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CD146: biosynthesis and production of a soluble form in human cultured endothelial cells

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Abstract We previously identified the S-Endo 1-associated antigen (CD146), an endothelial member of the immunoglobulin superfamily with a characteristic V-V-C2-C2-C2 Ig domain structure. In cultured human endothelial cells, we investigated its biosynthesis by immunoprecipitation and pulse-chase labeling. CD146 was synthesized as a 100 kDa precursor form, which was processed into a 120 kDa mature form. In the culture media of endothelial cells, we observed a CD146 soluble form that was about 10 kDa smaller than cell-associated CD146. In parallel with soluble forms of other members of the immunoglobulin superfamily, soluble CD146 could modulate and control the functions of the molecule.

Key words: Human endothelial cell; Immunoglobulin superfamily; Adhesion molecule; Precursor processing; Soluble CD146

1. Introduction

Vascular endothelium expresses a number of cell adhesion molecules classified by their molecular and functional characteristics [1,2]. One major group is the immunoglobulin superfamily (Ig SF) including several members such as intercellular adhesion molecule-1 and -2, vascular cell adhesion molecule-1, and platelet endothelial cell adhesion molecule-1 (CD31) [3,4]. Among the members of Ig SF, we have identified the S-Endo 1-associated antigen [5], recently clustered as CD146 [6]. Originally described as a marker of melanoma progression [7,8], this membrane glycoprotein (of around 120 kDa) is expressed in all types of human endothelial cells [9]. This molecule shares homology with a subgroup of well-established adhesion molecules composed of five Ig domains (V-V-C2-C2-C2) [10–12]. Its belonging to this subgroup and recent studies suggest that CD146 may function in adhesion and in intercellular recognition. First, melanoma cells adhere to the purified molecule [13]. Second, CD146 expression increased homotypic adhesion between cDNA transfected melanoma cells [14]. Third, CD146 is concentrated in the endothelial intercellular junctions with an increased expression of the molecule at the cell-cell contact. Fourth, a codistribution of CD146 and adherent junction proteins in early stage of confluence suggests that it plays a role in initiating the cell-cell interaction (submitted). However, although CD146 may play a role in the vascular adhesion, little is known about its biochemical characterization.

In this report, we investigated the CD146 biosynthesis in cultured human endothelial cells since membrane glycoproeins are often synthesized as a precursor molecule [15]. Moreover, we evaluated whether a soluble CD146 form was produced by endothelial cells since it is known that soluble forms of adhesion molecules can modulate and control cell adhesion [16]. We found that endothelial cells, first, express a precursor form of CD146 and, second, produce a soluble CD146.

2. Materials and methods

2.1. Monoclonal antibodies

The mouse monoclonal IgG1 antibodies used were S-Endo 1 [17] raised against CD146, anti-CD31 (Immunotech, Marseilles, France), and ST4 raised against CD4 (Biocytex, Marseilles, France).

2.2. Cell culture

Endothelial cells (HUVECs) were harvested from human umbilical cord vein as previously described [18]. HUVECs were maintained in RPMI 1640 containing 20% fetal calf serum (FCS). Myeloid U937 cells, CD146 negative, were maintained in RPMI 1640 supplemented with 10% FCS. To detect soluble CD146, supernatants of confluent cultured cells were filtered on 0.22 μm filters, centrifuged at $100\,000 \times g$ for 90 min and concentrated about 10-fold with a Centrisart (100 000) (Sartorius, Göttingen, Germany). Samples were stored at −20°C.

2.3. Western blotting

Confluent cells were solubilized for 20 min at 4°C in RIPA buffer containing protease inhibitors as previously described [5]. Briefly, lysates were centrifuged at $100\,000 \times g$ for 30 min. Supernatants were electrophoresed on a 6.7% SDS-polyacrylamide gel, under reducing conditions (8% β_2-mercaptoethanol) or non-reducing conditions and transferred on nitrocellulose membranes. Membranes were incubated for 1 h with 1 μg/ml of S-Endo 1 and for 45 min with 1:5000 horseradish peroxidase anti-mouse Ig (Amersham, les Ulis, France). The blots were revealed with chemiluminescent substrate (Valbiotech, Paris, France).

2.4. Metabolic labeling and pulse-chase experiments

HUVECs (6×10^6 at about 75% of confluence) were preincubated with DME medium without methionine and cysteine for 1 h at 37°C and then metabolically labeled with 25 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (Amersham) for 4 h [19]. For biosynthetic studies, starved HUVECs were pulsed with 1 mCi/ml [^{35}S]amino acids for 15 min and chased for 1 h and 4 h in DME medium supplemented with methionine and cysteine. After lysis in RIPA buffer containing protease inhibitors, antigens were immunoprecipitated by using mAbs and anti-mouse IgG1 sepharose beads (Pharmacia, St. Quentin Yvelines, France). They were then eluted with Laemmli's buffer and analyzed on a 6.7% SDS-polyacrylamide gel. Gels were fixed (40% methanol, 10% acetic acid) and dried before exposure on a Kodak X-ray film at −80°C. In some experiments, immunoprecipitated CD146 was incubated at 37°C overnight with endoglycosidase H (4 mU, Boehringer Mannheim, Meylan, France) in 50 mM sodium citrate pH 5.5, 1% Triton X-100, 0.1% β_2-mercaptoethanol, 1 mM PMSF.

