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Adipocytes derived fibrinolytic components in peritoneum – a pilot study

Rapid Communication

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Abstract: The proteins of the fibrinolytic system – urokinase plasminogen activator(uPA), tissue plasminogen activator (tPA)and plasminogen activator inhibitor type IPAI-I) – play important roles in fibrotization in various organs and including peritoneum. To study the cellular localization of PAI-1, tPA and uPA within the adipose tissue of the peritoneal membrane in patients at the onset of peritoneal dialysis(PD) we determined the initial expression of these proteins in relationship to multiple clinical variables. Methods: routinely performed parietal peritoneal biopsies in 12 patients undergoing peritoneal catheter implantation were examined. We used formalin-fixed, paraffin-embedded specimens for immunohistochemical localization of these proteins along with the stereological point-counting method for quantification of their expression within the peritoneal adipose tissue. Results: strong positive mutual correlation between the expression of PAI-1 and both uPA (SpearmanR=0.66) and tPA (R=0.59) as well as between the expression of uPA and tPA (R=0.77) was found without any relatioship to BMI, age, peritoneal transport characteristic or diabetes status. Conclusion: Adipose tissue within the peritoneum is capable of producing fibrinolysis regulators (independently on clinical parameters) thus possibly affecting the fibrotization and function of peritoneum as dialysis membrane. The effect of dialysis solution dosing, composition and other dialysis related factors should be clarified in future studies.

Keywords: *Peritoneal dialysis • Adipose tissue • Histology • Morphometry • PAI-1 • tPA • uPA*

1. Introduction

The most important mechanisms underlying ultrafiltration failure during peritoneal dialysis (PD) are the thickening and fibrosis of submesothelial connective tissue and changes in the number and structure of peritoneal blood vessels [1]. The proteins of the fibrinolytic system that include urokinase plasminogen activator

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(uPA), tissue plasminogen activator (tPA) and their inhibitor, plasminogen activator inhibitor type I (PAI-I), play important roles in the complicated process of fibrotization in various organs and in the peritoneum [2,3]. Moreover, epithelial to mesenchymal transition, which is the pathophysiological mechanism involved in fibrotization within the peritoneal membrane, is related to changes in fibrinolytic capacity [4,5]. Human peritoneal mesothelial cells are capable of producing fibrinolytic system components, the release of which can be affected by various factors such as inflammatory mediators [6,7]. Moreover, PD solution itself has the ability to affect the secretion of fibrinolytic system components from mesothelial cells and/or the entire peritoneum [2].

Abdominal adipose tissue is metabolically active and has been shown to produce fibrinolytic system components depending upon the body mass index (BMI) and hyperinsulinemia [8,9]. Despite the importance of the expression of fibrinolytic system components in the process of fibrosis, no quantitative histological data are available in human patients receiving PD concerning the expression of these fibrinolytic regulators within the subserosal peritoneal membrane. Our aim, then, is to describe the cellular localization of PAI-1, tPA and uPA in submesothelial adipose tissue and to quantify expression of these proteins in relation to the basic clinical variables of the patients in our study at the onset of PD. The following null hypotheses have been proposed and tested:

H_0(A) There is no correlation among expression of fibrinolytic proteins within adipose peritoneal membrane tissue.

H_0(B) The area fraction of PAI-1, tPA and uPA within peritoneal adipose tissue is independent from the basic clinical variables – BMI, age or peritoneal permeability – of the patients at the onset of PD.

2. Material and methods

2.1. Set of patients, collection of tissue samples and histological procedure

We examined biopsy specimens of the parietal peritoneum taken from 12 patients (8 men, 4 women) with a median age of 60 years (range 30-72 years) and a median body mass index (BMI) of 22.7 (range 17.9-35.9) who were undergoing implantation of a peritoneal catheter. The peritoneal permeability in our cases was assessed by the dialysate-to-plasma creatinine ratio (D/P creatinine) upon PD initiation. The median D/P creatinine was 0.69 (range 0.48-0.89). Four patients had diabetes mellitus (DM) and were treated with subcutaneous insulin.

We used formalin-fixed, paraffin-embedded sections for immunohistochemical localization of uPA, tPA and PAI-1 expression. For indirect immunohistochemistry, we used the polyclonal goat anti-human antibodies against uPA, tPA and PAI-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the Goat ABC staining system (Santa Cruz Biotechnology Inc.), according to the manufacturer's instructions.

