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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 797–801
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A sandwich ELISA for the estimation of human syndecan-2 and syndecan-4 in biological samples

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Accepted 2 October 2003

Abstract

Syndecans are integral membrane proteoglycans containing both chondroitin and heparan sulphate side chains. Syndecans are shed from the cell surface, and may be detected in cellular supernatant or body fluids. In this study, we wished to design ELISA methods for syndecan-2 and syndecan-4, with the future aim of analyzing syndecans in large number of patient materials. Briefly, sandwich ELISAs for human syndecan-2 and syndecan-4 were designed employing monoclonal mouse antibodies as capture and commercially available polyclonal rabbit anti-syndecan as secondary antibodies. Although no quantified standard was available, relative estimation of syndecan-2 and syndecan-4 levels was possible in a small pilot material. The paper presents the ELISA method for these proteoglycans. We suggest that this may be a useful tool in the analysis of patient materials and in the detection of syndecans during proteoglycan purification.
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Keywords: ELISA; Syndecans; Proteoglycans; Mesothelioma

1. Introduction

A number of different functions have been ascribed to the family of syndecans, four integral membrane proteoglycans. Many of these functions depend on ligand binding reactions involving the side chains while others relate to the protein core. Syndecans are shed from the cell surface, and can be detected in the extracellular matrix, in body fluids or in cell culture supernatants. These released fragments contain the heparan sulphate side chains, and therefore retain many of the ligand binding capacities. The biologic activities, in which these proteoglycans are involved, include cell-extracellular matrix adhesion and growth factor binding [1]. Syndecan-2 is also designated fibroglycan, and is highly expressed in areas of high morphogenetic activity such as epithelial-mesenchymal interfaces [2]. Syndecan-4 is expressed in almost all tissues, and functions cooperatively with integrins in the process of cell spreading, focal adhesion assembly and actin stress fiber assembly [3].

Our group has studied the expression of syndecan-2 and syndecan-4 in mesothelioma. We found that

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0731-7085/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00569-7

mesothelioma cells express both these syndecans, and that higher expression is seen in the epithelioid phenotype [4]. Also, we have demonstrated that the expression pattern of syndecans may be useful to distinguish between malignant mesothelioma, adenocarcinoma, and reactive benign mesothelium [5]. An enzyme-linked immunosorbent assay (ELISA) against syndecan-1 already exists in a commercial form, and has been employed for the analyses of large clinical materials [6]. We wished to design ELISA methods for syndecan-2 and syndecan-4, with the future aim of analyzing several syndecans in larger materials from patients.

In this study, we describe the ELISA method for syndecan-2 and syndecan-4, and the result of our pilot analyses.

2. Materials and methods

2.1. Antibodies

Monoclonal antibodies against syndecan-2 (10H4) and syndecan-4 (8G3) were developed by David et al. [2,7]. Polyclonal rabbit anti syndecan-2 (M-140) and rabbit anti syndecan-4 (H-140) were purchased from Santa Cruz Biothechnology. Horse-radish peroxidase (HRP) conjugated goat anti-rabbit IgG (62-6120) was purchased from Zymed.

2.2. ELISA

Monoclonal mouse anti syndecan-2 antibody 10H4 and anti syndecan-4 antibody 8G3 were diluted in phosphate-buffered saline (PBS) to a concentration of 5 μg/ml. 50 μl of diluted antibody was added to each well of a Nunc maxisorp 96-well plate. As a control, wells were incubated with PBS alone. Plates were left over night at 4 °C.

The following day, liquid was flipped off and all wells blocked with 150 μl of 0.5% (w/v) bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Wells were washed three times with 200 μl PBS, also containing 0.05% (w/v) Tween-20 (PBS-T).

Effusions sent for cytological analysis at the Department of Pathology, Huddinge University Hospital, were routinely pelleted and supernatants were collected and stored frozen at −20 °C without additives. All samples were thawed and diluted 1:2 in the dilution buffer (0.1% (w/v) BSA in PBS with 0.05% (w/v) Tween-20). One hundred microliter of diluted sample was added to duplicate wells. Plates were incubated for 2 h at room temperature.

After three additional washes with PBS-T, 50 μl of secondary antibody diluted 1:800 in dilution buffer was added to each well, for syndecan-2 (M140) and syndecan-4 (H140). Plates were incubated for 1 h at room temperature. After washing, plates were finally incubated for 1 h at room temperature with HRP-conjugated antibody, diluted 1:3000 in dilution buffer.

