EXTENDED REPORT

Identification of the advanced glycation end products Nε-carboxymethyllysine in the synovial tissue of patients with rheumatoid arthritis

S Drinda, S Franke, C C Canet, P Petrow, R Bräuer, C Hüttich, G Stein, G Hein

Background: Generation of advanced glycation end products (AGEs) is an inevitable process in vivo and can be accelerated under pathological conditions such as oxidative stress. In serum and synovial fluid of patients with rheumatoid arthritis (RA) raised AGE levels have been found.

Objective: To determine the presence of Nε-carboxymethyllysine (CML; marker of oxidative stress) in RA synovial tissue by immunohistology.

Methods: Frozen synovial tissue samples from 10 patients with RA and eight controls (four patients without joint disease and four patients with osteoarthritis (OA)) were treated with rabbit-anti-CML-IgG and goat-antirabbit-IgG. Immunostaining was visualised by streptavidine-alkaline phosphatase (chromogen fuchsin). Cell differentiation was performed with antibodies against CD68, CD45RO, and CD20.

Results: CML was detected in the synovial lining, sublining, and endothelium in 10/10 RA and 4/4 OA synovial specimens. In RA some macrophages (CD68+) and T cells (CD45RO+) showed positive immunostaining for CML, whereas B cells were negative. Staining in OA synovial sublining was weak compared with RA.

Conclusions: CML was detected for the first time in RA and OA synovial tissue. Different patterns of immunostaining in RA and OA and the presence of CML on macrophages and T cells, suggest a role for CML in the pathogenesis of RA. This might be due to presentation of new epitopes which can maintain or even trigger an autoimmune response.

Advanced glycation end products (AGEs) are formed during the Maillard reaction by non-enzymatic glycation and oxidation of proteins constituting a heterogeneous class of structures. The generation of AGEs is an inevitable process in vivo and their accumulation in different tissues has been implicated in the process of aging and also in the pathogenesis of several pathological conditions, including diabetes, atherosclerosis, Alzheimer’s disease, renal failure, and renal replacement treatment with maintenance haemodialysis.

AGE modification can lead to tissue damage through alterations of tissue protein structure and function, and stimulates cellular responses mediated by a specific receptor (RAGE).

Not only glucose but also reactive carbonyl intermediates derived from the Maillard reaction (3-deoxyglucosone), as well as the products of sugar oxidation (arabinoxy, glyoxal, methylglyoxal) and lipid peroxidation (malondialdehyde) are precursors causing chemical modification of proteins. Oxidative stress accelerates the formation of these products.

The well characterised AGE Nε-carboxymethyllysine (CML) represents a chemically modified amino acid and originates in vivo from carbohydrate as well as from lipid derived precursors. It has been suggested that CML is a general marker of oxidative stress and tissue damage through protein alteration. Oxidative stress plays an important part in acute and chronic inflammatory diseases, including rheumatoid arthritis (RA).

Recently reported studies have provided evidence that the AGE pentosidine is raised in the articular cartilage as well as in the serum and synovial fluid of patients with RA. Furthermore, the raised autoantibody level against CML in patients with diabetic renal failure indicates that CML accumulated in vivo serves as an immunological epitope. On the other hand, many findings in RA indicate an antigen driven process in this disease, but a specific antigen has not yet been found.

To obtain more detailed information on the accumulation of AGE modified proteins in RA, this study aimed at investigating whether CML modifications are present in the synovial tissues of patients with RA.

MATERIALS AND METHODS

Synovial tissue samples obtained by therapeutic synovectomy from 10 patients with RA (mean age 57 years, range 33–83) fulfilling the 1987 revised ACR criteria were examined in this study. Synovial specimens obtained at post mortem from four patients who had no history of joint disease (mean age 52 years, range 21–86) and from four patients (mean age 74 years, range 66–80) with osteoarthritis (OA) served as controls. The OA samples were obtained during the surgical implantation of a knee endoprosthesis. All patients were non-diabetic. Table 1 shows the patient characteristics.

