CALCITONIN-GENE-RELATED PEPTIDE (CGRP) AND GLYCOSAMINOGLYCANS (GAGs) IN RHEUMATOID ARTHRITIS

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ABSTRACT
There are important interactions between nervous system and rheumatic diseases. CGRP plays an important role in neural regulation of inflammatory synovial cell functions. In rheumatoid arthritis (RA), degrading cartilage releases increased amounts of GAGs.

In this study the blood and synovial fluid (S.F.) concentrations of calcitonin gene-related peptide (CGRP) and glycosaminoglycans (GAGs) were measured in patients with rheumatoid arthritis (R.A.). Significantly higher levels of CGRP and GAGs in S.F. were found when compared to the controls. However, plasma CGRP and serum GAGs showed no significant difference between R.A. and controls. A positive correlation was found between S.F. levels of CGRP and GAGs. Our results suggested that S.F. levels of CGRP and GAGs can be used as a specific battery of markers for R.A inflammatory processes and cartilage degradation.

INTRODUCTION
Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease predominantly affecting the peripheral joints (Hermann et al., 1998). It is now well established that proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) are involved in the inflammatory process leading to destruction of cartilage and bone and, eventually, to disability (Arend and
CALCITONIN-GENE-RELATED PEPTIDE (CGRP) etc...

Dayer, 1995, Feldmann et al., 1996). This pathologic process is partly counterbalanced by the production of mediators with antiinflammatory activities, such as IL-10 and cytokine inhibitors, including soluble TNF receptors and the IL-1 receptor antagonist (Hermann et al., 1998). The precise role of other cytokines abundantly detectable in RA joints remains a subject of controversy (Hermann et al., 1998).

Several hypotheses have been advanced to explain the pathogenesis of inflammation and joint damage in R.A. (Bensouyad et al., 1990). Long standing clinical observations suggest important interactions between nervous system and rheumatic diseases (Farndale et al., 1986).

The release of CGRP from peripheral nerve endings with other neuropeptides resulting in vasodilation, increased microvascular permeability and plasma extravasation was suggested by Bensouyad et al. (1990). So the release of CGRP from synovial nerve terminals seems to enhance or alternatively, protect the synovium from continuing damage (Hernanz et al., 1993, Kidd et al., 1990). A more recent study (Takeba et al., 1999) suggested a neural regulation of inflammatory synovial cell functions by CGRP.

CGRP is a 37 amino acid polypeptide chain, which arises by alternative processing of the calcitonin gene mRNA transcript (Lundy et al., 1999). The peptide is highly conserved in different mammalian species, between which 26 amino acids are homologous. It has α and β forms, the β-form differs from the corresponding α-form by one and three amino acids in rats and humans, respectively (Farndale et al., 1986). The structure of human (CGRP comprises an N-terminal disulphide bonded loop (amino acids 2 to 7), a well defined α-helix (amino acids 8 to 18) and a turn type conformation (amino acids 19 to 21) leads into an area of predominantly disordered structure before terminating in a carboxy-terminal amide group (Farndale et al., 1986).

Normal cartilage consists mainly of collagen and proteoglycans (protein core and GAG side chains). In normal cartilage, proteoglycans turn over slowly and as the degraded molecules are released they are
replaced by newly synthesized components (Bensouyad et al., 1990). In R.A. higher concentration of GAGs in S.F. was found, as degrading cartilage releases increased amount of GAGs (Bensouyad et al., 1990).

The objective of this study is the measurement of blood and S.F. levels of CGRP and GAGs to elucidate the possible involvement of CGRP in the inflammatory process as well as the use of GAGs as an indicator of increased cartilage degradation in R.A. Also to find any possible correlation between CGRP and GAGs which can be used as a more specific marker in R.A.

**SUBJECTS AND METHODS**

This study was conducted on 32 subjects. They gave written consents to share in the study. They were classified into two groups.

