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ORIGINAL ARTICLE

Urokinase and its receptor in follicular and inflammatory cysts of the jaws

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OBJECTIVE: Proteases are considered critical in peri-cystic tissue degradation required in jaw cyst expansion. We studied the expression of the plasminogen activation system (plasminogen activators; their inhibitor type-1, PAI-1; the receptor for the urokinase-type plasminogen activator, uPAR) in follicular and inflammatory cysts of the jaw, to identify a possible role of this system in jaw cyst enlargement.

MATERIALS AND METHODS: Jaw cysts were collected by therapeutic enucleation. ELISA and casein zymography were used to measure and characterize plasminogen activators in cyst fluid. By immunohistochemistry we examined the presence of uPAR in cyst walls and inflammatory cells, and by Western blotting the molecular forms of uPAR within the cyst fluid.

RESULTS: Inflammatory cysts fluid contained higher amounts of plasminogen activators of the urinary-type (uPA), and lower amounts of PAI-1, when compared to follicular cysts fluid. Epithelial layers of both types of cysts and inflammatory cells expressed uPAR. Native 3-domain uPAR was scarcely detectable within cysts, where its cleavage was accounted for by uPA.

CONCLUSION: These data suggest a plasminogen activation-dependent mechanism of cyst enlargement, where only the outward uPAR expressed on epithelial cells and on inflammatory cells direct the peri-cystic protease cascade, in a way similar to tumor enlargement within tissues.

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Keywords: jaw cysts; proteases; protease inhibitors; uPA; uPAR

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Introduction

Jaw cysts represent a pathology with high incidence (Shear and Speight, 2007). Most jaw cysts arise from odontogenic epithelial residues after tooth development. From an etio-pathogenetic point of view, inflammatory and developmental jaw cysts are distinguishable. Inflammatory cysts originate from the proliferation of odontogenic epithelial residues triggered by endodontal and/or parodontal inflammation. Developmental cyst formation is promoted and develops within odontogenic tissue itself from an unexplained event occurring during embryogenesis. Different cyst morphology, their aggressiveness, the frequency of relapse, and osteolytic activity depend on the embryologic phase during which the triggering event occurs. The more precocious the epithelial development abnormality is, the more undifferentiated and aggressive the cyst will turn out (Stoelinga and Bronkhorst, 1988). The primordial highly aggressive keratocyst, which develops from an undifferentiated odontogenic epithelium (Shear and Speight, 2007), and the follicular (dentigerous) cyst, which originates from a well differentiated epithelium, may be located at the opposing ends of the aggressiveness/differentiation scale. Little is known about the mechanisms of their expansion. It is commonly considered that intense bone resorption produced by activated osteoclasts may favor the intra-osseous expansion of the lesions (Tay *et al*, 2004). From a general point of view, proteolysis is required in order to gain space within compact tissues. The broad-spectrum protease plasmin, generated by the pro-enzyme plasminogen following activation by plasminogen activators (PAs), plays a central role in this process since it cleaves connective tissue proteins both directly and indirectly, by activating the pro-forms of matrix metalloproteinases (MMPs) (Birkedal-Hansen, 1995). Plasminogen can be converted into plasmin by two different PAs: the tissue-type PA (tPA) and the urokinase-type PA (uPA). Specific plasminogen activator inhibitors, namely PAI-1 and PAI-2, as well as a cellular receptor for uPA (uPAR) modulate

and direct the activity of the plasminogen activation system (Del Rosso *et al*, 2008). Fibrinolytic activity has been shown in homogenates of radicular cysts (Sugimura *et al*, 1976). Immunohistochemical studies have demonstrated the presence of tPA and PAI-1 in the lining epithelium, connective tissue, inflammatory infiltrates and endothelium of radicular cysts, showing that the expression of both tPA and PAI-1 increases with the grade of inflammation (Tsai *et al*, 2004). Here we have examined the protease/protease inhibitor composition of intra-cystic fluid of follicular and inflammatory-type odontogenic cysts as related to detection of uPAR in its native (domains D1 + D2 + D3) and truncated form (domains D2 + D3), in order to identify a uPAR-driven mechanism of jaw cyst expansion. By immunohistochemistry we have also studied uPAR distribution among cell components of cyst walls.