Sample preparation

Frozen synovial tissues (2 µm sections) were mounted on polylysine coated slides (Menzel, Germany), fixed in acetone, and blocked in 4% skimmed milk for 20 minutes. Slides were then incubated with rabbit-anti-CML-IgG (concentration 4 g/l, dilution 1:10 000; kindly provided by Roche Diagnostics, Germany) for one hour. Biotinylated goat-antirabbit-IgG conjugated with streptavidine/alkaline phosphatase was used for immunostaining in RA and OA and the presence of CML on macrophages and T cells, suggest a role for CML in the pathogenesis of RA. This might be due to presentation of new epitopes which can maintain or even trigger an autoimmune response.

Abbreviations: AGEs, advanced glycation end products; CML, Nε-carboxymethyllysine; IL, interleukin; OA, osteoarthritis; RA, rheumatoid arthritis; RAGE, receptor for AGEs; TNFα, tumour necrosis factor α; TRX, thioredoxin.
detection (concentration 1 mg/ml, dilution 1:400, incubation time 40 minutes; DAKO Denmark). Immunostaining was visualised using the streptavidine/alkaline phosphatase technique with the chromogen fuchsin. Negative controls included (a) omission of the primary antibody; (b) treatment of sections with rabbit immunoglobulin of irrelevant specificities at the same concentration as the primary antibody; (c) non-arthritic synovial tissue (table 1).

For cell differentiation we used antibodies against CD68 (macrophages; DAKO Denmark), CD20 (B lymphocytes; DAKO Denmark), and CD45RO (activated T lymphocytes; DAKO Denmark). Tissue samples were examined in two staining steps: in the first step slides were incubated with rabbit-anti-CML-IgG (see above), staining was visualised by the alkaline phosphatase anti-alkaline phosphatase technique (chromogen neufuchsin). In the second staining step, slides were treated with antibodies against CD20 and CD68 by applying the peroxidase technique (chromogen diaminobenzidine) or CD45RO using the streptavidine-biotin-immunogold-silver technique.

For monostaining the semiquantitative measurement was assigned to the following ranges: no positive cells, <5% positive cells, 5–10% positive cells, 10–25% positive cells, >25% positive cells.
cells, 30–60% positive cells, >60% positive cells, and not applicable. For double staining no measurement was made.

**RESULTS**

CML was detected in 10/10 RA synovial specimens. CML-specific staining was localised in the synovial lining and in numerous sublining and stroma cells as well as in the endothelium of synovial vessels (fig 1). Some macrophages (CD68+) and activated T cells (CD45RO+) showed positive immunostaining for CML (figs 2 and 3), whereas B cells were negative (fig 4). About 10% of CML positive cells were found in macrophages and about 30% in activated T cells. In contrast, in control tissues from patients without joint disease no signal was detected in synovial lining and sublining, and only weak to moderate immunostaining was seen in vascular endothelium cells (table 2). No staining was seen in tissue sections

<table>
<thead>
<tr>
<th>Patient No/disease</th>
<th>Immunohistology</th>
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<tbody>
<tr>
<td>Staining in synovial lining</td>
<td>Staining in synovial sublining</td>
</tr>
<tr>
<td>1/RA</td>
<td>NA</td>
</tr>
<tr>
<td>2/RA</td>
<td>+++</td>
</tr>
<tr>
<td>3/RA</td>
<td>++</td>
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<tr>
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<td>5/RA</td>
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<tr>
<td>6/RA</td>
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<td>7/RA</td>
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</tr>
<tr>
<td>9/RA</td>
<td>NA</td>
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<tr>
<td>10/RA</td>
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</tr>
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<td>15/Control</td>
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<tr>
<td>17/Control</td>
<td>–</td>
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<td>18/Control</td>
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Semiquantitative: –, no positive cells; +/-, <5% positive cells; ++, 30–60% positive cells; +++ , >60% positive cells; NA not applicable.

**Figure 3** RA synovial tissue with activated T cells, which shows double staining: CML (red) and CD45RO+ (black) (×40).

