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Fibronectin on the bronchoalveolar surface in children with recurrent obstructive bronchitis

B Nagy*, Éva Katona**, J Erdei**, L Maródi*, E Székely***, T Márialigeti***, L Karmazsin*, J Fachet**

Department of Paediatrics*, Institute of Pathophysiology**, Debrecen University Medical School, Debrecen, and Paediatric Institute, Szabadsághegy***, Budapest, Hungary

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Fibronectin is normally present in the lower respiratory tract. Significantly increased levels of it were detected in the lavage fluid in patients with interstitial lung diseases. Because this molecule appears to mediate a number of components of the inflammatory process, we evaluated the status of fibronectin in plasma and bronchoalveolar lavage in patients with recurrent obstructive bronchitis when signs of severe chronic mucosal inflammation were observed bronchoscopically. There was no considerable difference in plasma concentrations of fibronectin obtained from healthy children and patients. A comparison of lavage fibronectin/albumin ratios with plasma fibronectin /albumin ratios suggested significant local production, especially when the lavage and plasma ratios were measured in the same patients. Phagocytic activity of alveolar macrophages and blood granulocytes from the same patients was enhanced at both concentrations of fibronectin used. This concentrations referred to values quantified in the lavage fluid. The metabolism of fibronectin seems to be an important factor in tracing the inflammation process not only in adults with chronic interstitial lung diseases, but also in children with recurrent obstructive bronchitis.

Fibronectin is an adhesive and opsonic glycoprotein found in extracellular fluids, on basement membranes and cell surfaces [28, 31]. Two major forms have been identified: cellular and plasma fibronectin [1, 28]. Cellular fibronectin mediates many cell-matrix interactions including cell-to-cell and cell-to-substrate contacts [4, 24]. Plasma fibronectin is important in wound healing, maintenance of cellular and extracellular integrity and augmentation of macrophage and neutrophil phagocytosis [6, 7, 8, 9]. Both forms of fibronectin protect against microvascular occlusion and altered vascular permeability [2, 24].

Decreased plasma levels of fibronectin have been described during episodes of shock, trauma, burn and, in association with sepsis, disseminated intravascular coagulation, and pulmonary dysfunction [24]. Depressed plasma fibronectin concentrations were also measured in healthy term and premature newborns and infants under 12 months of age, in comparison with normal adult values [2, 33]. This

deficiency was correlated with reticuloendothelial system hypofunction and changes in vascular permeability in respiratory distress syndrome [33].

Fibronectin is normally present in the lower respiratory tract, too [20]. This macromolecule is a major product of alveolar macrophages. It is secreted rapidly and localized at cell membrane binding sites for gelatin-coated particles [28]. It is hypothesized that this glycoprotein plays an important role in an opsonin-independent phagocytic mechanism that is specific for particles and clearance of collagenous debris, injured platelets, immune complexes and some bacteria [9, 28, 31]. In addition, fibronectin has been implicated as an important product for remodelling of damaged alveolar structures [24]. Significantly increased levels of fibronectin were detected in the lung lining fluid in patients with interstitial lung diseases which were correlated with the degree of severity of inflammation and derangements of the pulmonary extracellular matrix in these disorders [20].

Recurrent obstructive bronchitis is a symptom complex in infants and early childhood caused by various disorders, frequently respiratory viral and/or bacterial infections. In serious cases signs of chronic mucosal inflammation can be observed bronchoscopically in symptom-free periods, too. This persistent mucosal inflammation is considered as a basic cause of the recurrent obstructive symptoms [26]. Recently we have demonstrated the morphological and functional characteristics of the lavagable cells from these patients emphasizing the great influx of macrophages, the decreased phagocytic and killing activity of the mononuclear phagocytes, and the high percentage of bacteriologically positive lavage samples [17].