2.2. Experimental Procedures and Statistical methods

Histological quantification: Through the use of a 40x objective optical microscope, we captured six image fields from each biopsy specimen in a systematic uniform random manner that allowed all portions of the adipose tissue an equal chance for selection and quantification. In these micrographs, we quantified the area fraction (A_A) of immunohistochemically positive cell profiles per area of the adipose tissue by using the stereological point-counting method according to Equation 1. An example using the expression of PAI-1 is as follows:

$$AA(PAI\text{-}1, adipose\ tissue)=estA\ (PAI\text{-}1)/estA\ (adipose\ tissue), \quad (1)$$

Where *estA (PAI-1)* is the estimated area of the profiles of cells positive for PAI-1 and *estA (adipose tissue)* is the estimated area of adipose tissue (used as reference space). The area estimates were obtained by counting the intersections between the testing grid (the Ellipse software, ViDiTo, Kosice, Slovak Republic) and PAI-1-positive cells or adipose tissue, respectively. The countable event was defined as any immunohistochemical positivity for PAI-1 in the cytoplasm of adipocytes as well as in the interstitial blood vessels, pericytes or other connective tissue cells constituting the adipose tissue. The area estimate *(estA)* was calculated by multiplying the counted intersections within the area corresponding to each point of the testing grid. In a similar manner, we estimated the area fraction of uPA and of tPA within the same reference of adipose tissue. When we compared the estimated area with the actual reference area (based on the use of planimetry after outlining the boundaries of the adipose tissue samples manually), the relative difference between estimated and actual total area of the adipose tissue was 0.81%. The area fraction of each fibrinolytic regulator within the adipose tissue was then expressed as the mean value based on all six micrographs sampled per fibrinolytic regulator.

Statistics: The data were processed with Statistica Base 9 (StatSoft, Inc., Tulsa, OK, USA).

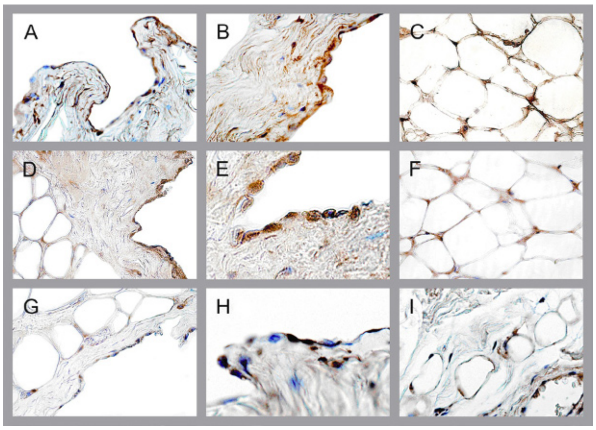
Nonparametric statistics, i.e., the Spearman rank order correlation and Wilcoxon matched pairs test, were used. For paired samples of adipose peritoneal tissue (PAI-1 – tPA, PAI-1–uPA and tPA – uPA), we used the Wilcoxon matched pairs test. The Spearman rank order correlation was used to measure statistical relationships between continuous variables. Values were considered statistically significant for $p < 0.05$.

3. Results

All three proteins were found in mesothelial cells, in the submesothelial collagenous connective tissue, in blood microvessels and in adipocytes (Figure 1). The area fraction of tPA was higher than the fraction of uPA ($p<0.01$) and PAI-1 ($p<0.05$), whereas the fraction of PAI-1 was higher than the fraction of uPA ($p<0.05$). The quantitative data are summarized in Table 1 and Figure 2. The area fractions of all three proteins were correlated with each other (Table 2), i.e., the null hypothesis $H_0(A)$ was rejected.

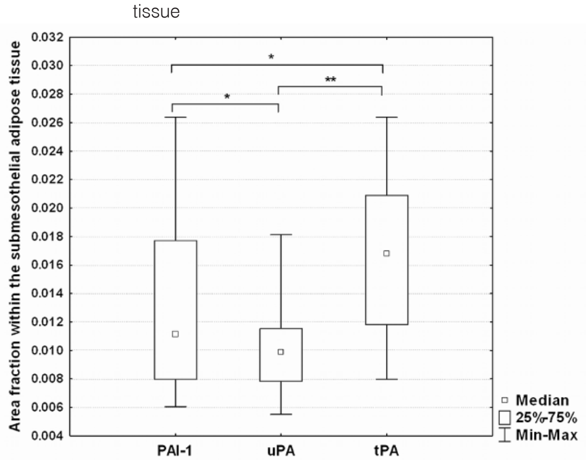
No correlation was found between A_A of these fibrinolysis-related antigens and BMI, age or peritoneal permeability. There was no difference between A_A of fibrinolysis-related antigens in patients without or without DM, and therefore, we did not reject the hypothesis $H_0(B)$.