**Figure 4** RA synovial tissue with follicle of B cells (brown staining). CML positive cells are organised outside the follicle (×20).

**Figure 5** OA synovial tissue showing CML expression (red) in the lining layer, the subsynovial interstitium, and in endothelial cells (×20). Overall, fewer inflammation cells are present compared with RA tissue.
after omission of the primary antibody or treatment with rabbit immunoglobulin, demonstrating the specificity of the anti-CML-antibody used. Synovial tissues from patients with OA showed, in the synovial lining and vascular endothelium, a staining pattern and intensity comparable with that of RA samples, but the number of lymphocytes and macrophages was lower than in RA tissue. Staining in OA synovial sublining was weak compared with RA synovial tissue (fig 5).

**DISCUSSION**

Besides the physiological occurrence of AGEs, these changed protein structures seem to play an important part in different diseases.\(^5\)\(^6\) As far as we know, this study is the first to show that CML accumulates in RA synovial tissue. CML was detected in all samples of synovial tissue from patients with RA.

CML is an AGE which can be used as a marker of oxidative stress.\(^7\)\(^8\) In RA, inflammatory changes and destruction of joints are seen.\(^9\) The accumulation of CML in RA synovial tissue might be the result of oxidative stress during local and systemic inflammation. The generation of AGEs is an inevitable process in vivo; yet, on the other hand, effects of inflammation, such as oxidative stress, can cause different “metabolic changes”, leading to the mutation of key regulatory genes. This may help to transform inflammation into chronic disease.\(^10\) In RA, a disease with a probable autoimmune pathogenesis, the triggering factors of the immunological process are still unknown and up to now a specific antigen is still missing. Yang et al showed that in older patients AGES represent new epitopes and contain new antigenic structures,\(^11\) thereby possibly contributing to the generation of autoimmune responses.\(^12\) This theory is supported by the findings of Miyata et al and Takahashi et al, who found a correlation between the inflammatory activity and concentration of AGES measured in the urine and blood of patients with RA.\(^13\)\(^14\) However, our data do not indicate a quantitative association between the degree of CML-specific staining and activity of RA.

Michaelsson et al showed the importance of collagen II glycosylation for T cell recognition.\(^15\) AGE modified proteins in contrast with “normal” proteins can also activate macrophages and stimulate secretion of interleukin (IL) 1, IL6, and tumour necrosis factor \(\alpha\) (TNF\(\alpha\)).\(^16\) This interaction is possible through binding of AGES on the receptor for AGES (RAGE).\(^17\) The presence of RAGE on macrophages and endothelial cells has been shown by Schmidt et al.\(^18\) In RA synovial tissue we could detect CML in vascular endothelium and on CD68+ and CD45R0+ cells (macrophages and activated T cells) using a double staining technique. AGES may influence secretion or activation of the last two cell types. This might directly influence the disease progress in RA: IL1, IL6, and TNF\(\alpha\) accelerate bone resorption and may participate in cartilage degradation, both phenomena seen in RA.\(^19\)\(^20\) More over, Miyata et al have shown that AGE modified proteins support production of collagenase and degradation of different cartilage collagens.\(^21\) So AGES may be a result of inflammation and possibly maintain, or even accelerate, the inflammation process by changing the antigen structure of different tissue compartments, or by activating the secretion of inflammation mediators. The fact that a possibly consequence of inflammation can maintain the inflammation itself can also be seen in the recent findings on the relation between thioreodoxin (TRX) and oxidative stress. TRX is a cellular reducing catalyst induced by oxidative stress. The effect of TRX on TNF\(\alpha\) induced IL6 and IL8 production using rheumatoid synovial fibroblast cultures was examined by Yoshida et al.\(^22\) The extent of IL6 and IL8 production in response to TNF\(\alpha\) was greatly augmented by TRX compared with TNF\(\alpha\) alone. Maurice et al found that TRX is significantly increased in the synovial fluid and synovial tissue of patients with RA in comparison with levels in patients with other joint diseases.\(^23\)

In contrast, no immunostaining was found in the synovial lining or sublining of the control patients without a history of joint disease.