After washing, *o*-phenylenediamine dihydrochloride (OPD) tablets were dissolved according to the manufacturers instructions (one tablet in 3 ml of distilled water) and immediately prior to analysis, 0.5 μl of 30% (w/w) H_2O_2/ml of OPD solution was added to catalyze the reaction. Fifty microliter of the ready

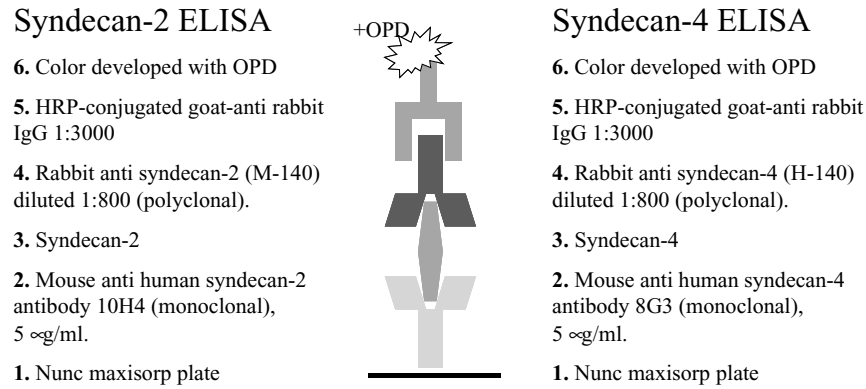

Fig. 1. Summary of the ELISA method for syndecan-2 and syndecan-4. For details, see materials and methods.

OPD solution was added to all wells. The reaction was stopped with 50 μl of 1 M H_2SO_4, and absorbance read at 490 nm. The ELISA methods are summarized in Fig. 1.

3. Results

Initially, four samples (two effusions, serum from one patient and dilution fluid alone) were added to wells coated with 10H4, 8G3 or PBS as a control. Wells coated with PBS alone subsequently passed through identical steps as those incubated with monoclonal antibody. As seen in Fig. 2, only one of these samples (ascites fluid from a patient with liver cirrhosis) resulted in absorbance above the background level. This sample was positive in wells coated with 10H4 and 8G3, but did not have increased absorbance in wells coated with PBS, showing the specificity of the reaction. The intra-assay coefficient of variation was less than 10%.

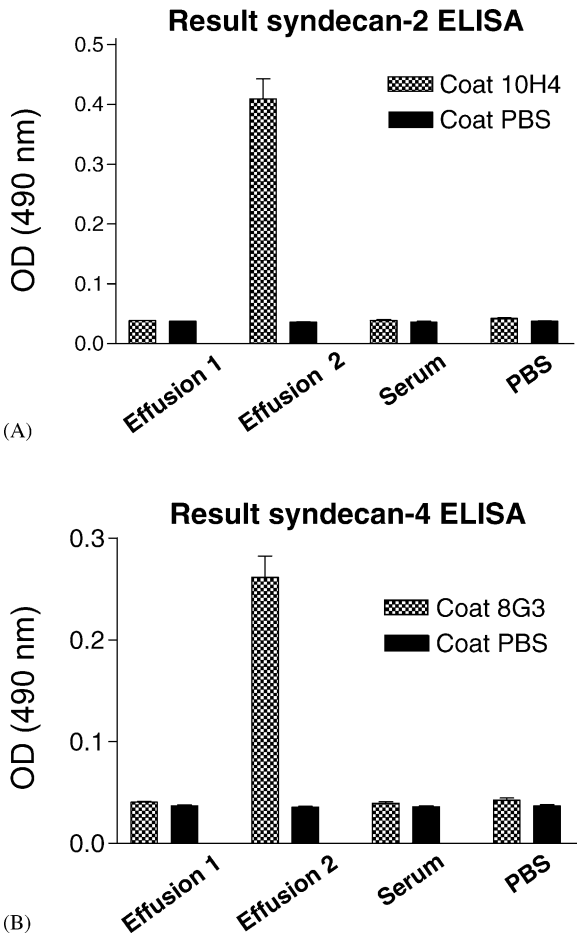

Fig. 2. Description of the results of a pilot study. Effusion two contained detectable levels of syndecan-2 and syndecan-4 by this ELISA, and was employed as a relative standard for subsequent analyses.