Materials and methods

Choice of patients, collection and scoring of biological samples

Fourteen patients, nine males and five females (age range 28–80), selected on the basis of radiographic and clinical evidences for the presence of jaw cysts, provided their written consent to participate to the study. Procedures were performed according to 1975 Helsinki Declaration (revisioned in 1983) on human experimentation. Cysts were classified according to clinics, pathology and histology. Each cyst was enucleated as a whole, the cyst liquid content was drawn by syringe needle aspiration and specimens were then prepared for histology and immunohistochemistry, after assessing the continuous wall of the cyst. The rate of inflammation was evaluated in one section of each cyst specimen stained with hematoxylin and eosin and observed at 200 × magnification, in four consecutive microscopic fields, starting from the cystic lining epithelium and proceeding toward the connective tissue. Each specimen was graded and recorded as follows for the percentage of inflammatory cells in each field: −, absence of inflammatory cells; +, less than 25%; ++, between 25% and 50%; +++, between 50% and 75%; ++++, between 75% and 100%. With the exception of three 'follicular cysts', all the others were 'inflammatory', with a varying degree of inflammation (Table 1).

Zymography of plasminogen activators and determination of uPA, tPA, PAI-1

The liquid content of each cyst was centrifuged and aliquots of each sample were analyzed for total protein content by the BCA protein assay kit (Pierce, Thermo Fisher Scientific, IL, USA). Aliquots containing the same amount of protein (40 μg) were subjected to a standard sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE 10%) under non-reducing conditions and subsequent zymography, as previously described (Serratì *et al*, 2006). For zymography, migrated proteins were transferred onto 0.45 μm pore-size nitrocellulose filter (Bio-Rad Laboratories, Richmond, CA, USA) in a 0.04 mol l^{-1} phosphate buffer (pH 6.5), under a current of 0.6 A during a 1.5-h run. The nitrocellulose filter was removed and placed on an indicating agar layer containing casein (0.1%) and plasminogen (1 μg ml^{-1}). In this assay, plasminogen activators diffusing out of the polyacrylamide gel activate the plasminogen present in the layer of insoluble protein substrate. The plasmin thus generated causes localized lysis of the substrate. After 10 h of incubation at 37°C, when clear bands of lysis were visible in the cloudy casein background, corresponding to the position of plasminogen activators in the polyacrylamide gel, zymograms were dried and stained with Ponceau S solution (Sigma, St Louis, MO, USA). Zymographies were also performed in the presence of 1 mmol l^{-1} amiloride (Sigma) incorporated in the agar-casein substrate, a substance that specifically inhibits u-PA (Vassalli and Belin, 1987), in order to identify both uPA and tPA activity. Plasminogen activator inhibitor type-1 (PAI-1), uPA and tPA were measured by a specific ELISA on aliquots of the cyst fluid (ELITEST PAI-1, Hyphen Biomed, Neuville Sur Oise, France;

Table 1 Comparison of uPA and PAI-1 with the histotype and inflammation degree of follicular and inflammatory cysts

Patient	*Sex*	*Age*	*Cyst histotype*	*Inflammation*[b]	*PAI-1*[a]	*uPA*[a]
1	M	28	Inflammatory	++++	0.57 ± 0.13	4.46 ± 0.91
2	M	62	Inflammatory	++++	0.75 ± 0.2	5.53 ± 0.86
3	M	80	Inflammatory	+++	0.85 ± 0.2	3.28 ± 0.68
4	M	71	Inflammatory	+++	0.63 ± 0.09	3.22 ± 0.56
5	F	55	Inflammatory	++	0.88 ± 0.12	2.95 ± 0.48
6	M	35	Inflammatory	+++	0.77 ± 0.15	3.66 ± 0.75
7	M	66	Inflammatory	+	0.69 ± 0.11	2.26 ± 0.49
8	M	45	Inflammatory	++	0.56 ± 0.08	3.01 ± 0.51
9	F	51	Inflammatory	++++	0.64 ± 0.17	5.95 ± 1.10
10	F	70	Inflammatory	+++	0.81 ± 0.21	3.47 ± 0.86
11	M	47	Inflammatory	++	0.66 ± 0.16	2.12 ± 0.33
12	F	56	Follicular	−	3.91 ± 0.6	1.26 ± 0.21
13	M	53	Follicular	−	3.38 ± 0.5	0.98 ± 0.18
14	F	29	Follicular	−	3.56 ± 0.7	1.03 ± 0.16

[a]Values expressed as ng per mg protein of the cyst fluid.
[b]Symbols represent the percentage of inflammatory cells in each field: −, absence of inflammatory cells; +, less than 25%; ++, between 25% and 50%; +++, between 50% and 75%; ++++, between 75% and 100%.

