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Vimentin cleavage in end-stage renal disease is not related to apoptosis

Research Article

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Abstract: Anti-vimentin auto-antibodies contribute to chronic allograft nephropathy. They exist in sera of end-stage renal disease patients on hemodialysis (ESRD) already before renal transplantation. We found recently that a 49 kDa vimentin fragment is increased in lymphocytes of ESRD patients which is presented on the cell surface. *In vitro* studies showed that such a fragment is formed during apoptosis by active caspase-3. We hypothesized that vimentin degradation in leukocytes of ESRD patients correlates to caspase-3 activation *in vivo*. Lymphocytes and monocytes were isolated from ESRD patients and from healthy volunteers and analyzed for vimentin expression and caspase-3 activation. In addition, apoptosis was induced *in vitro* and quantified by flow cytometry. ESRD monocytes have shown only the full length 60 kDa vimentin isoform. ESRD lymphocytes, however, showed in addition a strongly increased expression of the 49 kDa vimentin in all samples. Caspase-3 activation was found in 60% of ESRD lymphocytes and 66% of ESRD monocytes but not in healthy volunteers. UV-mediated induction of apoptosis was not associated with vimentin degradation. These experiments could confirm increased vimentin degradation in ESRD lymphocytes. However, we could not validate any correlation to apoptosis.

Keywords: *Vimentin • Apoptosis • Caspase-3 • End-stage renal disease*

1. Introduction

The intermediate filament Vimentin is an important part of the cytoskeleton in many cell types. It supports the position of cell organelles in the cytosol and provides cells with flexibility and resilience [1]. There is growing evidence that endogenous post-transplant auto-antibodies against vimentin are associated with transplant-associated coronary artery disease one and more years after heat transplantation and chronic allograft nephropathy (CAN) after kidney transplantation [2,3]. The presence of anti-vimentin antibodies in these patients is associated with vimentin-specific auto-reactive $CD8^+$ T cells [4]. We have shown in a previous study that anti-vimentin antibodies exist in sera of uremic patients on chronic hemodialysis already before renal transplantation [5]. In addition, we found that lymphocytes of hemodialysis patients express a different pattern of vimentin isoforms than lymphocytes of healthy volunteers. Lymphocytes normally express vimetin predominantly as a 60 kDa isoform, which represents the full length protein. Similar predominant expression of the full length protein was observed also in other human leukocytes with an apparent molecular weight of 58 kDa in neutrophils [6] and 62 kDa in monocytes [7]. In addition, healthy lymphocytes express a 49 kDa isoform, but at a much lower level. In contrast, lymphocytes from hemodialysis patients show a reduced expression of the 60 kDa isoform, a strong increase of the 49 kDa isoform and several additional isoforms with lower molecular weight indicating vimentin degradation in these cells. Vimentin is normally present only inside the cell and thus not accessible to anti-vimentin antibodies. In a recent study we found that the 49 kDa isoform is specifically transferred to the sur-

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face of lymphocytes in response to cell activation with concanavalin A [8]. This exposure of vimentin is associated with a higher binding of patient sera with high titers of anti-vimentin antibodies. Interestingly, anti-vimentin auto-antibodies bind specifically to the 49 kDa and the 60 kDa full length protein and not to other isoforms of vimentin [5]. It is therefore important to investigate the reason for the formation of the 49 kDa vimentin isoform. One potential reason could be apoptosis. Apoptosis is a type of programmed cell death in which caspase-3 plays an important role as an effector protease [9,10]. The inactive 35 kDa caspase-3 is degraded in an active 17 kDa caspase-3 fragment which proteolytically facilitates selective lysis of structural and functional proteins [11]. Byun et al. observed in MCF7 cells that active caspases 3 and 7 cleave the 57 kDa full length protein at Asp85 in a 48 kDa fragment [12]. Because the accuracy of the molecular weight determination by Western blotting depends strongly on the use of gel electrophoresis system, we propose that these two isoforms corresponds to our 60 kDa and 49 kDa isoform, respectively. Uremic patients on hemodialysis show a decline in leukocytes especially in lymphocytes. There are several studies which indicate that this is due to an increased susceptibility of these cells to apoptosis [13-16].