**Group 1 (R.A. group):** comprised 22 patients (16 women, 6 men) attending to the outpatient clinic of Rheumatology, Mansoura University Hospital. They had newly diagnosed definite or classical rheumatoid arthritis fulfilling the revised criteria of the American College of Rheumatology (Arnett et al., 1988) which stated that rheumatoid arthritis is diagnosed if at least four of seven criteria are present. These seven criteria are shown in table (1).

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Comment</th>
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</thead>
<tbody>
<tr>
<td>1. Morning stiffness.</td>
<td>• Duration &gt; 1 h lasting &gt; 6 weeks.</td>
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<tr>
<td>2. Arthritis of at least three areas.</td>
<td>• Soft tissue swelling or exudation lasting &gt; 6 weeks.</td>
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<tr>
<td>3. Arthritis of hand joints.</td>
<td>• Wrist, metacarpophalangeal joints or proximal interphalangeal joints lasting &gt; 6 weeks.</td>
</tr>
<tr>
<td>4. Symmetrical arthritis.</td>
<td>• At least one area, lasting &gt; 6 weeks.</td>
</tr>
<tr>
<td>5. Rheumatoid nodules.</td>
<td>• As observed by a physician.</td>
</tr>
<tr>
<td>6. Serum rheumatoid factor.</td>
<td>• As assessed by a method positive in less than 5% of control subjects.</td>
</tr>
<tr>
<td>7. Radiographic changes.</td>
<td>• As seen on anteroposterior films of wrists and hands.</td>
</tr>
</tbody>
</table>

MANSOURA MEDICAL JOURNAL
Their ages ranged from 19 to 67 years (mean 46), and the mean duration of the disease was 9 years. They were being treated with non-steroidal anti-inflammatory drugs, anti-rheumatic drugs and mild analgesics. All patients were instructed to stop any drug treatment for one month before taking blood and S.F. samples. No smoking was allowed during the day before. Any patient would be excluded from the study if he had any of the following criteria.

a- Under steroid therapy (either intra-articular or systemic).
b- History of or known endocrinial disease.
c- Chronic liver disease.
d- Cardiac, respiratory or renal diseases.

Group II (Control group): comprised ten healthy volunteers (8 women, 2 men). Their ages ranged from 17 to 66 years (mean 44). They were submitted to history taking and thorough clinical and laboratory investigations to exclude other inflammatory joint diseases, diabetes mellitus and malignancies.

Parallel samples of blood and S.F. were collected in all subjects after overnight 8h fasting. S.F. were obt-
tained from the knee joint when arthrocentesis was indicated for therapeutic reasons.

Blood and S.F. samples for CGRP assay were taken into ice cooled polypropylene tubes containing K$_2$EDTA (1 mg/ml of blood or synovial fluid) and aprotinin (500 KIU/ml of blood or synovial fluid (Hernanz et al., 1993), centrifuged in cooling centrifuge at 6000 rpm for 20 minutes and the plasma samples were separated immediately and stored at -20°C until assay.

Serum samples for GAG assay were collected and separated from whole venous blood and stored in aliquots at -20°C. S.F. samples for GAG assay were immediately mixed with citrate (10% V/V) and centrifuged (Farndale et al., 1986). The cell free supernate was stored at -20°C until assay.

Blood and S.F. samples were analyzed for:

1. CGRP by radioimmunoassay by the kits from Peninsula Laboratories, Inc., (Taylor Way, Belmont, California, USA). The assay is based upon the competition
between labeled 125I peptide and unlabeled peptide (either standard or unknown) binding to a limited quantity of specific antibody. As the concentration of standard or unknown in the reaction increases, the amount of 125I-peptide able to bind to the antibody decreases. By measuring the amount of 125I-peptide bound as a function of the concentration of the unlabeled peptide in standard reaction mixtures, it is possible to construct a "standard curve" from which the concentration of the peptide in unknown samples can be determined.