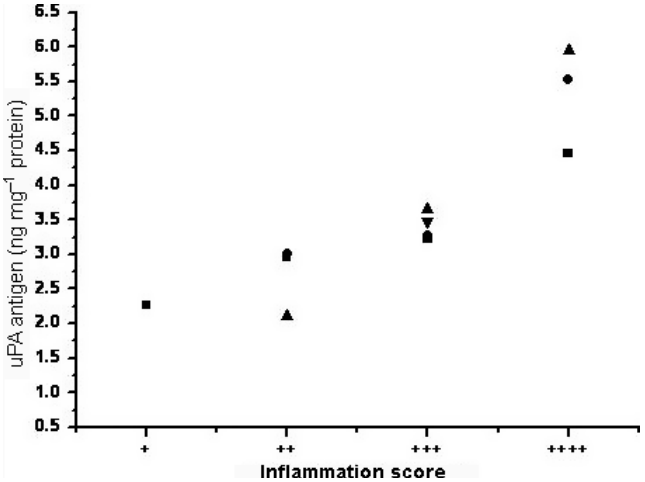

Figure 1 Relationship between uPA of the cyst fluid and the degree of inflammation. The figureshows the amount of uPA, measured by ELISA in the cyst fluids of inflammatory cysts (expressed as ng per mg of protein content of the cyst fluids), as related to the degree of inflammation (scored as described under the section 'Materials and Methods'). Each case, corresponding to a single patient, is represented with a different symbol in the plot. Refer to Table 1 for patient reference

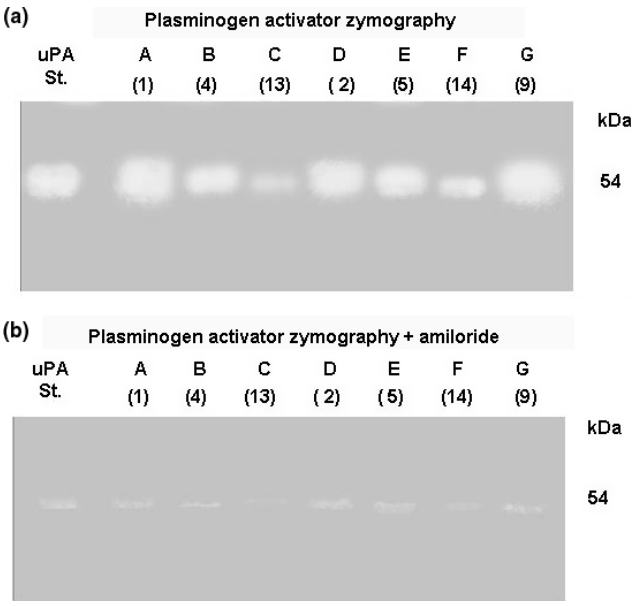

Figure 2 Zymography of plasminogen activators. (**a**) Representative zymography of plasminogen activators in the absence of amiloride. Aliquots of selected cyst liquid, containing 40 μg protein, were subjected to PAGE, blotting and casein zymography, as described under the section 'Materials and Methods'. Capital letters on the top of each lane identify applied samples, while numbers between parentheses refer to the patient number, as reported in Table 1. Samples A, B, D, E and G show the caseinolytic activity of inflammatory cysts, while samples C and F show the activity of follicular cysts. uPA St., standard uPA; numbers on the right indicate reference molecular weight. (**b**) Representative zymography of cyst fluid performed as described in (**a**), in the presence of 2 mmol l^{-1} amiloride, which specifically inhibits uPA activity, incorporated in the casein. Letters, numbers and symbols are the same as in (**a**)

IMUBIND, American Diagnostica, Montreal, Canada), following the instructions of the manufacturers. The amount of the relevant antigens was reported as ng per mg total protein present in the cystic fluid (Table 1). Each sample was analyzed three times and results were expressed as mean value ± s.d.