In this study, the status of fibronectin was evaluated in plasma and bronchoalveolar lavage fluid of patients with recurrent obstructive bronchitis and the effect of this glycoprotein on the phagocytic activity of alveolar macrophages and blood granulocytes in the presence of two concentrations of fibronectin was measured, for fibronectin seems to mediate a number of components of the inflammatory process.

Materials and Methods

32 children with recurrent obstructive bronchitis ranging in age from 1/2 to 6 years were studied who had suffered from severe episodes of obstructive bronchitis for months but they were symptom-free for at least two or three weeks at the time of the examinations. Allergic hypersensitization, focal infections, immunodeficient status, cystic fibrosis, malformation of heart and large vessels, gastro-esophageal reflux and other disorders which could be associated with obstructive symptoms, were excluded previously. Obvious signs of respiratory infections were rarely detected during obstructive episodes and serologic evidence of virus infections (Adeno-, Influenza A, B, Parainfluenza, Respiratory syntitial, Coxsackie B viruses) and Mycoplasma pneumoniae were developed in only 12 per cent of patients.

After obtaining consent from parents, bronchoscopy was performed for diagnostic

indications to reveal possible bronchial malformations, chronic foreign body, etc. using rigid tube bronchoscope (Friedel Bronchoscope model MLW Medizinische Geräte Berlin GRD). The right lower or middle lobe was lavaged as described elsewhere [17]. No side effects were observed at all. Bronchial washings were tested bacteriologically.

The recovered fluid was centrifuged at 800*g* for 10 min at 4 °C and the supernatant was decanted and immediately frozen at −40 °C until investigation. Each aliquot was used only once. Cells were resuspended in Hank's balanced salt solution (HBSS), counted and a cytocentrifuge preparatum was made. Viability of cells was determined by trypan-blue exclusion.

Quantification of fibronectin levels in plasma and lavage fluid

Plasma samples, anticoagulated with 3.5% sodium citrate, were collected from healthy children (n = 33) and patients (n = 18) on the morning of bronchoscopy. No child had a history of a recent infection or chronic disease in the group of healthy children. They were seen as outpatients. Plasma samples were centrifuged at 1200g for 15 min to remove cells and frozen at −40 °C until use.

Polyclonal anti-fibronectin antibodies

Rabbits were immunized with 1 mg purified human fibronectin in complete Freund's adjuvant distributed at several sites intracutaneously on the depilated back of the animals. The procedure was repeated at weekly interwals, until sufficient antibody activity was achieved. Rabbits were bled two weeks after the last boosting. Rabbit anti-fibronectin sera were purified by affinity chromatography on a fibronectin-Sepharose 4B column [23].

Monoclonal anti-fibronectin antibodies

Balb/c mice were primed subcutaneously with 100μg antigen in complete Freund's adjuvant. The animals were boosted two times with 100 μg antigen in saline intraperitoneally, in 3 weeks intervals. Fusion was performed 3 days after the last injection. Immune spleen cells were fused with 6-thyoguanine-resistant myeloma cell line, Sp-2/O according to a modification [25] of the method described by Köhler and Milstein [13].

The isotype of the monoclonal antibody 9/3 E9 used in this study is IgG_{2b}. It was labelled with horse radish peroxidase using the procedure of Wilson and Nakane [30].

The Solid-phase EIA procedure

Polystyrene microtiter wells (Nunc 96-well plate, Denmark) were coated with 50 μl of purified rabbit anti-human fibronectin antibodies diluted to 1 μg/ml in sodium carbonate buffer (0.1 M, pH: 9.6), and were then incubated overnight (16—20 h) at 37 °C. Immobilization was terminated by a 30 min incubation with 100 μl of phosphate buffer (PBS: 0.01 M, pH: 7.2) containing 0.05% (v/v) Tween 20 and 0.5% (w/v) BSA, and 0.5 M NaCl followed by washing twice with PBS-Tween 20. The wells then received 50 μl of the appropriate dilution of test specimen or fibronectin standard diluted in PBS-Tween 20 containing 0.5 M NaCl and 0.5% (w/v) BSA, and allowed to stand for 60 min at 37 °C. After 4 washings 50 μl of the peroxidase labelled monoclonal anti-human fibronectin antibodies, diluted in PBS-Tween 20 containing 0.5 M NaCl and 0.5% (w/v) BSA, were added to each well and incubated for 60 min at 37 °C. The washing procedure was repeated as above and the substrate, 50 μl of 0.003 M ortho-phenilenediamine and 0.3% (v/v) H_2O_2 in citrate-phosphate buffer (0.075 M, pH: 5) was then added to the wells. After an incubation for

