Comparison of xanthine oxidase levels in synovial fluid from patients with rheumatoid arthritis and other joint inflammations

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ABSTRACT

الأهداف: البحث عما إذا كانXOR الموجود في الغشاء المفصلي يتحرر، وتحديد نشاطه في السائل المفصلي وإلى إيجاد علاقة محتملة بين مستويات XOR وخطورة المرض عند المصابين بالتهاب المفاصل الرثويRA والغير المصابين بالمفاصل الرثوي.

الطريقة: تمت الدراسة في مختبر علم المناعة بجامعة فرحات عباس– سطيف – الجزائر خلال الفترة من 2001 إلى 2008. تم الحصول على عينات السائل المفصلي (SF) بعد موافقة المرضى من المستشفى الجامعي بسطيف، و من بالغين يعانون من التهاب المفاصل الرثوي(n=36) RA، وآخرين يعانون من التهابات مفصلية أخرى (n=52). بعد الكشف عن XOR في السائل المفصلي بواسطة المقايسة المناعية الإنزيمية (ELISA) و الأرتباط المناعي النقطي باستعمال أجسام مضادة ل XOR البقري كجسم مضاد أول، تم تقدير كمية الإنزيم بواسطة capture ELISA.

النتائج: بينت النتائج وجود XOR في كل السوائل المفصليةSF. أما نتائج ELISA، فبينت نسبا تصل إلى 0.762 و 0.143 mg/mL في السوائل المفصلية SF لكل من المصابين ب RA والالتهابات الأخرى على الترتيب. في أغلب الحالات وجد أن أكثر من 50% من الإنزيم المفصلي موجود في شكله المؤكسد. كما لوحظ تناسب موجب بين مستويات الإنزيم وخطورة الالتهاب المفصلي حيث أن المصابين ب RA لهم نسب من الإنزيم أعلى بكثير من المصابين بأمراض مفصلية أقل خطورة.

خاتمة: تبين النتائج المتحصل عليها أن الانزيم يمكن أن يكون داخلا في الالتهابات المفصلية ربما بإنتاج الأنواع الأكسيجينية النشيطة.

Objectives: To search whether xanthine oxido-reductase (XOR) present in the synovium is also liberated, to determine its activity in synovial fluid and to establish a possible relationship between XOR levels in rheumatoid arthritis (RA) and non-RA patients.

Methods: This study was carried out in the Laboratory of Immunology, University Ferhat Abbas, Setif, Algeria from 2001-2008. This study is a retrospective controlled study matching cases with RA to non rheumatoid joint inflammations. Synovial fluid (SF) samples were collected with consent of the patients, at Setif University Hospital, from adults suffering from RA (n=36) or only with joint inflammations (n=52). After its detection in SF with indirect enzyme-linked immunosorbent assay (ELISA) and dot-immunobinding, using anti-bovine XOR as first antibodies, XOR was assayed with capture ELISA.

Results: Xanthine oxidoreductase is found in all studied SF. Capture ELISA showed levels up to 0.762 and 0.143 mg/mL in SF of RA and other joint inflammations patients, respectively. In most cases, more than 50% of synovial XOR is present as oxidase form. Positive correlation was observed between enzyme level and the disease severity since RA patients had a significantly high enzyme amount compared to patients with other less severe arthritic pathologies.

Conclusion: These results suggest that the enzyme could well be involved in joint inflammation probably by producing reactive oxygen species.

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In mammalians, xanthine oxidoreductase (XOR) catalyses the final step in purine catabolism to produce uric acid, whereas in other species uric acid can be oxidized further to urea.[1] This enzyme occurs in 2 interconvertible forms, xanthine dehydrogenase (XDH, EC 1.1.1.204) and xanthine oxidase (XO, EC 1.1.3.22). Both forms can reduce molecular oxygen, whereas, only the XDH form uses nicotinamide adenine dinucleotide (NAD) as electron acceptor. Reduction of oxygen leads to superoxide anion ($O2^{-}$) and hydrogen peroxide (H_2O_2) production. The potential of XOR to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), increases the interest in this enzyme as a pathologic agent.[2,3] Over the last decade, ROS have been, increasingly, cited as intermediates in normal signal pathways.[4] The well that characterized XOR is from the bovine milk is present at high levels. Human milk XOR is of special interest as, surprisingly, in comparison to the bovine milk and rat liver XOR, it has a very low activity towards reducing conventional substrates, such as hypoxanthine and xanthine. Such propriety was largely attributed to inactive molybdenum center.[5,6] Using both light and electron microscopy immunohistochemical procedures, Jarasch et al,[7] showed that the enzyme is spread throughout the cytoplasm of bovine capillary endothelial cells. This was also found to be the case in the rat pulmonary endothelial cells.[8] Ishikawa et al[9] reported the enzyme to be exclusively cytosolic with no association with intracellular organelles such as endoplasmic reticulum, Golgi apparatus, lysosomes or peroxisomes.