Western blotting

Aliquots of cyst fluid, containing 50 μg protein, or 50 ng recombinant human uPAR (R&D Systems, Inc., Minneapolis, MN, USA), were subjected to polyacrylamide slab gel electrophoresis and blotted as described (D'Alessio *et al*, 2004). The blotted membrane was incubated with 5% skim milk in 20 mM Tris buffer, pH 7.4, for 1 h at room temperature to block non-specific binding and then probed with a mouse monoclonal antibody directed against the domain D1 (MON R-3) or D3 (MON R-4) of uPAR (BioPorto Diagnostics A/S, Gentofte, Denmark) overnight at 4°C. After incubation with horseradish peroxidase-conjugated donkey anti-mouse IgG (1:5000) for 1 h (Amersham Biosciences, Piscataway, NJ, USA), immune complexes were detected with the enhanced chemoluminescence (ECL) detection system (Amersham Biosciences). The membranes were exposed to autoradiographic films (Hyperfilm MP; Amersham Biosciences) for 1–30 min.

Immunohistochemistry

Specimens of the cyst walls were fixed for 24 h in 4% buffered formaldehyde and included in paraffin. Tissue sections of cyst specimens, 3–5 μm thick, were placed on pretreated glass slides, de-waxed and immersed in 3% methanol–hydrogen peroxide (H_2O_2) solution for 10 min to block endogenous peroxidase activity. The following primary antibodies were employed: mouse polyclonal anti-human CD87 (uPAR) (American Diagnostica, Montrèal, Quebec, Canada) (500 μg ml^{-1}), and mouse monoclonal anti-CD45 (leukocyte common antigen) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (200 μg ml^{-1}). Primary antibodies were diluted 1:40 and incubated overnight with tissue sections in a moist chamber at 4°C. For the secondary antibody we used the prediluted SuperPicture kit (HRP-Broad Spectrum, cat. 87-8963, Invitrogen, Celbio, Pero, Italy). 100 μl of the solution was incubated 30 min at 25°C. A standard streptavidin–biotin detection system (Vector, Burlingame, CA, USA) was carried out. Isotype Ig controls were used in parallel with primary antibodies (staining specificity). Antigen-bound primary antibody was visualized by diaminobenzidine and a nuclear counterstaining with hematoxylin was performed.

Statistical analysis

Results are expressed as means ± s.d. for (n) experiments.

Results

uPA and PAI-1 determination and zymography of plasminogen activators

Table 1 shows the amounts of PAI-1, and uPA measured by ELISA in aliquots of the cyst fluid. Tissue-type plasminogen activator (tPA) was undetectable in our