30 min at room temperature the reaction was terminated by addition of 50 μl 4 N H_2SO_4 The absorbance values were measured at 405 nm. The detection limit of the assay was 30 ng/ml of fibronectin.

To compare fibronectin levels in lavage (n = 32) among different study groups and lavage fluid with plasma levels, albumin was measured by bromcresolgreen photometry [21, 22]. All fibronectin/albumin data were expressed as micrograms per milligram.

Assessment of phagocytosis by alveolar macrophages and granulocytes

Human granulocytes from heparinized, differential centrifuged and dextran-treated blood were obtained from healthy children [5]. Cell preparations were washed in HBSS and samples of 200 μl of 5×10^6 granulocytes/ml were used for phagocytosis of S. aureus. The decrease in the number of viable extracellular bacteria was measured [11, 14].

Alveolar macrophages derived from recovered lavage fluid (n = 5) were washed and suspended in HBSS containing 0.1% gelatin to a concentration of 10^7 cells/ml. To prevent attachment of the cells to glass surfaces, all experiments were performed with siliconized glassware or plastic tubes.

Preparation of human fibronectin

Human fibronectin was prepared from fresh, citrated human plasma by affinity chromatography on gelatin-Sepharose 4B and arginine-Sepharose 4B columns [29]. Protein concentration was determined by the absorbance at 280 nm using the extinction coefficient: $E^{1\%}_{280\,nm,\,1\,cm} = 13.5$ [18].

Phagocytosis was determined in the presence of serum of healthy donors with blood-group AB and at two concentrations of fibronectin: 110 μg/ml, 220 μg/ml. Both values of fibronectin used as an opsonin referred to concentrations quantified in lavage fluid.

Results

The mean recovery of lavage fluid was 42.2% of instillate for the group of patients. The average recovered cell count was 8×10^6 per patient and mean values for differential counts of recovered cells were: 97% alveolar macrophages, 2.4% lymphocytes and 1% granulocytes. No samples contained blood contamination at all. Viability of cells was 98%. The bronchial washings were bacteriologically positive in 62.1% of patients: Streptococcus in one third of cases, other bacteria (S. aureus, E. coli, H. influenzae, Klebsiella) only in a few cases.

Plasma levels for fibronectin were significantly lower in the group of healthy infants between 1—6 months of age than in older children (Fig. 1). No differences existed between groups over the age interval of 1—6 years. Comparing the values of normal children reported previously by others, our data were generally lower except for those found in the group 7—12 months of age [15]. There was no considerable difference in plasma concentrations of fibronectin obtained from the 1/2—6-year-old group of healthy children and patients with recurrent obstructive bronchitis 1/2—6 years of age. This correlation was demonstrated more strikingly by ratios of fibronectin/-albumin (Fig. 2).