Here we investigate whether the alterations in the vimentin expression pattern in PBMCs of uremic patients on hemodialysis is related to apoptosis in this cell population via cleavage by activated caspase-3. To prove this hypothesis we extracted PBMCs from the blood of patients on hemodialysis and analyzed their vimentin protein and active caspase-3 expression pattern. Although we could confirm the previously shown multiple vimentin isofoms in patients' lymphocytes a connection to a caspase-3 activity could not be validated.

2. Subjects and methods

2.1 Patient characteristics

Five chronic hemodialysis patients on the renal transplantation waiting list at the General Hospital of Vienna (3 male, 2 female; age: 30-65, mean 47) and 5 healthy volunteers (3 male, 2 female; age: 23-53, mean 43) were enrolled at the General Hospital of Vienna and an informed consent was obtained from each. The study was approved by the local ethics committee.

2.2 Cell isolation and treatment

PBMCs were isolated by standard Ficoll-Paque gradient centrifugation (PAA, Vienna, Austria) from EDTA treated blood within two hours after blood withdrawal. Obtained PBMCs were washed with phosphate buffered saline without calcium and magnesium (PBS) supplemented with 0.5% BSA (bovine serum albumin). Separation of monocytes from lymphocytes was achieved using Magnetic Activated Cell Sorting with CD14 microbeads according to the manufactures protocol (Miltenyi Biotec., Bergisch-Gladbach, Germany). After separation of monocytes and lymphocytes both were lysed in 100 mM Tris-Cl (pH 9.5) containing 1% SDS and stored at −70°C as needed. For the in vitro apoptosis experiment isolated lymphocytes were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine (PAA, Wien, Austria) and 10 % FCS (Linaris, Wertheim-Bettingen, Germany) at 37°C and 5 % CO_2. Subsequently cells were treated with 300 mJ/cm² UV-C radiation (GeneLinker UV chamber, Bio-Rad, Hercules, CA). Then cells were cultured for the indicated period of time. After this incubation cells were collected, washed twice in PBS, lysed in protein lysis buffer as described above. To quantify apoptosis a small amount of cells were stained with annexin V-FITC apoptosis detection kit from BD Pharmingen (Franklin Lakes, NJ) following the manufacturers protocol and analyzed by flow cytometry (Gallios, Beckman Coulter, Brea, CA).

2.3 Western blot

For Western blotting experiments 10 µg per lane lymphocyte or monocyte protein extracts were separated on 14% SDS-polyacrylamide gel, transferred onto a nitrocellulose and visualized using a ruthenium-(II)-tris (bathophenanthroline disulfonate) (RuBPS). Membranes were then incubated either with rabbit anti-human vimentin polyclonal antibody (Gene Tex, Irvine, CA) or with mouse anti-human caspase-3 (Enzo Life Sciences, Farmingdale, NY) in PBS containing 2% milk and 0.3% Tween-20 for 1.5 hrs. Bound anti-vimentin antibodies were detected with a Cy5-labelled anti-rabbit IgG antibody (Jackson Immuno Research Labartories, West Grove, PA). Bound caspase-3 antibodies were detected with a horseradish peroxidase conjugated anti mouse IgG antibody (Pierce, Rockford, IL) in the presence of Supersignal West Femto Detection System (Pierce, Rockford, IL). Nitrocellulose membranes were scanned using a Typhoon TRIO scanner (GE Healthcare, Uppsala, Sweden).