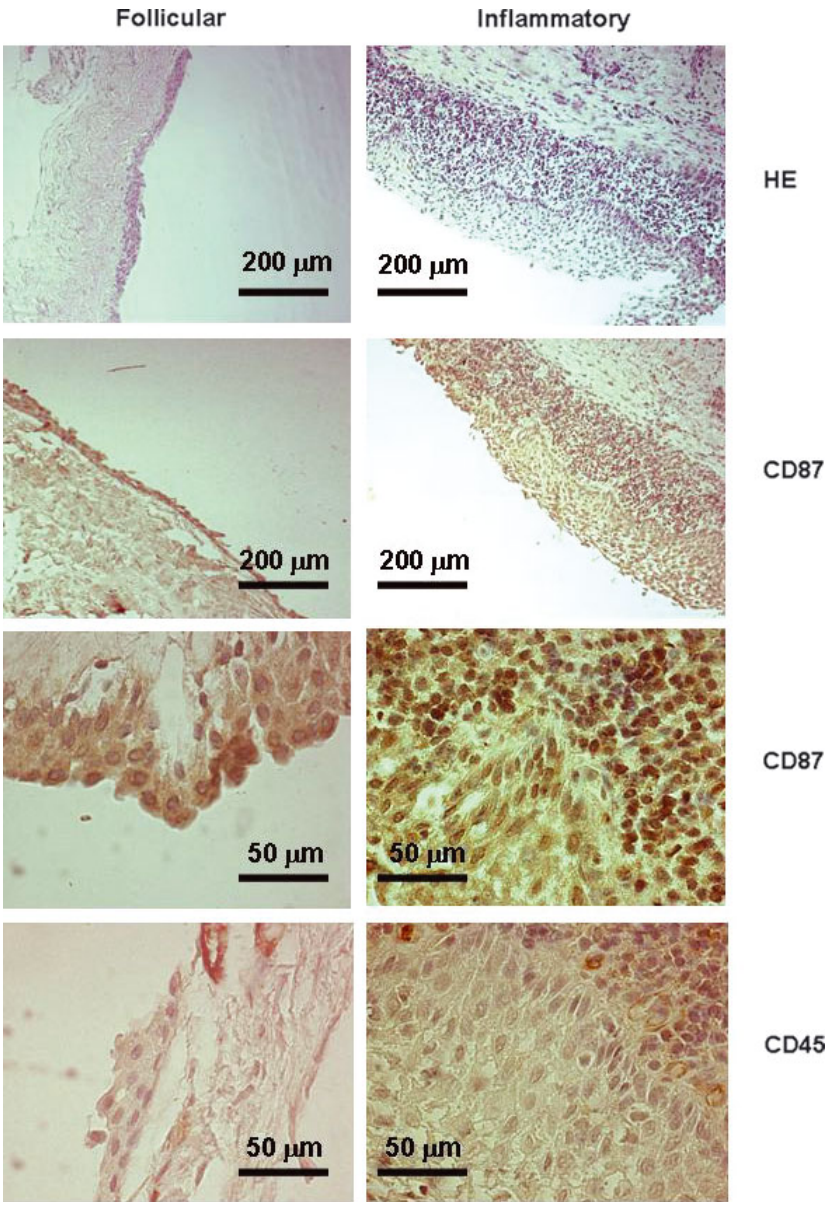

Figure 3 Immunohistochemistry of cyst walls. Specimens of the cyst walls were prepared by therapeutic surgical enucleation of each cyst and prepared for immunohistochemistry as described. The scale of magnification is reported within each micrograph. The top of each lane of micrographs indicate the cyst type, while symbols on the right indicate: HE, hematoxylin–eosin staining; CD87, immunocytochemistry performed with anti-CD87 (uPAR) antibody; CD45, immunocytochemistry performed with anti-CD45 (leukocyte common antigen) antibody

samples. Plasminogen activator inhibitor type-1 (PAI-1) was more concentrated within follicular cysts than in inflammatory ones (median values: 3.56 and 1.68 for follicular and inflammatory cysts, respectively), while uPA showed an opposite trend (median values: 1.03 and 3.25 for follicular and inflammatory cysts, respectively). In the inflammatory cysts, the uPA level was almost perfectly linearly related to the degree of inflammation (Figure 1). Since the ELISAs used in this study to measure the PA content recognize antigenic epitopes unrelated with the enzyme activity, we performed zymographies to reveal whether the measured molecules showed a detectable proteolytic activity within the cyst fluids, as well as the nature of the PA (uPA or tPA). Figure 2a shows the zymography of aliquots of liquid obtained from selected cysts of inflammatory and follicular types. It is evident that all cysts expressed PA activity, and that areas of caseinolysis were more intense in inflammatory than in follicular-type cysts. PA activity observed in all the specimens was ascribed to uPA on the basis of co-migration with standard human uPA (54 kDa) and of amiloride sensitivity of the lytic activity, which is restricted to the uPA-type enzyme (Figure 2b) (Vassalli and Belin, 1987). We did not find any tissue plasminogen activator (tPA) enzyme activity in the cyst fluid, in agreement with tPA antigen determination by ELISA.

Immunohistochemistry of uPAR (CD87) and leukocyte common antigen (CD45)

The immunohistochemistry study of uPAR distribution among the tissue components of the cysts is shown in Figure 3. A strong positivity to the polyclonal anti-CD87 antibody, which recognizes both full-length (D1 + D2 + D3) and truncated (D2 + D3) human uPAR, was present in the non-keratinizing squamous

