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Immunoglobulin isotype isolated from human placental extract does not interfere in complement-mediated bacterial opsonization within the wound milieu

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ABSTRACT

The wound healing potency of an aqueous extract of placenta can be evaluated through the presence of numerous regulatory components. The presence of glycans was detected by thin layer chromatography and fluorophore-assisted carbohydrate electrophoresis. Mass spectrometric analysis revealed the existence of multiple fragments of immunoglobulin G (IgG). IgG was present in the extract at a concentration of 25.2 ± 3.97 μg/ml. IgG possesses anti-complementary activity by diverting the complement activation from target surface. Thus, effect of placental IgG on complement–bacteria interaction was investigated through classical and alternative pathway and the preparation was ascertained to be safe with respect to their interference in the process of bacterial opsonization.

1. Introduction

Immunoregulatory effects of human placental extract along with wound healing ability, hormonal regulation, prevention of recurring respiratory infections, asthmatic bronchitis, etc. are clinically well established [1–7]. Consistent maintenance of fetal-maternal nutrient exchange through placenta endures the organ to have remarkable therapeutic potential. This is exhibited through multiple independent mechanisms in order to exhibit the cumulative effect. An aqueous extract of human placenta, the preparation of which has been elaborated in the text, plays an imperative role in various stages of healing. Studies have demonstrated its role in modulation of cytokine induction during different phases of healing, effective stimulation of collagen synthesis during regeneration and epithelialisation and placental growth factor (PIGF) as an accelerator of granulation tissue maturation [8–10]. Characterization of this placental extract lead to the identification of numerous biological regulators such as growth factors, receptors, glycosaminoglycans and PDRNs [11–19]. These findings indicate that placental extract plays a definitive role in wound repair through multiple regulatory mechanisms.

The role played by complex carbohydrates in wound repair is an active topic of investigation since the carbohydrate moieties of glycoconjugates are often important as recognition determinants in cellular interactions, modulation of immunogenicity etc. [20,21]. A previous study showed that in a *Pax6*$^{+/-}$ mouse model of Aniridia-related keratopathy, mutations in glycoconjugate composition of the cell surface might lead to an abnormal cellular migration phenotype [22]. This could cause impaired re-epithelialisation which in turn could elevate risk of infection, inflammation and undermine normal stromal remodeling. Another study suggested that cell surface proteins which are N-glycosylated with terminal fucosylation mediated wound closure of airway epithelial monolayer by promoting cellular adhesion and migration [23]. These observations have important implications in understanding the critical role played by the glycans moieties in augmenting wound repair.

Injury to the skin is equivalent to an immunological crisis since the sub-epidermal exposed tissue is vulnerable to bacterial colonization [24–28]. The innate immune system, which is composed of phagocytes and the complement system, provides the primary line of defense against invading pathogens [29]. The complement system which is composed of more than 50 plasma and membrane

Abbreviations: IgG, immunoglobulin G; ATP, adenosine triphosphate; NAD$^+$, nicotinamide adenine dinucleotide; CNBr, cyanogens bromide; PNGase F, peptide N-glycosidase F; BHI, Brain–Heart Infusion; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; BCIP, 5-Bromo 4-Chloro 3′ indolylphosphate; NBT, nitro-blue tetrazolium chloride; G6PDH, glucose-6-phosphate dehydrogenase; BSA, bovine serum albumin; EDTA, ethylenediamine tetra acetic acid; EGTA, ethylene glycol tetra acetic acid

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associated proteins, is a key mediator of inflammatory and immune responses [30–32]. It generates a flux of active signaling molecules that includes C1q, C3, C4 and C5, which help to orchestrate the recruitment of phagocytes from blood. The complement activation proceeds primarily through two pathways: classical and alternative. Another pathway involved in the complement activation is the Mannan-Binding Lectin (MBL) pathway. MBL triggers complement similar to C1q. All these pathways are activated by different molecules but in the end, they all converge to generate the same set of effector molecules [33]. The inflammatory response promotes the recruitment of neutrophils which cause the debridement of necrotic and apoptotic cells and eliminate infectious agents from the wound bed. Role of complement in wound healing has been exemplified by the involvement of C3a and C5a in promoting liver regeneration [34]. C1q, another member of the complement cascade, possesses proangiogenic activity along with the increase in tissue strength, inflammation, fibroblast migration and collagen deposition post application of C3 on wounded surface [35–37]. In wound physiology, bacteria compete for the nutrients in the wound milieu and cause significant destruction to the wound matrix [38]. Wound debridement is an effective method to physically remove the dead, devitalized tissue and reduce the bacterial bioburden. On the other hand, the complement system, based on its ability to interact with bacteria, provides an element of natural immunity. In order to evaluate the effect of promoter/inhibitor molecules on the complement mediated opsonization of bacteria, *pseudomonas aeruginosa* was used as the chosen strain because of its common availability in infected wound matrix. It is an opportunistic gram negative pathogen which causes serious infections in patients with severe burns, wounds injuries [39,40].

This study was initiated to study the role of glycoconjugates present in the placental extract in wound healing. Multiple chromatographic and biophysical evidences indicated the presence of IgG as a major constituent of glycoprotein content in human placental extract. IgG is known to be a preferential acceptor of activated C3 and might interfere in the process of opsonization of bacteria inside the wound milieu [41,42]. Thus, effect of placental IgG on complement-bacteria interaction was investigated, through classical and alternate pathways, to elucidate its role in complement regulation.