3.1. Analysis of serum and effusions

In order to compare levels of syndecan-2 and syndecan-4 between different samples, the above-mentioned ascites fluid (that resulted in a high absorbance to in both anti syndecan-2 and anti syndecan-4 coated wells) was designated as a "standard". This standard was diluted 1:2, 1:4, 1:8, 1:16 and 1:32 in dilution buffer in duplicate wells. Two additional wells were filled with dilution buffer alone. Linear standard

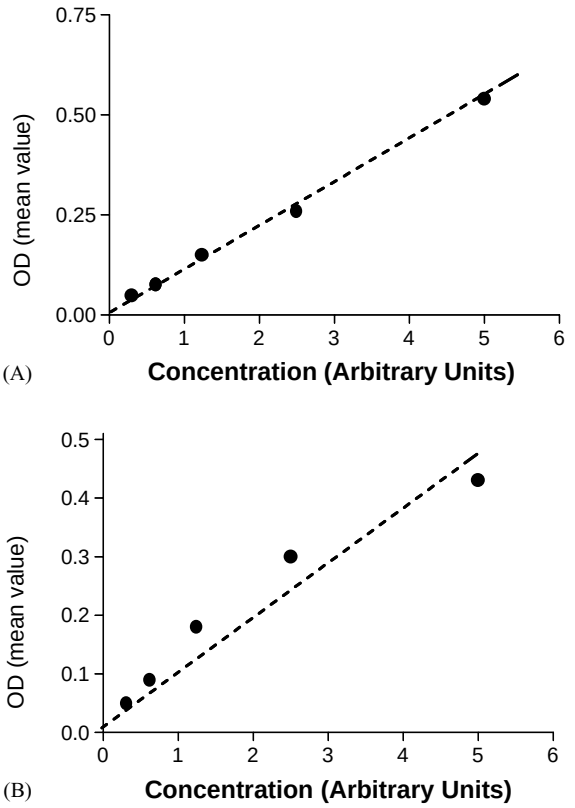

Fig. 3. Standard curve obtained for syndecan-2 (A) and syndecan-4 (B) with serial dilutions of sample 2 in Fig. 2.

Table 1
Results of pilot ELISA analyses of eight effusions

Sample description	Concentration	
	Syndecan-2 (U/ml)	Syndecan-4 (U/ml)
#1 Ascites fluid	0.62	Below detection limit
#2 Ascites fluid	Below detection limit	Below detection limit
#3 Pleural fluid	Below detection limit	Below detection limit
#4 Pleural fluid	>5	>5
#5 Pleural fluid	Below detection limit	Below detection limit
#6 Ascites fluid	0.55	Below detection limit
#7 Pleural fluid	0.50	0.45
#8 Pleural fluid	0.45	0.52
Standard (5 arbitrary units), 25% ethanol added	0.90	0.80

curves were achieved for syndecan-2 and syndecan-4 by this method (Fig. 3). The highest concentration of standard was defined as five arbitrary units (U), the lowest detectable level was 0.32 U. The linearity within the entire interval studied and the moderate absorbance at the highest concentration both indicate a wider analytical range for this assay.

We went on to examine the presence of syndecan-2 and syndecan-4 in eight effusions. Also, the highest concentration of standard (5 U) was supplemented with 25% ethanol and run as a sample in duplicate. Results are reported as arbitrary units (see Table 1). As shown, five out of eight effusions had detectable levels of syndecan-2. There were detectable levels of syndecan-4 in three out of eight fluids. The presence of 25% ethanol in samples, without further purification of the proteoglycan, inhibited the detection of syndecan-2 and syndecan-4.

4. Discussion

To our knowledge, this is the first report to demonstrate that syndecan-2 and syndecan-4 can be analyzed by an ELISA method, directed against the human core protein of these proteoglycans. For future work, these ELISAs may be useful tools in evaluating syndecan content in large patient materials or during the process of proteoglycan isolation and purification.

Although the number of samples in this study was too small to allow meaningful correlations between syndecans and disease characteristics, our small pilot experiments allowed us to make several observations. First, the assay was simple to use, and had a low intra-assay coefficient of variation. We found that syndecan-2 and syndecan-4 levels were highly variable in the effusions. The assay is easily run in one day, simplifying analyses of large patient materials.

However, this method has drawbacks. First, as the antibodies are directed against the core protein, measured levels do not necessarily reflect the intact proteoglycan content. Also, the presence of ethanol, which is often added to effusion fluids, almost completely inhibits detection of the core protein. Sample handling and storage should therefore be further evaluated. In this study, we employed a standard of aliquoted effusion known to have a high level of reactivity against both syndecans. Thereby, we cannot define the absolute amounts or the lower detection limit of our assays. Future efforts should therefore be put into purification and quantification of syndecan-2 and syndecan-4 proteins to use as defined standards in this ELISA.

5. Conclusion

In summary, we describe a fast and simple sandwich ELISA for the relative estimation of human syndecan-2 and syndecan-4 levels in vitro. After preparation and quantification of standards, this assay could be used to measure absolute levels of these proteoglycans in various biological samples.

Acknowledgements

This work was supported by the Swedish Cancer Funds (projects no. 2485 and 4577) and the Swedish Heart and Lung Foundation.

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