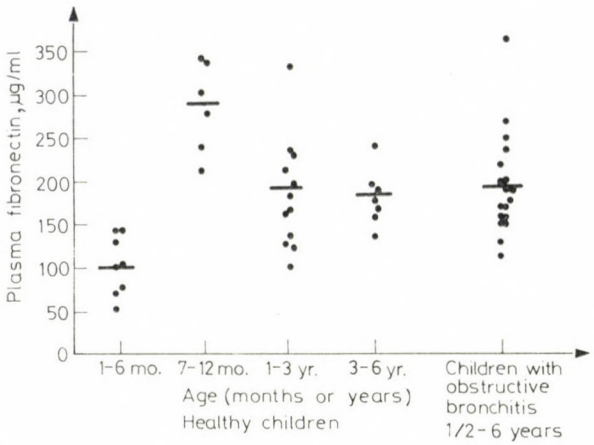

FIG. 1. Plasma fibronectin levels measured in healthy infants and children as well as patients with recurrent obstructive bronchitis. Each data-point represents a single patient. A horizontal line denotes the mean for the group

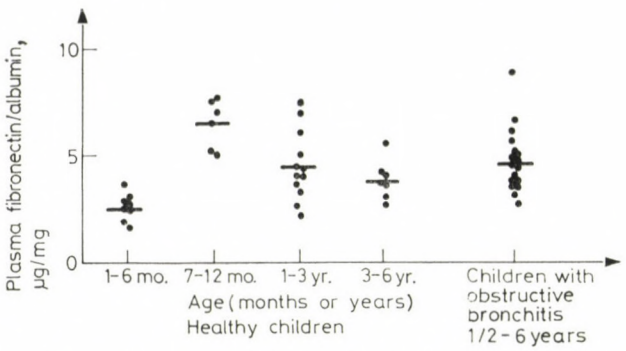

FIG. 2. Ratios of plasma fibronectin/albumin in healthy children and our patients are expressed as µg/mg. Means are represented by horizontal bars

Comparison of lavage with plasma ratios of fibronectin/albumin revealed that the lavage fluid ratios were elevated, even in sample-pairs obtained from the same patients suggesting the local concentration of this glycoprotein (Fig 3). In contrast, lavage fluid ratios of fibronectin in our patients were significantly higher than in normals and very similar to ratios of patients with interstitial lung diseases demonstrated by Rennard and Crystal [20]. (A true control group of children has not been investigated for ethical reasons.)

The capacity of alveolar macrophages to phagocytose S. aureus was enhanced in presence of each concentration of fibronectin, but the number of non-ingested viable bacteria in supernatant was higher than in the presence of AB serum (Fig. 4). This augmentative effect was similar at both amounts of fibronectin used. Concentrations of fibronectin used in the phagocytic assays nearly correspond-

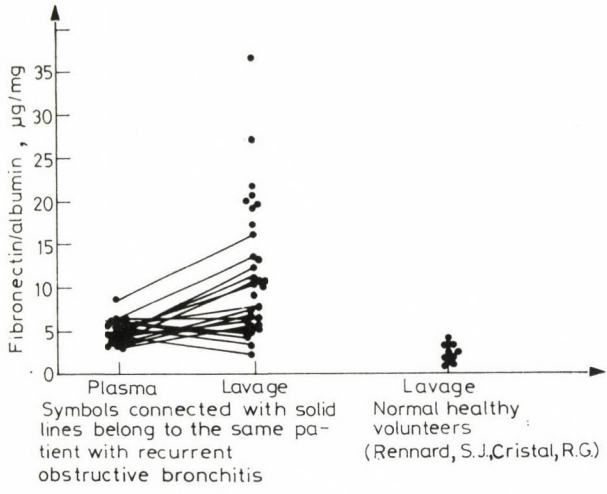

FIG. 3. A comparison of fibronectin/albumin in plasma and lavage fluid is shown for patients with recurrent obstructive bronchitis suggesting the local production or concentration of fibronectin on the bronchoalveolar surface. In contrast, lavage fluid ratios of fibronectin in our patients were also significantly higher than in normal adults (mean age 27.6 ± 4.6 years, three of them were smokers) demonstrated by Rennard and Crystal [20]