Figure 1. Figure 2. PAI-1, tPA and uPA in human peritoneal membrane.



Panels A, B and C: Immunohistochemical detection of PAI-1 in mesothelium and submesothelial layer (A - original enlargment 100x, B - 400x) and in adipocytes (original enlargment 100x). Panels D, E and F: Immunohistochemical detection of tPA in mesothelium and submesothelial layer (A - original enlargment 100x, B - 400x) and in adipocytes (original enlargment 100x). Panels G, H and I: Immunohistochemical detection of uPA in mesothelium and submesothelial layer (A - original enlargment 100x, B - 400x) and in adipocytes and vessel walls (Panel I-original magnification 100x).

Figure 2. Area fractions of PAI-1, uPA and tPA within the adipose tissue



4. Discussion

4.1. Fibrinolytic components within peritoneal membrane

Table 1. Patients and results of quantification of fibrinolytic regulators. Age, presence/absence of diabetes mellitus (DM), body mass index (BMI), dialysate-to-plasma creatinine ratio (D/P creatinine), and area fractions A_A of PAI-1, uPA, and tPA within the submesothelial peritoneal adipose tissue. For publication, the data were rounded. One value is missing due to insufficient sustainability, and the value is replaced by a – sign.

Age	DM	BMI	D/P creatinine	A_A(PAI-1, adipose tissue)	A_A(uPA, adipose tissue)	A_A(tPA, adipose tissue)
64	DM	27.4	0.69	0.008	0.007	0.010
72	non-DM	22.1	0.88	0.016	0.012	0.021
41	DM	18.0	0.69	0.008	0.018	0.026
61	non-DM	30.8	0.67	0.010	0.009	0.010
62	DM	23.2	0.81	0.020	0.010	0.026
67	non-DM	21.3	0.55	0.012	0.010	0.016
40	non-DM	22.3	0.48	0.006	0.005	0.012
36	DM	22.9	0.89	0.010	0.008	0.017
30	non-DM	23.5	0.65	0.015	0.010	0.020
31	non-DM	22.7	0.77	0.008	0.007	0.008
60	non-DM	22.5	0.81	0.020	0.012	0.019
35	non-DM	22.1	0.49	-	0.010	0.014

Table 2. Spearman ranked order correlations between the quantitative parameters. The data were pooled across the patients. A_A stands for the area fraction of each fibrinolytic regulator (PAI-1, uPA, tPA) within the adipose tissue. Autocorrelations and repeating values are replaced by a – sign. Marked (*) correlations are significant at $p < 0.05$.

	A_A(PAI-1)	A_A(uPA)	A_A(tPA,)
A_A(PAI-1,)	-	*0.71	*0.65
A_A(uPA,)	-	-	*0.72

As the endothelial cells and smooth muscle cells in the microvessels are known to produce uPA, tPA and PAI-1 [10], we used their positivity as a standard for the quantification of the fibrinolytic regulators. As adipose tissue is rich in microvessels, the area fractions of fibrinolytic proteins (Table 1, Figure 2) consist of the cumulative value of positivity in the endothelial cells, smooth muscle cells, and adipocytes. Although PAI-1 expression in adipocytes has already been reported [11], the importance of peritoneal adipocytes is still a neglected area of study in regard to the local regulation of fibrinolytic system activity that modulates the balance between fibrotization and fibrinolysis in submesothelial connective tissue.

