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Original article

A cellular model system for expression studies of coagulation proteins

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Abstract

Introduction: The development of novel antithrombotic agents directly affecting gene expression requires well established, reliable and useful in vitro model systems for initial validation of drug effects. Since most proteins involved in coagulation are synthesized by the liver, the hepatoblastoma cell line Hep G2 is introduced, here, as a model system to test nucleic acid based coagulation inhibitors. **Methods:** Hep G2 cells were characterized with respect to prothrombin, tissue factor and factor VIII expression in dependence of cell culture conditions. Reliable enzyme linked immuno sorbent assays as well as viability tests were introduced that allow drug screening procedures with multiple probes in microplate format. Furthermore, a multiplex PCR-procedure has been presented that offers the possibility to simultaneously detect the effects of a selected compound on two coagulation proteins in comparison to a house keeping gene. **Results:** Hep G2 cells were not affected in viability by cell culture conditions, while proliferation and the expression patterns of some coagulation factors were affected by the adhesion factor collagen. The prothrombin expression characteristics allowed us to choose a specific time point for the transfection of Hep G2 cells with prothrombin specific antisense oligonucleotides. Antisense oligonucleotides inhibited prothrombin expression independent from culture conditions and the effects were detected on protein-and mRNA-level. **Discussion:** Nucleic acid based agents require cellular in vitro model systems since they affect the process of gene expression and not the gene product. Hep G2 cells are a useful model to study effects of novel nucleic acid based coagulation inhibitors with an antisense mechanism of action on protein and mRNA level.
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1. Introduction

Current coagulation inhibitors are agents that interfere with post-translational modification of coagulation factors like warfarin or inhibit coagulation directly by binding to proteins like heparin, involved in coagulation. With increasing knowledge about nucleic acid based agents we envisaged a new class of coagulation inhibitors targeting coagulation factors on mRNA level with an antisense mechanism of action. Phosphorothioate modified antisense oligodeoxynucleotides (PS-ODNs) are especially qualified for this purpose. The phosphorothioate modification enhances nuclease stability but does not lead to a loss of sequence specificity (Agrawal & Lyer, 1995). PS-ODNs are therefore capable of recognizing their target by Watson-Crick base pairing. Recent studies showed furthermore the high bioavailability of PS-ODNs and that they are accumulated in the liver which is the main source of coagulation factors in man (Sands et al., 1994; Soni et al., 1998). The organ specific accumulation of PS-ODNs qualifies this type of nucleic acid based agents for the potential use as anticoagulants in vivo. We therefore sought a model system that allows the initial validation of PS-ODNs targeting specific coagulation factors.

Hepatocytes are often used as liver models since they correspond to 92.5% of the total liver cell volume. However the cultivation of primary hepatocytes for experimental procedures is laborious and problematic, because most cells survive only for few days and the hepatocyte specific cell functions decline after a few hours in culture (Reid & Rojkind, 1979). Therefore cellular systems are needed that are easier to handle and maintain constant cell function during cultivation. It has been proven that the hepatoblastoma cell line Hep G2 offers hepatocyte specific cell function (Bouma, Rogier,

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Verthier, Labarre, & Feldmann, 1989; Knowles, Howe, & Aden, 1980) that allow for example the study of effects of agents on the synthesis of prothrombin (Jamison, Burkey, & Degen, 1992; Karpatkin, Finlay, Ballesteros, & Karpatkin, 1987). Furthermore antisense oligonucleotides can be delivered into Hep G2 cells by liposomal transfection (Wu et al., 2001) and efficiently suppress gene expression (Kishima et al., 2002; Varga et al., 2001). Although the fact that Hep G2 cells express prothrombin (Fair & Bahnak, 1984), factor VIII (Ingerslev, Christiansen, Heickendorff, & Munck-Petersen, 1988) and tissue factor (Wojta et al., 1994) is well known, there is little data on the expression characteristics of these proteins by Hep G2 cells, which is a crucial factor limiting development of an efficient procedure for antisense mediated inhibition of gene expression.

We decided to characterize the expression of the possible antisense targets prothrombin, factor VIII and tissue factor on antigen level during 15 days and two different culture conditions. Collagen serves as a substratum for primary hepatocytes and is essential for their successful cultivation (Rubin, Oldberg, Höök, & Öbrink, 1978; Seglen & Fossa, 1978). We wanted to know if collagen affects the expression characteristics of our target proteins to choose the cultivation method that offers the best conditions for our experiments.