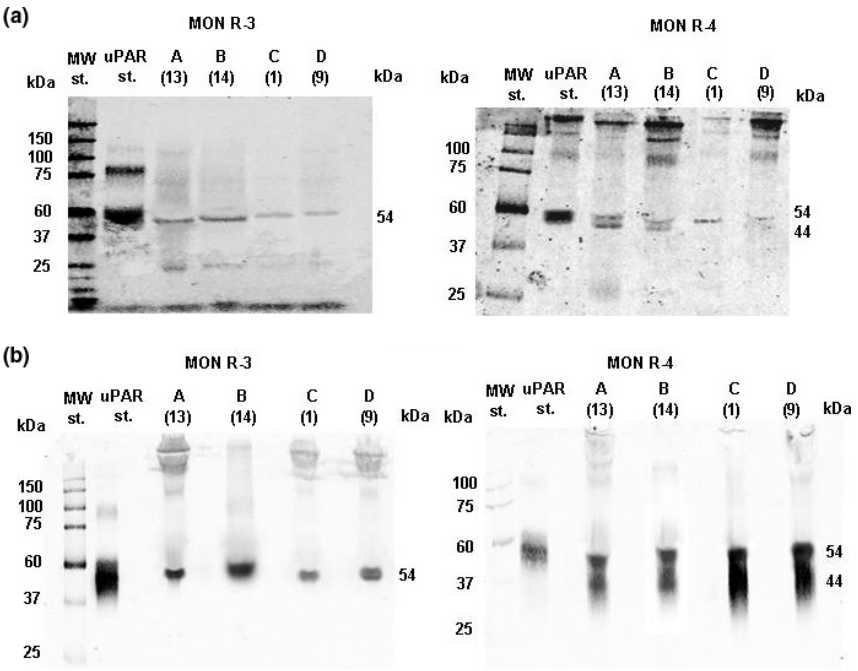

Figure 4 Degradation of uPAR by cyst fluid. (**a**) Western blotting of cyst fluid (50 μg protein) with MON R-3 (left) and MON R-4 (right). Capital letters on the top of each lane identify applied samples, while numbers between parentheses refer to the patient number, as reported in Table 1. uPAR St., standard uPAR (50 ng). Numbers on the left refer to the molecular weight of reference standards (expressed in kDa), while numbers on the right refer to the molecular weights of the blotted bands. (**b**) Western blotting of standard uPAR (50 ng) incubated with cyst fluid and blotted with MON R-3 (left) and MON R-4 (right). uPAR St., standard uPAR (50 ng). For symbols and numbers, refer to (**a**)

cyst-lining epithelium, in both inflammatory and follicular cyst. Peri-cystic inflammation was particularly prevalent in inflammatory cysts, and mononuclear inflammatory cells were all positive to anti-CD87 (uPAR) and anti-CD45 (leukocyte common antigen) staining.

Intra-cystic liquid-dependent uPAR cleavage

Figure 4a shows a Western blotting of uPAR of cyst fluid performed by using the anti-uPAR MON R-3 and R-4. MON R-3 recognizes an uPAR epitope present on uPAR domain 1 and therefore identifies only full-length (D1 + D2 + D3) native uPAR, while MON R-4 reacts with an etitope present on uPAR domain 3 and can thus identify both full-length (D1 + D2 + D3) and truncated (D2 + D3) uPAR. Trace amounts of the full-length receptor were detectable mainly in the fluid of follicular cysts with both antibodies. Upon immunoblotting with MON R-4, inflammatory cysts showed both the native (54 kDa, D1 + D2 + D3) and the truncated (44 kDa, D2 + D3) forms. It is noteworthy that the truncated form was almost undetectable in the fluid of follicular cysts. Figure 4b shows a Western blotting of standard full-length uPAR incubated overnight at 37°C with cyst fluids and immuno-blotted with MON R-3 and R-4. The results obtained with MON R-3 indicated a lower uPAR degradation upon incubation with follicular cysts fluid. MON R-4, which reacts with uPAR D3 and recognizes both full-length and truncated uPAR, indicated that the uPAR cleaved form (D2 + D3) was detectable mainly in inflammatory cysts, and that cleavage was proportional to the proteolytic activity expressed by the cystic liquid. In the attempt to identify the main protease family responsible for uPAR cleavage, we incubated cyst liquids and uPAR in the presence of protease inhibitors. Figure 5a shows that the mixture of inhibitors (against metallo proteases, serine proteases and cysteine proteases) and aprotinin (against serine proteases), inhibited uPAR cleavage, while Ilomastat (a general inhibitor of metallo proteases), and *N*-ethyl-maleimide (for cysteine proteases) were ineffective. These data, as well as the shown amiloride sensitivity of uPAR cleavage (Figure 5b), point to uPA as the main uPAR-degrading enzyme in follicular and inflammatory cysts.