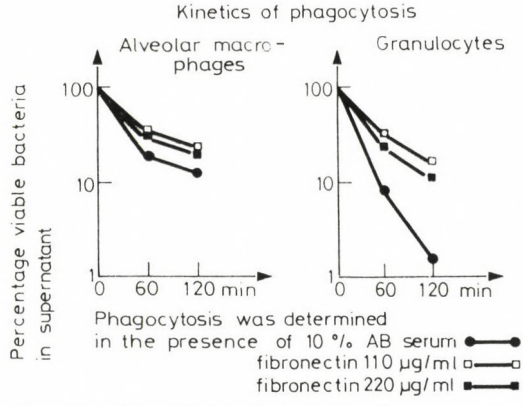

FIG. 4. The phagocytic capacity of alveolar macrophages was enhanced at each concentration of fibronectin used, but the percentage of non-ingested bacteria in supernatant was higher than in the presence of AB serum

ed to plasma levels and their halves, but lavage fluid from our patients contained much more elevated levels. Phagocytic activity of granulocytes from the same patients was slightly increased in the presence of fibronectin concentrations compared to that found in the case of alveolar macrophages. The opsonic effect of AB serum during ingestion of S. aureus by granulocytes resulted in a considerable decrease in the number of viable extracellular bacteria.

Discussion

There are several possible mechanisms to explain the presence of fibronectin on the bronchoalveolar surface of the human lung. Intact plasma fibronectin with a molecular weight of 440 000 can permeate the alveolar-capillary structures from plasma [27] and it is also produced locally by lung fibroblasts and alveolar macrophages [3, 19, 31]. Plasma and cellular fibronectin are indistinguishable antigenically, but subtle differences between them have been described [32]. Fibronectin is primarily a soluble secretory product of human alveolar macrophages in vitro whereas fibroblasts synthesize it as a component of an insoluble extracellular matrix [1, 28].

In normal adult individuals the level of fibronectin/-albumin in lavage averaged 25% of that of plasma [20]. Increased levels of fibronectin in the lower respiratory tract of patients with interstitial lung diseases were detected [20] which is derived mainly from local production and concentration. Tissue fibronectin or its fragments can be released to the epithelial surface by the effect of proteolytic enzymes [16]. Increased degradation and accelerated turnover of fibronectin in the lung are characteristic of interstitial disorders. Alveolar macrophages are expanded in number in these diseases and capable of producing increased amounts of fibronectin [32].

Fibronectin on the epithelial surface of the lower respiratory tract in patients with chronic lung inflammation may be a byproduct and may not relate to the pathogenesis [20], but may play an important role in the remodelling of the lung parenchyma. It may also play the role of an opsonin for phagocytosis of matrix debris after injury and that of a chemoattractant for fibroblasts [24], as well as an adhesive protein and cell modulator for certain cell types [10].

In this study, plasma fibronectin levels and fibronectin/albumin ratios in healthy children and patients with recurrent obstructive bronchitis were similar or slightly lower than in normals [15]. A comparison of lavage fibronectin/albumin ratios with plasma fibronectin/albumin ratios suggested significant local production, especially when the lavage and plasma ratios were measured in the same patients.

Phagocytic activity of alveolar macrophages and blood granulocytes from the same patients was enhanced at fibronectin concentrations of 110 and 220 μg/ml which was due to a considerable non-immune opsonin effect of this glycoprotein. Concentrations of fibronectin used were in correspondence with endogenous conditions. The phagocytosis-enhancing activity is probably achieved by fragmented fibronectin through the recognition unit for particulate activators of the phagocytes. Active fragments may be generated by proteolysis from intact plasma fibronectin molecules [16].

Finally, alveolar macrophages have an opsonin independent phagocytic

mechanism and these cells may be able to augment their own phagocytic capacity to produce fibronectin. The metabolism of fibronectin appears to be an important factor in tracing the inflammatory process and in monitoring the morphological and functional outcome of lung diseases not only in adults with chronic interstitial lung diseases, but also in children with recurrent obstructive bronchitis and severe chronic signs of mucosal inflammation.

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B Nagy MD
P. O. B. 32
H-4012 Debrecen Hungary