preS1 preparations on surface expression of SCCA. The following preparations were used: a) synthetic myristyl–preS1(2–119) protein (adw2 subtype) (De Falco *et al.*, 2001a); b) 4-preS1(21–47) tetrameric peptide, obtained starting from a polylysine core, as described (De Falco *et al.*, 2001b); or c) scrambled preS1(21–47) peptide used as a control (non-HBV peptide) NH$_2$-FDPNSPNWDNGFNALAQPDPHDGPFFL-COOH. This control peptide has been designed by scrambling the amino acid sequence of preS1(21–47) but maintaining its hydropathic profile, and was synthesized by solid phase chemistry on an automated peptide synthesizer. The non-HBV peptide was purified by semipreparative RP-HPLC and fully characterized by MALDI-TOF and amino acid analysis.

The preS1 synthetic protein and the peptides were dissolved at 7 µg ml^{-1} concentration in 3 ml PBS (pH 7·4) and added to HepG2 cells cultured in six-well plates (about 10^6 cells per well), as described above. As a control, cell plates were incubated with medium alone. After overnight incubation supernatant was removed, cells were washed with PBS containing 10 % FCS, mechanically detached using a scraper and surface expression of SCCA was assessed by FACS analysis, as described above.

A similar procedure was carried out with primary human hepatocytes, obtained from an explanted liver by collagenase perfusion (Rijntjes *et al.*, 1986) and seeded on culture plates pre-coated with human liver biomatrix. The approval for experimental use of the human liver was obtained from the Medical Ethical Committee. The induction experiments with preS1 preparations were carried out one day after seeding.

To verify whether the observed increase of expression of SCCA was associated with different efficiency in HBV binding and/or internalization, we tested virus per cell interaction capability in cultured cells treated or not treated with trypsin after pre-incubation with preS1 preparations, the non-HBV peptide or with medium alone. HepG2 cells, grown to semiconfluence in six-well plates, were pre-incubated overnight with medium alone or with medium containing 7 µg ml^{-1} of preS1 synthetic protein, preS1(21–47) tetrapeptide or non-HBV peptide. Medium was then removed and HBV particles were added at 4×10^8 genome equivalents ml^{-1} concentration in culture medium. After 18 h incubation at 37 °C the medium was removed, cells were washed twice with PBS–10 % FBS and cells were treated with 0·5 mg ml^{-1} trypsin, 0·5 mM EDTA for 5 min at 37 °C to analyse virus entry. Trypsin was removed and cells were washed twice with PBS–10 % FBS before cellular DNA extraction by phenol/chloroform procedure. Parallel experiments using medium alone instead of trypsin treatment were carried out to evaluate virus attachment.

To assess the efficiency of HBV attachment and entry into cells pre-incubated with preS1 preparations and treated with DMSO, similar experiments were carried out in parallel using HepG2 cells pretreated with 2 % DMSO for 18 h and washed twice with PBS–10 % FBS.

The amount of HBV DNA was assessed by PCR, starting from 1 µg of cellular DNA for each sample. The primers used for nucleic acid amplification were located in the core region of HBV genome (primer 1, 5′-TTGCCTTCTGACT-TCTTTCC-3′; primer 2, 5′-TCTGCGAGGCGAGGGAGT-TCT-3′) (Mantero *et al.*, 1991).

The internalized viral DNA was semiquantitatively evaluated by densitometry (Gel DOC 1000, Bio-Rad, program Quantity One, Bio-Rad) after agarose gel electrophoresis in TBE buffer (0·089 M Tris, 0·089 M boric acid, 1 mM EDTA, pH 8) containing 50 µg ml^{-1} ethidium bromide. Serial dilutions of a known amount of HBV DNA, amplified in parallel, were used as a reference standard and a roughly linear detection between 10^3 to 10^7 genomes ml^{-1} was obtained (data not shown).

The expression of SCCA was assessed by FACS analysis in cell samples obtained 24 h after seeding and the results obtained in both HepG2 cells and primary human hepatocytes were similar. The addition of DMSO treatment did not influence basal expression of surface SCCA, as shown in Fig. 1. Pre-incubation of cells with preS1 containing reagents increased surface expression of the serpin, while the scrambled peptide did not affect the basal cell fluorescence signal (Fig. 2), confirming that the observed biological effect was strictly sequence dependent. Intracellular staining for SCCA, carried out in parallel experiments with permeabilized cells, did not show a significant increase in the total fluorescence intensity after incubation with preS1 reagents, suggesting a redistribution of the serpin at the
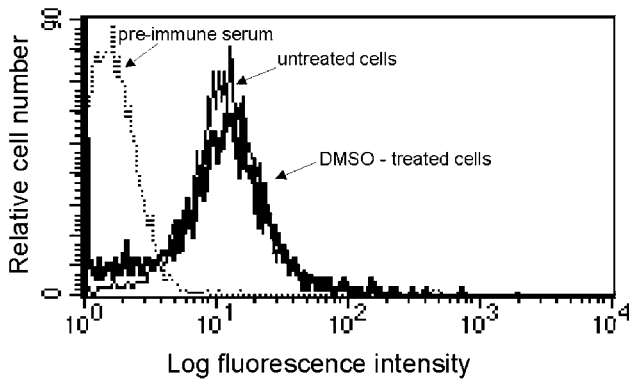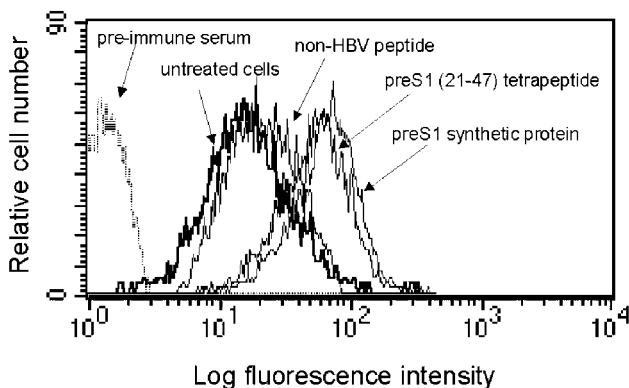
Fig. 1. FACS analysis of squamous cell carcinoma antigen (SCCA) expression in HepG2 cells treated with 2 % DMSO and in untreated cells. 10^6 DMSO-treated or untreated cells were mechanically detached 24 h after seeding and incubated with monospecific rabbit anti-SCCA variants antibody or with pre-immune serum, followed by FITC-conjugated goat anti-rabbit antiserum.