Using affinity purified and monoclonal anti-XOR antibodies, Rouquette et al[10] showed the enzyme to be cytoplasmic lines but with higher intensity in the perinuclear region in human endothelial and epithelial cell. By immunohistochemical approach using anti-bovine XOR antibody, Stevens et al[11] localized XOR in synovial endothelium and suggested that the enzyme could play a role in post-ischemic reperfusion of rheumatoid synovium contributing to the characteristic signs of radical attack present in synovial fluid (SF). In RA, a massive liberation of lysosomial enzymes such as hydrolases, collagenases, mucopolysaccharidases and elastase has been reported.[12] Xanthine oxidoreductase present in the synovium[11] could also be liberated from synovium and participate, by the production of ROS together with reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of the multinucleated giant cells,[13] in the destruction of the joint tissue. We report in this study the detection, by indirect ELISA and dot-immunobinding, of the XOR in SF of patients suffering from RA and other joint inflammations. Total and oxidase activity of the enzyme is also studied to determine its possible role in joint inflammation and bone erosion.

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Methods. This study was carried out in the Laboratory of Immunology at University Ferhat Abbas of Setif, Algeria from 2001-2008. Synovial fluid samples were collected from adults volunteers after consent of patients at the Setif University Hospital, Setif, Algeria, from 36 subjects suffering from RA according to the American College of Rheumatology (ACR) criteria,[14] among which 12 were rheumatoid factor positive (RA+) and 24 negative (RA-) and from 52 subjects with other joint inflammations without rheumatoid factor. Inclusion criteria are: age 25-60 years old, suffering from RA consisting of 3 out of 5 of: ≥5 swollen and ≥5 tender joints, erythrocyte sedimentation rate (ESR) ≥28 mm/hour and/or C-reactive protein (CRP) ≥15 mg /L, ≥45 minutes morning stiffness. Exclusion criteria are pregnancy, nursing, usual clinical exclusions including hematological, renal and cardiac diseases, active infection, smoking and cancer. Approval was obtained from the ethics local committee prior to the commencement of the study. Bovine xanthine oxidase used to prepare anti-XOR antibodies in rabbits[15] was purified from fresh bovine milk according to the protocol described by Sanders et al[5] and Baghiani et al.[16] The purity of enzyme was estimated using the following criteria; protein/flavin ratio (PFR): A280nm/A450nm), ultraviolet (UV) visible spectrum and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern.[17] Total xanthine oxidase activity in 50 µl of SF was spectrophotometrically measured at 295 nm (extinction coefficient of 9600 M-1 cm-1).[1] Assays were performed at room temperature in air-saturated 50 mM Na-bicine, pH 8.3, containing 100 µM xanthine in presence of 500 µM NAD+. The oxidase activity was determined in the same conditions as above but in the absence of NAD+.

Detection of the enzyme in SF were performed using Enzyme Linked Immunosorbent Assay (ELISA) where SF (1%, v/v), diluted in 0.1 M carbonate buffer pH 9.6, was coated into 96-well polystyrene plates (Costar, Spain) and the presence of XOR was detected using rabbit anti-human XOR antiserum (first antibody).[15] The second antibody was an affinity purified anti-rabbit IgG labelled with horse radish peroxydase (Sigma). Orthophenylene diamine (OPD) was used as substrate and the absorbance was measured, at 492 nm in each

well using a 96 well plate reader (Diagnostics Pasteur LP200). Dot immunobinding assay (DIBA) was used to confirm the presence of XOR in SF. In this method, 5 µl of diluted, 4 fold in phosphate buffer saline (PBS), SF was loaded into nitrocellulose sheet (Shleisher & Shull, Germany). All following steps were performed as described by Hawkes et al.[18] To detect the possible interference of proteins with peroxidasic activity, substrate (4-chloronaphtol) was directly added to the dotted SF omitting labelled antibodies. To quantify XOR, rabbit anti-human XOR serum, at appropriate dilution (1/20), was coated into 96 well microtiration plates. Synovial fluid was added to each well and XOR was detected with affinity purified human antibodies anti-human XOR (purified in our laboratory). After application of anti-rabbit IgG labelled with peroxydase, the OPD substrate was added, in dark, and the reaction was stopped after 10-20 minutes with 50 µl of 2 normal sulphuric acid. Xanthine oxidoreductase, with known concentration, was used to build up a standard curve.

Statistical analysis were carried out using Sigma Stat software. The probability value was considered significant at <0.05.

Results. Using indirect ELISA and DIBA (**Figure 1**), all SFs were XOR positive. Capture ELISA showed that the enzyme concentrations varied from one group of patients to another and results are represented in **Table 1**. Xanthine oxidoreductase levels are up to 0.762 mg/ml in SF of patients with RA, whereas patients with other joint inflammations revealed less than 0.143 mg/ml. **Figure 2** shows the distribution of XOR concentrations among all tested SF. It is noticed that a positive relationship was observed between enzyme levels and the disease severity since RA patients had a significantly high amounts (p=0.001) compared to patients with less severe joint inflammations. Results of enzymatic activity point out that up to 96% of SF XOR is present as oxidase form in most tested cases as showed in **Table 1**. Total XOR activity is inhibited by allopurinol as a specific inhibitor of XOR. Preliminary results show that all SFs tested contained NADH oxidase activity but lacking a specific inhibitor for the FAD active center of XOR, we are unable to confirm that this activity is due to xanthine oxidase.