2. GAGs according to the colorimetric method of Farndale et al. (1986).

Statistical analysis
The data of this study were statistically analyzed using the SPSS/PC Computer Package version 5 on an IBM compatible personal computer (SPSS Inc., Chicago IL). Unpaired student's t-test was used to evaluate the difference in each variable between the two groups. P value <0.05 is considered significant.

RESULTS
Table (2) shows that the S.F. concentrations of CGRP and GAGs are very highly significantly increased (P<0.001). However there were no significant differences in the levels of plasma CGRP and serum GAGs.

Table (3): shows that there is a positive correlation (r = 0.704 P<0.01) between S.F. levels of CGRP and GAGs.
Table (2) : CGRP (pmol/L) and GAGs (ug/ml) in blood and S.F. in both studied groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 10)</th>
<th>R.A. group (n = 22)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma CGRP Mean</td>
<td>14.12 ±2.18</td>
<td>16.49 ±2.32</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>S.F. CGRP Mean</td>
<td>13.39 ±1.77</td>
<td>32.39 ±1.89</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum GAGs Mean</td>
<td>85.3 ±1.711</td>
<td>83.93 ±1.912</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>S.F. GAGs Mean</td>
<td>55.86 ±2.423</td>
<td>169.22 ±19.87</td>
<td>&lt;0.001</td>
</tr>
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</table>

(P>0.05) : non significant. (P < 0.001): Very highly significant.

Table (3) : Correlation between blood and S.F. levels of CGRP and GAGs in both studied groups.

<table>
<thead>
<tr>
<th></th>
<th>S.F. CGRP</th>
<th>S.F. GAGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma CGRP</td>
<td>r</td>
<td>-0.065</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Serum GAGs</td>
<td>r</td>
<td>-0.173</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>S.F. GAGs</td>
<td>r</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
</tr>
</tbody>
</table>

r = Pearson correlation coefficient.
P>0.05: Non significant
P<0.01: Highly significant

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DISCUSSION

Although rheumatoid arthritis (RA) is believed to be primarily an inflammatory disease of synovium, there is a good possibility that the initiation of the rheumatoid process is triggered by the autoimmune reaction involving type II collagen in the articular cartilage as a consequence of an unknown aetiologic agent. Synovitis and other extra-articular features may be induced secondary to the immune complex formation in the subchondral area (Fujii et al., 1999).

This study showed that there was a very highly significant increase in S.F. level of CGRP in rheumatoid arthritis patients when compared to controls (Table 2, P<0.001) whereas no significant difference was found between plasma levels of CGRP in R.A. patients when compared to controls (Table 2, P>0.05). These results agree with the results found by Arnalich et al. (1994) and Hernanz et al. (1999).

This S.F. increase in the levels of CGRP can be explained by the hypothesis that certain neuropeptides particularly CGRP are released at increased concentrations from peripheral nerve endings into the joint cavity in arthritis patients, thus enhancing the inflammatory and immunological responses (Arnalich et al., 1994, Hernanz et al., 1999).

A more recent study suggested that CGRP provides a neural regulation of inflammatory responses in patients with R.A., as it inhibited the proliferation of synovial cells as well as the production of the proinflammatory cytokine and matrix metalloproteinase by R.A. synovial cells at the level of mRNA expression (Takeba et al., 1999). Furthermore, they suggested the possible clinical application of the neuropeptides.

This study also showed that there was a very highly significant increase in the S.F. level of GAGs in R.A., (table 2, P<0.001) whereas there was no increase in the serum level of GAGs in R.A. (table 2, P>0.05) when compared to S.F. and serum level of GAGs in controls respectively. These results agree with the results of Bensouyad et al. (1990).