Fig. 3. HBV DNA attachment and entry detected by semi-quantitative PCR in cellular DNA extracted from trypsin treated (white bars) and untreated (black bars) cells, pretreated or not with 2 % DMSO and incubated overnight with HBV particles at 4×10^8 genome equivalents ml^{-1} concentration. Cells were previously incubated with identical concentrations of non-HBV peptide, preS1(21–47) tetrameric peptide, synthetic myristyl–preS1 protein or with medium alone (untreated cells).
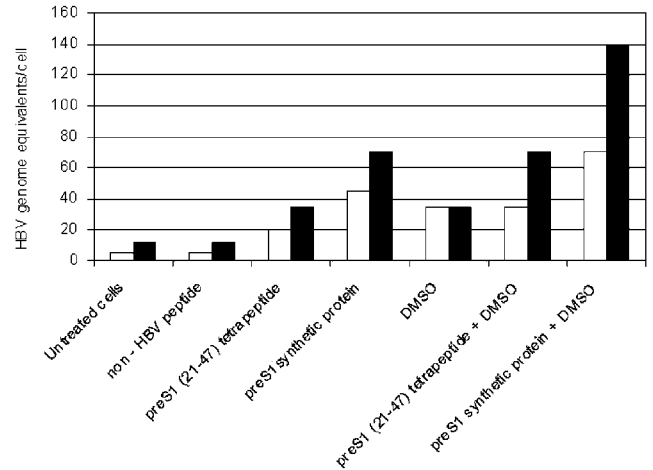
surface level, rather than an increase in overall expression of SCCA.

The preS1 preparations were able to induce a significant increase of surface SCCA expression, suggesting that the (21–47) sequence of the viral envelope was responsible for the observed event. The fluorescence gain achieved using the preS1 synthetic protein was not statistically different when compared to the results obtained using the preS1 tetrameric peptide; however the effect of preS1 synthetic protein was more remarkable when the different efficiency in HBV binding and/or internalization was evaluated after surface SCCA induction.

As shown in Fig. 3, an increased binding and percentage

Fig. 2. FACS analysis of surface squamous cell carcinoma antigen (SCCA) expression in HepG2 cells after 24 h incubation with identical concentrations (7 μg ml^{-1}) of preS1 synthetic protein, preS1(21–47) tetrameric peptide or of a preS1(21–47) scrambled control peptide (non-HBV peptide).

of internalization was documented in cells pre-incubated with preS1 tetrameric peptide and the amount of HBV genomic equivalents was duplicated after pre-incubation with synthetic preS1, while non-HBV scrambled peptide did not affect HBV per cell interaction. The greatest effect of synthetic preS1 protein compared to preS1 peptide is probably due to its higher affinity for surface SCCA, as a consequence of partial folding of this myristylated synthetic protein, previously documented by circular dichroism measurement (De Falco et al., 2001a).

DMSO treatment alone induced an increased efficiency of HBV internalization, since all the virus bound to the cell surface was internalized, compared to the other controls, whereas about 50 % of the attached HBV particles were internalized. DMSO treatment and pre-incubation with preS1 preparations showed an additive effect on HBV binding and internalization, indicating that the mechanism involved in the improved capability of virus entry probably depends on different membrane modifications that co-operate in HBV internalization. Several reports have identified the HBV preS1(21–47) envelope sequence as the major attachment epitope (Neurath et al., 1986; Pontisso et al., 1989; Le Seyec et al., 1999). However, recent findings have reported that the small HBsAg contains a secondary attachment site that recognizes a distinct receptor on the cell membrane, supporting the hypothesis of a multivalent and cooperative mechanism of virus attachment to the cell surface, as occurs for many other viruses (Paran et al., 2001). The fact that DMSO was able to increase virus binding by activating this putative additional receptor, is also supported by our findings, whereas the confirmed increase in

efficiency in HBV internalization after DMSO treatment was not associated with modifications in the expression of the primary serpin receptor on the cell surface.

SCCA has recently been found to be overexpressed in hepatocellular carcinoma (HCC) (Pontisso *et al.*, 2003), as occurs in other tumours of epithelial origin (Kato, 1996). Whether the SCCA surface overexpression induced by the preS1(21–47) motif is one of the mechanisms by which HBV aids HCC development is still an open question and studies are currently ongoing in our laboratories.

In conclusion, the HBV preS1(21–47) sequence is able to induce overexpression of SCCA at the cell surface facilitating virus entry, whilst the increased efficiency of HBV entry following DMSO addition is not mediated by SCCA.

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