2.5. Peptide mapping

This mapping was performed according to the Cleveland modified procedure, using *Staphylococcus aureus* V8 protease [20]. Briefly, [^{35}S]-labeled antigens immunoprecipitated and separated on SDS-PAGE were excised from dried gel and transferred into the stacking of a second gel. Proteins were digested for 30 min with comigrated protease in reducing conditions. Peptide maps were then analyzed.

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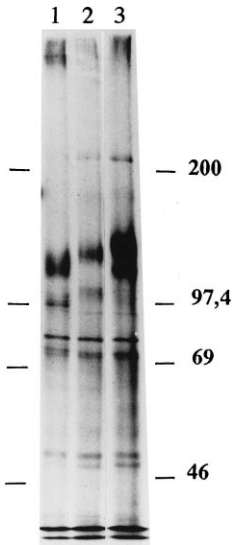

Fig. 1. Immunoprecipitation of CD146. [^{35}S]-labeled HUVECs were immunoprecipitated with S-Endo 1 in non-reducing (lane 1) and reducing conditions (lane 2) and with anti-CD31 in reducing conditions (lane 3), followed by SDS-6.67%-PAGE analysis.

3. Results

In previously Western blot experiments on HUVEC lysates, we found that S-Endo 1 specifically recognized a major protein of about 120 kDa (CD146), and a minor protein of about 100 kDa presenting a variable intensity [5]. Metabolic labeling was performed on HUVECs followed by specific immunoprecipitation (Fig. 1). Under non-reducing conditions, two proteins of 120 kDa and 100 kDa were immunoprecipitated with S-Endo 1 (lane 1). Under reducing conditions, their electrophoretic mobility was respectively increased to 130 and 110 kDa (lane 2), indicating intracatenar disulfide bonds. As control, two different proteins (135 and 125 kDa) were revealed when labeled HUVECs were immunoprecipitated with CD31 under reducing conditions (lane 3). Clearly, our results indicate that the 100/110 kDa protein (p100) specifically precipitates with CD146, the 120/130 kDa protein (p120). These data raised two hypotheses: either p100 coprecipitated with CD146 because it reacts directly with S-Endo 1, or p100 is physically associated with CD146. To distinguish between these hypotheses, several experiments were performed. First, we dissociated the labeled complex in 0.1% SDS for 5 min at 100°C and precipitated it with S-Endo 1 (Fig. 2). p100 was revealed in undissociated (lane 1) as well as dissociated (lane 2) immunoprecipitated CD146, indicating that p100 shared with p120 a common epitope recognized by S-Endo 1. Second, we performed partial proteolysis of p100 and p120. The comparison of peptide maps showed structural similarities (Fig. 3), suggesting that the two proteins are partially identical. Third, S-Endo 1 immunoprecipitation performed after cross-linking experiments indicated that CD146 is expressed at the cell surface as a monomeric structure of about 120 kDa (data not shown). Fourth, to determine whether p100 and p120 represent two steps of maturation of the same protein, HUVECs were pulsed 15 min and chased for various times in reducing conditions (Fig. 4). Pre-CD146 was detected within 15 min at a molecular weight of 110 kDa (lane 1). Significant levels of mature form of 130 kDa appeared after 1 h of chase (lane 2). After 4 h, only the mature form persisted (lane 4). In addition, when labeled HUVECs were treated with endo H, which cleaves high-mannose carbohydrate moieties, only the precursor form presented a significant faster migration (Fig. 4, lane 3). As controls, CD31 immunoprecipitated a protein of

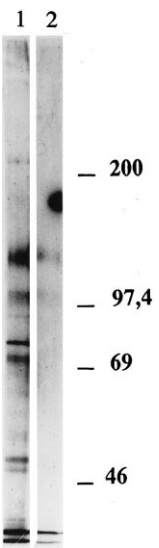

Fig. 2. Dissociation of the CD146-labeled complex. The CD146 immunoprecipitated with labeled HUVECs (lane 1) was dissociated in 0.1% SDS for 5 min at 100°C. It was immunoprecipitated a second time with S-Endo 1 (lane 2) and analyzed by SDS-PAGE.