4.2. PAI-1 in chronic renal failure

Plasminogen activator inhibitor-1 (PAI-1) is mentioned as an acute phase reactant. In a study by Costa [12] patients with chronic renal disease (CRD) undergoing hemodialysis (HD) had an elevated tPA/PAI-1 ratio compared to controls. This study pointed out that an association between fibrinolytic cell function and increased inflammatory markers exists in patients witih CRD. An Irish study [13] dealt with PAI-1 activity in such patients and its relationship with alteration in metabolic and cytokine parameters. Groups of patients with CRD not undergoing dialysis were subdivided into low proteinuric (LP) and high proteinuric (HP) groups and compared with patients on continuous ambulatory peritoneal dialysis (CAPD) or hemodialysis (HD) and healthy volunteers. No group had significantly higher PAI-1 values than the controls. This finding was the reverse of interleukin-6 (IL-6), the mediator of acute phase response, in that IL-6 was significantly elevated in the HD and CAPD groups. Elevation of IL-6 was described as related to obesity and chronic inflammation and influenced by a diet high in fat [14]. Another study examined patients with CRD according to diet, being low (L) or high (H) in advanced glycoxidation end products (AGE, i.e., L-AGE or H-AGE). L-AGE diet showed a significant decrease in PAI-1 level [15] compared to H-AGE diet. This led to a suggestion that the level of PAI-1 is modulated by diet as well.

PAI-1 correlates with BMI and triglycerides [13]. It appears that PAI-1 is related to the common metabolic abnormalities of obesity rather than to chronic renal disease, but because of its genetic variability (polymorphism in the promoter region of PAI-1 gene), PAI-1 may react variably to triglycerides and acute-phase cytokines by differential binding of nuclear transcription factors.

4.3. Adipocytes and fibrinolytic system

The expression of PAI-1, uPA and tPA within peritoneal adipose tissue was mutually correlated, showing that tPA has the highest expression and uPA the lowest (Figure 2). The heterogeneity of cell types expressing uPA, tPA and PAI-1 in the peritoneal membrane (Figure 1) indicates that further studies focusing on the interchange of these proteins among different cell types and the networking of regulatory pathways in peritoneal membrane are needed. This would be a promising step towards understanding the role the fibrinolytic system plays in fibrotization and ultrafiltration failure during peritoneal dialysis. An increase in peritoneal permeability at the onset of PD is linked to a higher risk of peritoneal membrane failure during PD. It has been reported in patients with a higher comorbidity. In such patients, higher plasmatic levels of PAI-1 and its greater secretion from subcutaneous adipose tissue have been reported [16].

4.4. Inflammatory products of adipocytes

Adipocytes are not only one of the sources of local fibrinolytic proteins, but they also produce their regulators, such as TNF-α, IL-6 and TGF-β, thus forming local autocrine, paracrine and endocrine signals. Profibrotic factor TGF-β is also produced by mesothelial cells influenced by peritoneal dialysis fluids [18]. TGF-α, IL-6 and TGF-β are known as modulators of PAI-1 expression. In human cultivated adipocytes, PAI-1 expression has been stimulated with TGF-β. The TGF-β pathway may be influenced again by TNF-α [17].

4.5. Fibrinolytic system components in peritoneal membrane and the basic clinical variables

In our study, we did not find evidence of a relationship between the peritoneal permeability at the onset of PD and the extent of fibrinolytic protein expression in adipose tissue in the peritoneal membrane. We also did not find a relationship between the expression of fibrinolytic regulators in adipose tissue and BMI. This finding is in agreement with the study by Bouchard [18], but not with the findings of Alessi [8]. The differences between patients with and without DM could not be confirmed in our study due the small sample size, although it has been reported that hyperglycemia can lead to the increased expression of PAI-1 in adipose tissue [19]. We might also speculate about the participation of other regulatory pathways in the response of the peritoneal membrane tissues to PD. Low-grade inflammation accompanying uremia is the best known example.

5. Limitations of the study

With regard to the small number of patients in our study, our research generates hypotheses rather than provides final answers.We acknowledge this as one of the limiting factors of our study.

The age dispersion in the set is high, but it represents the typical population receiving dialysis without any specific selection and enables comparison of possible differences between younger and older patients in the group.

Similarly, we can take the highly dispersed BMI into consideration as a limitation, but it is important for a comparison of the potential differences between obese and lean individuals.

6. Conclusion

In our study, adipose tissue within the peritoneal membrane was capable of producing all three regulators of fibrinolysis (PAI-1, tPA, uPA) with strong mutual correlation between their area fractions. However, in our limited group of patients at the onset of peritoneal dialysis, we found no difference between fibrinolytic components and basic clinical variables. Longitudinal studies focused on the effects of different types of dialytic solutions and therapeutic approaches are needed in order to understand the importance of fibrinolytic proteins for the development of fibrosis and the alterations of the peritoneal membrane function during peritoneal dialysis.

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