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Endocan expression is increased in the placenta from obese women with gestational diabetes mellitus

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ABSTRACT

Introduction: Endocan, a member of the proteoglycan family, is involved in a wide range of diseases including obesity and diabetes. The aim of this study was to determine the effect of (i) maternal obesity and gestational diabetes mellitus (GDM) on placental endocan expression; and (ii) endocan knockdown on markers of inflammation.
Methods: Endocan mRNA and protein expression was determined in human placenta from (i) lean and obese and normal glucose tolerant (NGT) pregnant women (n = 10 patients per group); and (ii) women with GDM and BMI-matched NGT women (n = 40 patients per group). Primary villous trophoblast cells and HUVECs were used to assess the effect of endocan siRNA knockdown on pro-inflammatory cytokines.
Results: There was no effect of maternal obesity on placental endocan expression. Further, endocan expression was not different between lean NGT and BMI-matched women with GDM. However, endocan mRNA and protein expression was significantly higher in placenta from obese women with GDM when compared to BMI-matched NGT women. Knockdown of endocan in villous trophoblast cells and HUVECs had no effect on infection- or inflammation-induced expression and secretion of IL-6, IL-8 and MCP-1.
Discussion: Endocan expression is increased in the human placenta from obese women with GDM, and in response to pro-inflammatory stimuli. Loss-of-function studies in villous trophoblast cells and HUVECs revealed that endocan is not directly involved in the genesis or in the expression of pro-inflammatory cytokines induced by LPS or IL-1β. Further studies are required to elucidate the functional consequences of increased placental endocan expression in obese GDM pregnancies.

1. Introduction

Proteoglycans are complex macromolecules that consist of a protein core (a polypeptide) with one or more linear polysaccharide chains of glycosaminoglycans (GAGs), and are copiously found on the surface of cells, intracellular compartments and in the extracellular membranes [1]. Proteoglycans including decorin, biglycan, perlecan, syndecans and glypicans are macromolecules that contain a core protein to which sulphated GAG chains are covalently linked. These include Chondroitin Sulphate (CS), Dermatan Sulphate (DS) and Heparan Sulphate (HS). Hyaluronan (HA), a non-sulphated GAG is an exception to this definition, as it lacks a protein core. Proteoglycans play important roles in multiple biological pathways, including cell proliferation, adhesion and migration and are associated with pathological conditions such as inflammation, cancer, infection, homeostasis and angiogenesis [2,3]. They also modulate the operative behaviours of growth factors, chemokines and coagulation factors which in turn regulates vascular endothelium [3].

Endocan, also known as ESM-1 and discovered in 1996, is a proteoglycan secreted by endothelial cells and is composed of a 165 amino acid core protein with one DS chain attached to serine 137 on

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the core [4]. Endocan is encoded on the long arm of chromosome five with three exons and two intervening introns. Its protein core has two domains, an N-terminal cysteine rich domain that is 110 amino acids long and contains an endothelial growth factor (EGF)-like region and a phenylalanine-rich domain; and a C-terminal region that is free of cysteine and is 55 amino acids long. Endocan is a vasculoprotective molecule in normal physiological conditions; it promotes vasodilation, protects the endothelium from migration and proliferation of inflammatory cells, and plays a central role in the regulation of inflammation-induced endothelial dysfunction [5–10].

In addition to the well described role of endocan in endothelial cell function, recent studies suggest that it also plays a role in regulating inflammation [5,9]. As such, increased endocan expression has been reported in various inflammatory disease states including arthritis [8], cancer [11–14] and obesity [15]. In addition, endocan is regulated by pro-inflammatory cytokines including IL-1β and TNF-α [2], and the bacterial endotoxin lipopolysaccharide (LPS) [16], and serum endocan levels are correlated to the severity of sepsis [17]. Notably, endocan mRNA and protein expression is increased in placenta from women with preeclampsia [18]. Inflammation, a central feature of pregnancies complicated by maternal obesity [19–24] and GDM [25–27], can induce a robust placental inflammatory response [28,29]. Similarly, endotoxemia, which is higher in obese women compared to lean pregnant controls [30], can increase inflammation associated with obese and GDM pregnancies [29,30]. There is, however, no data on placental endocan expression in obesity and GDM-affected pregnancies, or its role in modulating inflammation induced by pro-inflammatory cytokines or bacterial endotoxin. Thus, the aims of this study were (i) to determine the effect of pre-existing maternal obesity and GDM on endocan expression in human placenta, and (ii) investigate if endocan is involved in the genesis of pro-inflammatory cytokines in human primary villous trophoblast cells and primary human umbilical vein endothelial cells (HUVECs) obtained from term uncomplicated pregnancies. For these studies, inflammation was induced by the pro-inflammatory cytokine IL-1β and the bacterial product LPS as they have been shown to induce placental inflammation [28,29].