2. Experimental procedures

2.1. Reagents

Fine chemicals were obtained as follows: Glucose, ammonium molybdate, sodium dihydrogen arsenate, sodium potassium tartrate, ATP, NAD^+, hexokinase, G6PDH, copper sulphate, diphenylamine, aniline, ovalbumin, Concanavalin A, CNBr activated Sepharose 4B, methyl-α-D-mannopyranoside, PNGase F from *Elizabethkingia miricola*, sodium cyanoborohydride, reference human IgG, subtilisin, protein-A conjugated alkaline phosphatase, protein-A agarose, BSA, protein A-alkaline phosphatase, p-nitrophenyl phosphate, EDTA, EGTA from Sigma, USA; *P. aeruginosa* (ATCC 51679), BHI, phosphoric acid, agar from Himedia; C_{18} zip-tip, silica gel TLC plates from Merck, Germany; periodic acid, acidic fuschin sulfate, sodium metabisulfite from Pierce, USA; ANTS from Invitrogen; nitrocellulose membrane, BCIP, NBT from Promega; veronal buffer saline from Lonza. Other reagents were of analytical grade and purchased locally.

2.2. Placental extract

The drug house M/s Albert David Ltd., Kolkata, India supplied aqueous extract of human placenta that is sold as a licensed drug under the trade name 'Placentrex'. Preparation of the extract holding the confidentiality of the manufacturer's proprietary terms has been described [11]. In short, the extract is prepared from fresh term pooled placentae using single hot (90 °C) and cold (6 °C) water extractions followed by sterilization under saturated steam pressure of 15 psi at 120 °C for 40 min. It is routinely tested for HIV antibody and Hepatitis B surface antigen. It contains 1.5% benzyl alcohol (v/v) as preservative which does not interfere with the experiments presented here. Collection and handling of the placenta and manufacturing of the drug were done under the export license of the drug controlling authority of India.

2.3. Analysis of free/bound carbohydrates

2.3.1. Nelson-Somogyi method for reducing sugars

To 0.5 ml of glucose solution (0.0125–0.150 mM) or test samples, 0.5 ml of Somogyi's alkaline copper tartrate reagent was added and kept in a boiling water bath for 10 min. The tubes were cooled to room temperature and 0.5 ml of Nelson's arsenomolybdic reagent was added. The tubes were incubated at 25 °C for 10 min and the $A_{620\ nm}$ was read. Somogyi's and Nelson's reagents were prepared after [43]. Colorimetric estimation of molybdenum blue at 620 nm generated a standard curve of glucose showing linear dependence between absorbance and concentration (R^2 = 0.996, where R^2 is the regression coefficient).

2.3.2. Coupled enzyme assay for glucose

The test sample was added to 0.1 M Tris–HCl, pH 7.5 containing 2.1 mM $MgCl_2$, 1 mM ATP and 1 unit/ml of hexokinase to a final volume of 1 ml and incubated at 25 °C for 30 min. Then 1.5 mM NAD^+ and 1 unit/ml of glucose-6-phosphate dehydrogenase was added and again incubated for 30 min followed by measuring NADH production at 340 nm [44].

2.3.3. Detection of glycoconjugates by TLC

Glucose and galactose (5 μl, 10 mg/ml) and placental extract (5 μl, 100×) were loaded on a silica gel TLC plate (E Merck, Germany). Chromatograms were developed with the solvent system chloroform: acetic acid: water (12:7.5:1.5). Diphenylamine-aniline-phosphoric acid spray reagent was used in the detection of glycoconjugates after heating at 105 °C for 2 h [45].

2.3.4. Fluorophore assisted carbohydrate electrophoresis (FACE)

The oligosaccharides present in placental extract were analyzed using FACE [46,47]. Firstly, the placental extract was deglycosylated using PNGase F (peptide-N-glycosidase F from *E. miricola*). Briefly, the extract was dissolved in 50 mM Na-phosphate, pH 7.5 containing 0.005 ml of denaturation solution (0.2% SDS with 0.1 M β-mercaptoethanol) and boiled at 100 °C for 10 min. The solution was cooled and 10 μl (50 units/ml) of PNGase F was added to the reaction mixture. The solution was incubated at 37 °C for 3 h. The released oligosaccharides were dried and labeled by adding 5 μl of 0.2 M ANTS in acetic acid/water (3:17, v/v) and 5 μl of 1 M Na-cyanoborohydride in DMSO. The mixture was incubated at 37 °C for 15 h. Oligosaccharides labeled with ANTS were subjected to 32% PAGE. The stacking gel was 8% acrylamide/0.6% bisacrylamide. The running buffer was 0.025 M Tris/ 0.192 M Glycine, pH 8.4 and the electrophoresis was run at a constant current of 15 mA for 2 h.

2.3.5. Quantification of glycoprotein

Glycoprotein content of the extract was estimated by Schiff-periodic acid staining procedure using ovalbumin as reference. Ovalbumin (10–200 μg) and placental extract (50 μl; 100× conc.) were electrophoresed in 15%/20% SDS–PAGE respectively and stained using GelCode® Glycoprotein Staining Kit (Pierce) [19]. ImageJ densitometric analysis was performed to quantify glycoprotein staining. It