3. Results

In a first experiment we prepared total cell lysates of lymphocytes and monocytes from healthy volunteers and analyzed them by Western blotting (Figure 1A). Both cell types showed a strong expression of the full length vimentin protein with an approx. molecular weight of 60 kDa. Lymphocytes showed an additional, much less expressed

49 kDa isoform. Next we analyzed the vimentin expression pattern in lymphocytes and monocytes of patients on chronic hemodialysis by Western blotting (Table 1 summarizes the clinical and anthrophometrical data of these patients). A clear band at approximately 60 kDa could be shown in all samples (Figure 1B). Interestingly, all five lymphocytic extracts from dialysis patients showed a strongly increased expression of the 49 kDa vimentin band. The monocytic extracts showed no additional bands at all. To find out whether lymphocytes in patients on dialysis undergo apoptosis *in vivo* we determined the activation of caspase-3 by Western blotting (Figure 1B, left panel). In three out of five patients a clear band could be detected at 17 kDa using specific antibodies against activated caspase-3. Such bands could not be seen in healthy volunteers (Figure 1A). Similarly, active caspase-3 was detected in monocytes of two patients but undetectable in monocytes from healthy volunteers. These data indicates that apoptosis can occur in lymphocytes as well as in monocytes of individual patients on hemodialysis. Active caspase-3 had no effect on the vimentin pattern in monocytes. Although, lymphocytes show disease-related changes in the vimentin pattern, it remains unclear whether they are related to caspase-3 because they were also observed in the absence of caspase-3 activation.

For further investigation of this question we performed additional *in vitro* experiments. Lymphocytes from a healthy volunteer were treated with UV and analyzed for apoptosis after different points in time by staining the cells with annexin V (ann V) and propidium iodide (PI) using flow cytometry. Immediately after irradiation more than 80% of all cells were still negative for ann V and PI indicating viable cells (Figure 2A open circles). This value decreased and fell below 10% 12h after irradiation (see also insert in Figure 2A which represents the flow cytometric analysis at 12h). From two to six hours post-radiation the reduction in viable cells is predominantly due to the formation of ann V+ / PI- cells, indicating early apoptosis. After six hours ann V+ / PI+ cells account for the strong majority of dead cells. Already after 4 h post-radiation a faint band of activated caspase-3 could be detected which grew clearer and stronger with each following point in time. (Figure 2B).

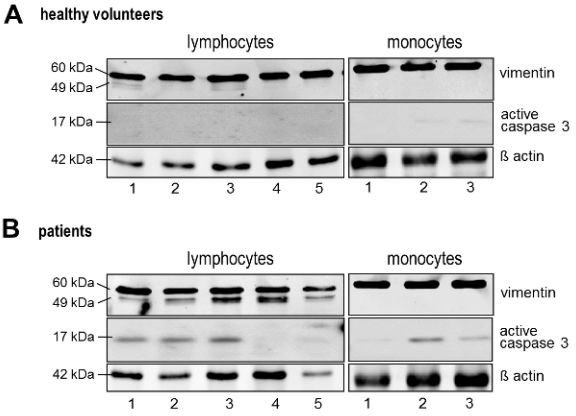

Figure 1. Vimentin expression in lymphocytes and monocytes of patients on hemodialysis. Peripheral blood mononuclear cells (PBMCs) were prepared from healthy donors (A) and patients on hemodialysis (B). Lymphocytes and monocytes were separated and protein extracts were analyzed for vimentin and for active caspase 3 content by Western blotting. Beta actin expression was analyzed for loading control.

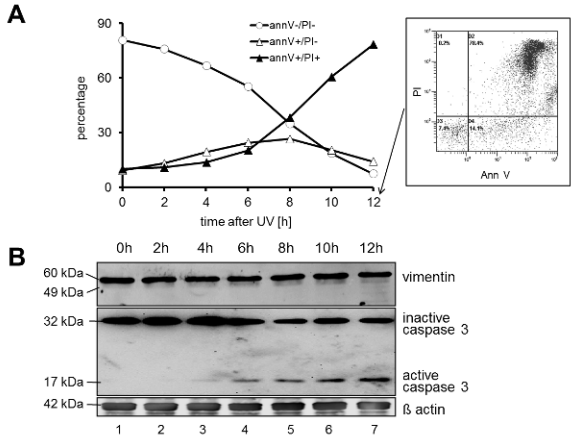

Figure 2. Effect of apoptosis on vimentin expression in primary human lymphocytes. Lymphocytes were prepared from a healthy donor and exposed to 300 mJ/cm² UV-C irradiation and cultured for different periods of time. (A) cells were analysed at the indicated points in time for binding of annexin V (ann V) and propidium iodide (PI). The insert on the right side exemplifies the results of such an analysis. (B) Western blot analysis of cells harvested at the indicated time after irradiation.