2. Materials and methods

2.1. Tissue collection

Approval for this study was obtained from the Mercy Hospital for Women's Research and Ethics Committee and written informed consent was obtained from all participating subjects. Women were invited to provide samples on the day of admission for surgery. All tissues were obtained at the time of term Caesarean section in the absence of labour to ensure there were no effects of labour on endocan expression. Indications for Caesarean section included repeat Caesarean section or breech presentation. Women fulfilling any of the following criteria were excluded; vascular/renal complication, multiple gestations, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute fetal distress and women with any other adverse underlying medical conditions.

Placenta with the attached umbilical cord was collected for two studies; expression studies using placenta villous tissue and cell culture studies using freshly isolated trophoblast cells and HUVECs. Placenta was obtained within 15 min of delivery. Placental lobules (cotyledons) were obtained from various locations of the placenta; the basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross-section. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. Tissues were washed extensively with PBS, and (i) immediately snap frozen in liquid nitrogen and immediately stored at −80 °C for analysis of protein expression by Western blotting as detailed below; or (ii) used for isolation of trophoblast cells as detailed below.

For the expression study, placenta was obtained from (1) normal glucose tolerant (NGT) women who entered pregnancy lean (BMI between 18 and <25 kg/m^2; n = 10 patients); (2) NGT patients who entered pregnancy obese (BMI ≥30 kg/m^2; n = 10 patients); (3) women with GDM who entered pregnancy lean (n = 20 patients); or (4) women with GDM who entered pregnancy obese (n = 20 patients). Women with GDM were diagnosed according to the criteria of the Australasian Diabetes in Pregnancy Society (ADIPS) by either a fasting venous plasma glucose level of ≥5.5 mmol/l glucose, and/or ≥8.0 mmol/l glucose 2 h after a 75 g oral glucose load at approximately 26–28 weeks gestation. Women with GDM were managed by diet alone (n = 20 patients; n = 10 lean and n = 10 obese) or insulin in addition to diet (n = 20 patients; n = 10 lean and n = 10 obese). Women diagnosed with GDM were prescribed insulin according to hospital guidelines for insulin therapy in GDM. Women were controlled by diet if their fasting glucose readings were maintained <5.5 mmol/l over a 2 week period post diagnosis. Women with fasting glucose readings >5.5 mmol/l over a 2 week period post diagnosis were placed on insulin for optimal glucose control. The relevant clinical details of the subjects in this cohort are detailed in Table 1.

2.2. Gene silencing of endocan in primary villous trophoblast cells and primary HUVECs

Isolation and purification of primary villous trophoblast cells was performed from placenta from NGT non-obese pregnant women. These cells were used for the siRNA studies detailed below. Placental villous cytotrophoblasts were isolated as previously described [28] by DNase/trypsin digestion and purified by separation on a Percoll gradient. Briefly, placental villous tissue (~25 g) was dissected and washed in saline and then digested three times in a HEPES-buffered salt solution containing 0.25% trypsin and 0.2 mg/ml DNAse. Tissue was shaken at 37 °C for 30 min. The cytotrophoblast cells were separated on a Percoll gradient and resuspended in standard cell culture medium (5.5 mM glucose, 44.5% DMEM, 44.5% Ham's-F12, and 10% fetal calf serum supplemented with antibiotics). The cells were plated on 24-well plates at a density of 5×10^5 cells per well. The cells were cultured for a total of 90 h at 37 °C in 8% O_2, 5% CO_2 atmosphere and the cell culture media was changed daily. Trophoblast cell purity was confirmed by high protein expression of cytokeratin-7 (epithelial cell marker), absence of vimentin (fibroblast cell marker) expression, and secretion of hCG (measure of biochemical differentiation).

Isolation and purification of primary HUVECs was performed from umbilical cord from NGT non-obese pregnant women. These cells were used for the siRNA studies detailed below. Isolation of HUVECs was performed as previously described [31]. Briefly, the umbilical vein was washed thoroughly with saline, and then incubated with 0.2% collagenase A for 10 min in a water bath. The detached endothelial cells were collected and resuspended in DMEM supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 10% FBS (v/v), 1% antibiotic-antimycotic (v/v), 1% endothelial cell growth supplement (ECGS) (v/v) and 1% heparin (v/v). The cell suspension was incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 21% O_2.

Transfection of trophoblast cells and HUVECs was performed as previously described [28] using endocan siRNA (siESM1) and negative control siRNA (siCONT) obtained from Ambion (Thermo Fisher Scientific; Scoresby, Vic, Australia). Briefly, cells were transfected with 150 nM siCONT or siESM-1 and 0.3% Lipofectamine