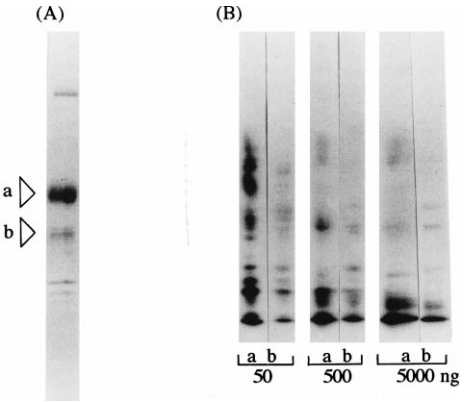

Fig. 3. Peptide mapping. A: The p100- (a) and p120- (b) labeled proteins were excised from the wet gel. B: The proteins (a and b) were incubated with increasing concentrations of protease and electrophoresed in order to obtain their peptide maps.

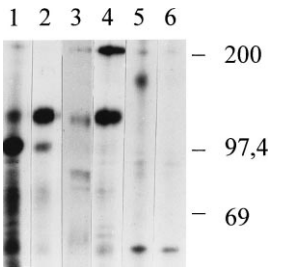

Fig. 4. Biosynthesis of CD146. HUVECs were pulsed with [^{35}S]amino acids for 15 min (lane 1) and chased for 1 h (lanes 2, 3) and 4 h (lanes 4, 5, 6). Cells were then immunoprecipitated with S-Endo 1 (lanes 1, 2, 4), anti-CD31 (lane 5) or ST4 (lane 6) and SDS-PAGE analyzed. Endo H treatment was performed on S-Endo 1 immunoprecipitated (lane 3).

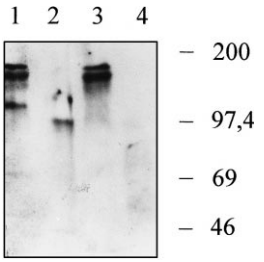

Fig. 5. Soluble CD146 detection. HUVEC supernatants were immunoprecipitated with S-Endo 1 (lane 2) and compared to the corresponding cell form (lane 1) after Western blotting. U937 lysate (lane 3) or supernatants (lane 4), used as controls, were treated under the same conditions.

about 135 kDa and irrelevant mAb did not reveal any band (lanes 5 and 6, respectively). Taken together, these results show that p100 and p120 represent two maturation states of CD146.

To examine whether a soluble form of CD146 might be synthesized by endothelial cells, we performed a two-site sandwich ELISA on HUVEC supernatants. Before concentration, samples of supernatants were filtered and ultracentrifuged to remove cell debris. The ELISA detected a soluble form of the molecule (data not shown). To verify the specificity of detection, Western blot analysis was undertaken on these supernatants previously immunoprecipitated by S-Endo 1 (Fig. 5). Soluble CD146, detected in the HUVEC culture media (lane 2), migrated more rapidly (around 105 kDa) than the corresponding cell-associated form (lane 1). As expected, no band was observed in lysates and supernatants of U937 cells used as negative control (lanes 3 and 4, respectively).

4. Discussion

We show that CD146 is synthesized by cultured endothelial cells as a precursor form (p100) further processed into a mature glycoprotein (p120) expressed at the cell membrane. We also demonstrated that CD146 is found in a soluble form (p105) in the culture media.

The pre-CD146 (p100) is supported by several lines of evidence. The two proteins immunoprecipitated by S-Endo 1, p100 and p120, shared common epitopes. Their peptide map revealed structural identities. Only the p120 is expressed at the cell membrane. Study of CD146 synthesis showed that the precursor form, p100, can be detected in as little as 15 min. It was then converted into p120 within 4 h. Analysis of the glycosylation pattern indicated that only p100 is sensitive to endo H digestion. Probably, high-mannose carbohydrate moieties allow p100 to be further processed in the Golgi apparatus [15], resulting in the mature form of CD146.

In a submitted manuscript, we described the progressive organization and accumulation of CD146 at the endothelial junction and reported the relative stability of its total amount. These results compared to the rapid kinetics of CD146 synthesis suggest a large turn-over of the molecule, which could be regulated by secretion or by proteolysis of the membrane form. Indeed, we observed a soluble CD146, about 10 kDa smaller than the corresponding cell-associated form. Mechanisms leading to the production of soluble CD146 are under investigation. Discovery of a soluble form in the culture media prompted us to look for soluble CD146 in normal human plasma and preliminary experiments are in agreement with its presence in healthy donors.

Soluble forms of adhesion molecules may function as competitive inhibitors of membrane-bound forms, thereby regulating cell adhesion [21]. This has been demonstrated for the soluble form of P-selectin, which is able to inhibit the adhesion of activated neutrophils to endothelial cells [22]. In the same way, soluble CD31 was able to inhibit the heterotypic adhesion mediated by the cell surface CD31 expressed on transfected cells [15]. As there is evidence that CD146 plays a role in cell adhesion [13,14], we can suppose that the soluble form acts in modulating and controlling CD146 functions during adhesive interactions of human endothelial monolayer.

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