Table 1.

patient_ID	sample_ID (Fig. 1)	sex	age	time on dialysis [years]	monocytes per µl	lymphocytes per µl	underlying disease
P1	9, 15	m	74	3	500	1700	interstitial nephritis
P2	10	m	38	4	400	1600	terminal renal insufficiency
P3	11, 16	f	66	10	500	2900	chronic pyelonephritis
P4	12	f	39	7	500	2200	terminal renal insufficiency
P5	14, 17	m	25	7	500	1400	Goodpasture syndrome

The concentration of inactive caspase-3 decreased correspondingly. At the end point at 12 h post-radiation we observed the highest expression of active caspase-3. The vimentin expression pattern before irradiation was nearly identical as in Figure 1A: a predominant expression of the 60 kDa full length protein and a faint band at 49 kDa. The latter band showed a very modest increase after induction of apoptosis. But it never reached expression similar levels as observed in patients' samples at undetectable caspase-3 activation (Figure 2B, lanes 4 and 5).

4. Discussion

The aim of this study was to examine whether vimentin degradation in PBMCs of hemodialysis patients is associated to caspase-3 activation. Although caspase-3 activation could be observed in lymphocytes as well as monocytes of these patients we could not confirm a relationship to an increase of low molecular weight vimentin isoforms *in vivo* or *in vitro*.

Light but clear bands of activated caspase-3 could be found in three out of five patients suggesting apoptosis in very few of the lymphocytes in these three patients. In contrast, lymphocytes from HVs showed no caspase-3 activation indicating, that the observations in patients' lymphocytes are disease-related. The observed apoptosis may be based on multiple causes in hemodialysis. Previous studies showed more than 3-fold increase in the numbers of apoptotic lymphocytes in end-stage renal disease patients on hemodialysis in comparison to healthy controls [17,18]. This apoptosis seems to be related to increased oxidative stress [17,18] which is potentially mediated by elevated levels of oxidized LDL [19]. The uremic disease itself [20] as well as the dialysis treatment [15] seem to contribute to the increase in apoptosis. In a similar fashion we analyzed monocytes for signs of apoptosis using the activated caspase-3 as marker. All of the patients and two out of three healthy volunteers expressed the protein indicating apoptosis in almost all of the samples collected. These data support another publication reporting of monocyte apoptosis in dialysis [21]. In spite of clear caspase 3 activation in monocytes, none of the monocytic preparations showed any degradation of vimentin. Byun and co-workers reported that isolated human full length 57 kDa vimentin protein is cleaved in a 48 kDa fragment when incubated with active caspase-3 [12]. Therefore we propose that monocytes possess a factor which protects vimentin against caspase 3-mediated degradation.

The situation for lymphocytes was less clear. All patients show clear bands with a lower molecular weight (approx. 49 kDa) in addition to the one clear vimentin band at 60 kDa. This additional isoform might derive from caspase-3 mediated proteolytic cleavage of the full length vimentin protein. Since we could find multiple vimentin bands in all patients but activated caspase-3 in only three of them we cannot support this hypothesis. On the contrary, our findings rather point towards an independence of the two parameters. For further investigation we induced apoptosis in lymphocytes in vitro and analyzed its effect on vimentin. With increasing size of the annexin V positive cell population the caspase-3 band became more and more clear. Yet the vimentin band in lymphocytes underwent no corresponding change. It can be concluded that vimentin seems resistant against degradation through caspase-3 activity in both PBMC populations. The detected additional vimentin isoforms in lymphocytes of hemodialysis patients must have other reasons, which have not yet been discovered.

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