Discussion. In rheumatoid joint disease conditions of ischemia-reperfusion prevail in the synovium. The resulting, repeated cycles, provide conditions for ROS production by XOR, which has been detected in endothelial cells of the synovium.[11,17] In comparison with normal synovial tissue, rheumatoid

Table 1 - Xanthine oxidoreductase (XOR) protein (µg/ml) and activity (pM/min.) in 50 µl of synovial fluids (SF).

Group of patients	XOR concentration	Total xanthine oxidase activity in 50 µl of SF	Percentage of oxidase in 50 µl of SF
Rheumatoid arthritis (n=36)	476.81 ± 223.35	162.29 ± 179.56	87.86 ± 90.51
Hydarthrosis (n=24)	65.43 ± 109.47	42.21 ± 51.26	65.44 ± 100.99
Hemarthrosis and other inflammations (n=28)	7.6 ± 6.45	15.65 ± 33.78	42.27 ± 76.12
Values are expressed as mean ± SD.			

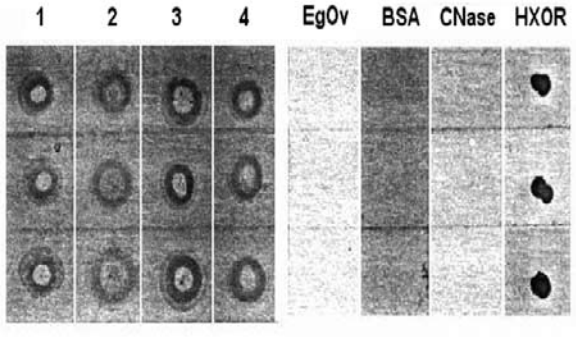

Figure 1 - Dot immuno-binding of 4 synovial fluids (1-4) coated on nitrocellulose sheet and tested with rabbit anti-xanthine oxidoreductase serum. Three proteins were used as negative controls (EgOv - egg ovalbumin, BSA - bovine serum albumin and CNase - carbonic anhydrase) and human xanthine oxidoreductase (HXOR) as positive control. All samples and proteins were coated in triplicate.

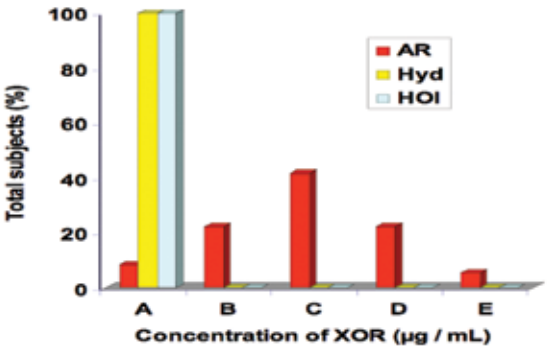

Figure 2 - Distribution of xanthine oxidoreductase concentrations among patients with rheumatoid arthritis (RA), hydarthrosis (Hyd) and hydarthrosis and other joint inflammations (OJI). A: 0-160 µg/mL, B: 161-320 µg/mL, C: 321-480 µg/mL, D: 481-640 µg/mL, E: >640 µg/mL.

synovium showed up to 60 times higher levels of XOR possibly because of upregulation of the enzyme by characteristically high levels of cytokines and the hypoxic nature of rheumatoid synovium.[19] Elevated levels of XOR in serum of patients with inflammatory and auto-immune rheumatic diseases have been equally reported.[20] The roles of XOR in cytokine induced bone erosion, promoting vasculitis and spreading disease and generation of nitric oxide (NO) under conditions of hypoxia have been well documented.[19] Under specified low O_2 conditions, XOR will metabolise dietary NO_3^- to NO_2^- and then may form NO, which can not be efficiently formed under hypoxic conditions by nitric oxide synthase (NOS), as this enzyme requires O_2.[21] The effect of NO in inflammatory conditions and its possible interaction with ROS to form peroxynitrite suggest that XOR may play a key role in diseases characterized by hypoxic episodes. In view of the clearly elevated level of synovial XOR in RA and the higher activity of the human enzyme towards NADH rather than xanthine, a chemiluminescence assay was used to detect NADH oxidase activity in the plasma of patients with RA and normal controls.[5] The enzymatic NADH oxidase activity was found to be significantly higher in sera of RA patients correlating well with biochemical markers,[19] and anti-XO antibodies detected in SF of these patients are shown to be in good correlation (positive) with the severity of the disease.[22]

We have reported here the presence of relatively high concentrations of XOR in SF especially of patients suffering from RA where the oxidase form of the enzyme is predominant. Therefore, there were some limitations with respect to the analysis that may affect the accuracy of these results due to sample size (RA^+ =12). This result showed that XOR is liberated with lysosomal enzymes from the synovium and could be involved in the cartilage destruction and bone erosion.[19] Preliminary tests show that the enzymatic NADH oxidase activity was found to be significantly higher in SFs of all patients. Xanthine oxidoreductase may well play an important role as source of ROS in joint inflammations and participate in self maintenance of the disease.

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