Discussion

We have observed that radicular (peri-apical) inflammatory cyst fluid exhibits a strong plasminogen activation activity, proportional to the degree of inflammation, that may be ascribed to the urokinase-type plasminogen activator (uPA). Follicular (dentigerous) cysts show a weaker uPA activity, which is further counterbalanced by high expression of the specific protease inhibitor PAI-1. In spite of the origin of the cyst, the non-keratinizing squamous cyst-lining epithelium expresses uPAR in the cells of the basal and suprabasal layer, which are in close contact with the peri-cystic tissue. The uPAR is also expressed by the peri-cystic inflammatory cells of inflammatory radicular cysts. Further, the intra-cystic liquid of both follicular and inflammatory cysts cleaved uPAR proportionally to the proteolytic uPA activity expressed by each type of cyst. We did not perform immunohistochemistry of uPA since it has been shown that uPA is very sensitive to fixation and its immunoreactivity is reduced as fixation time increases, which usually makes routine-fixed specimens unsuitable for uPA immunohistochemistry (Nielsen *et al*, 2001).

The radicular inflammatory cyst is the most common cyst of the jaw, which represents the last step in a

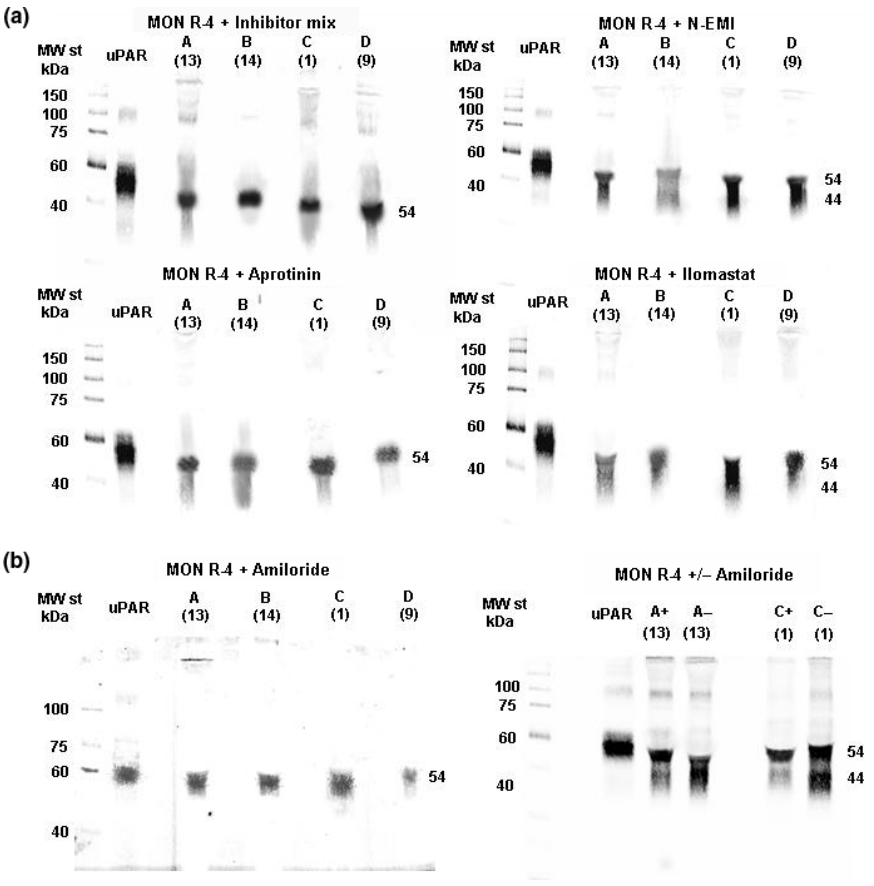

Figure 5 Characterization of uPAR-degrading activity in cyst fluids. (**a**) Western blotting of standard uPAR (50 ng) incubated with cyst fluid and blotted with MON R-4 in the presence of: a mixture of protease inhibitors (Ilomastat, 25 μM; N-EMI, 10 μM; aprotinin, 10 μg ml^{-1}) (upper left); a selective low molecular weight inhibitor of cysteine proteases (N-EMI, 10 μM, kindly provided from Prof. Francesco Paoletti, University of Florence) (upper right); a serine-protease inhibitor, Aprotinin, 1 μg ml^{-1} (Sigma, St Louis, MO, USA) (lower left); a general hydroxamate inhibitor of MMPs activity, Ilomastat or GM6001, 25 μM (Chemicon International, Temecula, CA, USA) (lower right). For symbols and numbers, refer to (**a**) and (**b**) of Figure 3. (**b**) Western blotting of standard uPAR (50 ng) incubated with cyst fluid and blotted with MON R-4 in the presence of amiloride, which selectively inhibits the enzymatic activity of uPA (left). The results are similar to those obtained with the mixture of protease inhibitors. The panel on the right shows the comparative amiloride-dependent inhibition of uPAR degradation by the cyst fluid proteases from patient samples A (follicular) and C (inflammatory). Symbols + and – refer to the presence and absence, respectively, of amiloride in the incubation medium. For all other symbols, refer to (**a**)