Detection of CML in control tissue samples from patients with OA indicates that the presence of AGES in higher concentration is not a specific phenomenon of RA and is not restricted to those diseases with autoimmune pathways. OA tissue samples showed in synovial lining and vascular endothelium a staining pattern and intensity comparable with that of RA samples, but staining in the synovial sublining was weak. This might be due to the lower number of lymphocytes and macrophages followed by a lower presence of AGE CML than in the RA tissue. To understand the role of AGES in autoimmune pathways further studies should examine whether the presence of AGES is followed by an invasion of immune competent cells, like macrophages or lymphocytes, or if AGES are only a “by product” in the autoimmune response.

**ACKNOWLEDGEMENTS**

We thank the German Ministry for Education and Research (grant FKZ 01 ZZ 9602) and Roche Diagnostics for their generosity in providing the rabbit-anti-CML-antibody.

**Authors’ affiliations**

S Drinda, S Franke, G Stein, G Hein, Department of Internal Medicine IV, Friedrich Schiller University Jena, Germany

C C Canet, P Petrov, R Bräuer, C Hüttich, Institute of Pathology, Friedrich Schiller University Jena, Germany

**REFERENCES**

Colchicum autumnale from Het Cruydtboeck by Rembertus Dodonaeus

The cover illustration (fig 1) shows a woodcut representing colchicum autumnale from the famous Cruydtboeck (Herbal) by Dodonaeus. Nowadays, Rembertus Dodonaeus is considered to be the founder of modern botany.

He was born in 1517 in Mechelen, at that time an important Flemish town and judicial capital of the Netherlands. After graduating in medicine at the University of Louvain at the age of 18, Dodonaeus returned to his native town to work there as a general physician. He had a special interest in botany and began describing in detail the external appearance, inflorescence, flowering time, and medical properties of herbs and plants. This meticulous work resulted in 1554 in the publication of the famous Flemish herbal Het Cruydtboeck. The book contains descriptions of more than 1000 herbs and plants and is illustrated with 715 woodcuts based on drawings by Peter van der Borcht. Later, the book was translated into Latin in order to reach a larger public.

The work of Dodonaeus was an inspiration for two other famous physician-herbalists in the early 16th century, Clusius (1526–1609) and Lobelius (1538–1616), who expanded and improved his work. From 1574 until 1577 Dodonaeus was the personal physician of Emperor Maximilian at the imperial court of Vienna. In 1582 he was nominated professor of medicine at the Leiden University in Holland where he died in 1585. Until Linnaeus (1707–1778) introduced the new classification of plants in 1735, Het Cruydtboeck remained a standard not only as a botanical work but also as a pharmacopoeia, and was improved and reprinted several times.

Colchicum autumnale (meadow saffron) was already known to the ancient Greeks for its beneficial effect on podagra, but also for its toxicity. The active components, the alkaloids, are extracted from the seed as well as from the tuber. Strikingly, the plant flowers in autumn, hence the specification “autumnale” in its name. Colchicum is said to refer to the Greek region Colchis, near the Black Sea from where the flower originates. Some even speculate that the sorceress Medea, daughter of the King of Colchis, used colchicum autumnale as a poison to kill her children as a revenge for the adultery of her husband Jason.

Acknowledgement

We thank Mr Bekkers and Mr van Druenen from the “Maaslands Antiquariaat” in the Stokstraat, Maastricht, The Netherlands, who kindly allowed us to take the photographs from their version of Het Cruydtboeck.

A Boonen
Department of Rheumatology, University Hospital Maastricht, The Netherlands

L van de Putte
Department of Rheumatology, University Medical Centre Nijmegen, The Netherlands

Correspondence to: Dr A Boonen, University Hospital Maastricht, PO Box 5800, 6202 AZ Maastricht, The Netherlands, Aboonen@sint-azm.nl