Here we describe a simple test system to assay cell viability of Hep G2 cells, and an ELISA method to quantify prothrombin, factor VIII and tissue factor in cell culture supernatant and cell lysate. These investigations can be done in microplate size and are suitable for screening with numerous samples.

Using prothrombin for validation we found that antisense oligonucleotides can be transfected into Hep G2 cells and are able to inhibit specifically the expression of a single clotting factor. Furthermore multiplex PCR analyses showed that the antisense effect is suitable to study feedback reactions on gene expression level within the coagulation cascade.

Our results show that Hep G2 cells are a reliable hepatic model to study effects on the expression of coagulation proteins which can be used as primary screening system during the development of novel antithrombotic therapeutics directly effecting gene expression, like antisense oligonucleotides.

2. Methods

2.1. Collagen coating of cell culture test plates

In a 24 well test plate (TPP, Trasadingen, Switzerland) 300 µl/well collagen type-I (Sigma, Steinheim Germany) solution (1 mg/ml in 0.1 M acetic acid) was incubated overnight at 4 °C and washed twice with 500 µl culture medium (see below).

For collagen coating of 96 well test plates (TPP), 100 µl/well collagen solution was used, and wells were washed twice with 200 µl culture medium.

2.2. Cultivation of HepG2 cells

The hepatoblastoma cell line Hep G2 (German Collection of Microorganism and Cell Cultures, Braunschweig, Germany) was cultured in RPMI 1640 Medium with Glutamax-I (Invitrogen, Karlsruhe, Germany) containing 10% fetal bovine calve serum (Biochrom AG, Berlin, Germany) and 80 mg/l gentamycin (Biochrom) and was passaged once a week after trypsination.

The cells were seeded for the experiments in collagen coated and uncoated test plates, with a density of 1×10^5 cells/well in 500 µl culture medium (24 wells) and 2×10^4 cells/well in 200 µl culture medium (96 wells). The cell culture medium was not changed during the experiment.

2.3. Characterization of cells

2.3.1. Total number of cells

Determination of the total number of cells was carried out with a Neubauer counting chamber. Cells were trypsinated and stained with 0.5% trypan blue (Chemapol, Prague, Czech Republic). Only unstained, living cells were counted.

2.3.2. Characterization of cell viability using WST-1 test

10 µl/well WST-1 reagent (Roche Molecular Biochemicals, Mannheim Germany) was added to cells in 96 well test plates and incubated 4 h at 37 °C. 50 µl of each well were transferred in a new 96 well test plate and the absorbance was determined at 450 nm with a microplate reader. This test is based on the conversion of a pale red tetrazolium to a dark red formazane by mitochondrial dehydrogenases. The formazane is released by cells in cell culture supernatant.

2.3.3. Cell viability determination using neutral red uptake assay

The culture medium of 96 well test plates was exhausted and 200 µl of RPMI 1640 medium (Invitrogen) with 80 mg/ml gentamycin (Biochrom) and 50 µg/ml neutral red (Fluka, Buchs, Switzerland) was added in each well and incubated 4 h at 37 °C. The neutral red containing medium was exhausted and cells were washed twice with 200 µl PBS (150 mM NaCl, 20 mM Na_2HPO_4). Neutral red was extracted from cells with 100 µl/well of an ethanol/acetic acid solution (50% ethanol, 1% glacial acetic acid). 50 µl of each well were put through a new 96 well test plate and the absorbance was determined at 540 nm in a microplate reader.

2.4. Quantification of protein expression using specific enzyme immunoassays (ELISA)

2.4.1. Preparation of samples for ELISA

The cell culture supernatant of 96 well test plates was exhausted and samples were stored at −20 °C. The remaining cells were lysed in 150 µl lysis buffer (0.05 M Tris, 0.1 M NaCl, 5 mM EDTA and 0.2% Triton X 100 (Sigma), at pH 7.6) 4 h at 37 °C. Cell lysates were stored at −20 °C.

2.4.2. Prothrombin ELISA

96 Well maxisorb immuno plates (Nalge Nunc International, Rochester, USA) were coated with 50 µl/well capture antibody (sheep anti-human prothrombin, Cedarlane, Ontario, Canada) at 5 µg/ml in coating buffer (15 mM Na_2CO_3, 35 mM

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