progression of inflammatory events following the formation of a peri-apical inflammatory lesion secondary to pulpal necrosis in a tooth (Shear and Speight, 2007). The follicular (dentigerous) cyst is the most common type of non-inflammatory odontogenic cyst and both types of cyst may reach very large volumes (Shear and Speight, 2007). It is known, as well, that follicular cysts may give origin to development of tumors (Scholl *et al*, 1999). The property of odontogenic cysts to enlarge at the expenses of jaw bone and gingival soft tissues has prompted studies on cyst-associated proteolytic enzymes able to degrade peri-cystic anatomical barriers. There is evidence that the fibrinolytic system may be related to jaw cyst enlargement (Sugimura *et al*, 1976). IL-1 increases the secretion of proMMP-9, proMMP-3 and uPA from cyst wall epithelial cells, and uPA converts pro-MMPs to active enzymes (Kubota *et al*, 2000). Other observations indicate the local production of tissue-type plasminogen activator (tPA) and of its inhibitor PAI-1 in radicular cysts, as related to the grade of inflammation (Tsai *et al*, 2004). The immunolocalization of parathyroid hormone-related protein (PTHrP) to the basal and suprabasal cells of the epithelial lining wall in dentigerous and radicular cysts led to speculate that PTHrP might modulate growth and bone resorption in odontogenic cysts and might act synergistically with IL-1 to increase bone resorption (Li *et al*, 1997).

Our data indicate that epithelial cells of both follicular and inflammatory cysts express uPAR. In inflammatory cysts also inflammatory cells express uPAR. Under normal conditions uPAR has a three-domain structure (D1 + D2 + D3): D1 is the N-terminal domain, D2 connects D1 to D3, and D3 is the C-terminal domain which anchors uPAR to the cell membrane through a glycosyl phosphatidylinositol (GPI) tail (Ploug *et al*, 1991). D1 is involved in uPA binding and in the interaction with vitronectin (VN) (Wei *et al*, 1994), but D2 and D3 are also required for high-affinity interaction (Hoyer-Hansen *et al*, 1997a). Cell surface uPAR can be cleaved within the D1–D2 linker region by several proteolytic enzymes and the cleavage leaves on the cell surface a two domains (D2 + D3) uPAR, a form which is expressed in various tissues and cell lines (Hoyer-Hansen *et al*, 1997b). uPAR truncation results in a decrease of uPAR-dependent cell invasion activity. We have shown that proteases of intra-cystic liquid cleave uPAR between domains 1 and 2. It is therefore likely that only the outer layers of the cystic epithelium in follicular cysts, and the peri-cyst inflammatory infiltrate in cooperation with the outer epithelial cells in inflammatory cyst, can perform an uPAR-driven degradation of the peri-cyst ECM, thus allowing cyst enlargement. uPAR-operated mechanisms may act synergistically with PTHrP and IL-1 to increase bone resorption.

Based on these findings we hypothesize a mechanism, accounting for both intra-osseous and soft-tissue enlargement of odontogenic cysts, based on the central role of the cystic epithelial layer and/or inflammatory cells-associated uPAR. The role of uPAR in this mechanism is similar to that described in tumor cell migration and invasion.

Our data also suggest that the use of inhibitors of proteases, as well as of uPA/uPAR interaction may have the chance to be useful in the control of enlargement of both radicular and dentigerous cysts of the jaw.

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