This S.F. increase of GAGs can be explained by three possibilities. First it can be the result of increased synthesis and release of R.A. cartilage GAGs, but this hypothesis needs an
evidence, as no other studies could prove it. Second the process which leads to removal of GAGs from the joint space is inhibited in patients with R.A. However, this is not supported by animal experiments, which showed that the clearance rate of GAGs from normal and arthritic S.F. was similar (half lives of 12 h and 14 h respectively) (Thomas et al., 1987).

The third possibility is that increased S.F. levels of GAGs is due to increased degradation of cartilage, which seems the most likely explanation. As animal experiments have shown that intra-articular injection of agents, which either inhibit the synthetic capacity of chondrocytes or their production of degradative enzymes, cause a net loss of cartilage and also increased GAGs concentration in S.F., at least in the short term (Pettipher et al., 1986, Dingle et al., 1987).

This study showed that there is a positive correlation ($r = 0.704$, $P<0.01$, Table 3) between S.F. levels of CGRP and GAGs. To our knowledge no other study tried to study this correlation. However, earlier studies failed to find a relationship between joint degradation indicated by increased S.F. levels of GAGs and immunological as well as inflammatory mediators in R.A. as rheumatoid factor, polymorphonuclear leucocyte number, myeloperoxidase concentration, free radicals and cytokines (Bensouyad et al., 1990), metalloproteinases and plasminogen activators (Blaser et al., 1996). Only one study found a correlation between S.F. level of GAGs and $C_3d$, but they reported that the explanation for this correlation remains uncertain (Bensouyad et al., 1990).

As regard the correlation between S.F. levels of CGRP and GAGs we suggest that this relation can be explained if we consider S.F. GAGs as an indicator of cartilage degradation (Bensouyad et al., 1990) and S.F. CGRP as a neural regulator of the pathophysiologic responses of patients with R.A., because CGRP can inhibit inflammatory agents at the level of mRNA expression and also can inhibit nuclear translocation, and phosphorylation of the transcription factor cAMP response element binding protein in RA synovial cells as proved by Takeba et al., (1999). This explanation makes it logic that increase of S.F. levels of GAGs must find an increased
regulation through the increased levels of neuropeptides especially CGRP. We think that this is the most believable point of view, taking into consideration the earlier studies (Bensouyad et al., 1990, Blaser et al., 1996). which failed to find relations between S.F. levels of GAGs with many parameters and others (Bensouyad et al., 1990) which failed to explain the single relation which was found with C3d.

In conclusion we suggest that S.F. levels of GAGs and CGRP are more important than their blood levels, and that the new correlation which was found between S.F. levels of GAGs and CGRP can be considered as a new and specific battery of markers for R.A. which helps in a most precise diagnosis and gives a recent line for clinical application of neuropeptides in R.A.

REFERENCES


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البيبتيد المتعلق بجين الكالسيتونين والجلوكوز أمينوجليكان
في حالات التهاب المفاصل الروماتويدية

المشتركون في البحث
محمد سعيد، إيمان الشبراوي
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كلية الطب - جامعة المنصورة - مصر

هناك تفاعل هام بين الجهاز العصبي والأمراض الروماتيزمية. ويلعب البيبتيد المتعلق بجين الكالسيتونين دوراً هاماً في التنظيم العصبي لوظائف الخلايا الزائبة المصاحبة بالالتهاب. استهدفت هذه الدراسة قياس مستوى كل من البيبتيد المتعلق بجين الكالسيتونين والجلوكوز أمينوجليكان في الدم وفاري السائل اللزلي لمرضى إنتفاخ المفاصل الروماتويدية وكذلك في أشخاص أصحاء، مع دعمات إيجاد علاقة بين مستوى البيبتيد المتعلق بجين الكالسيتونين ومستوى الجلوكوز أمينوجليكان في الدم والسائل اللزلي لهؤلاء الأشخاص. وتشمل هذه الدراسة على إثنتين وثلاثين شخصاً تم تقسيمهم إلى مجموعتين. الأولى مكونة من 22 شخصاً يعانون من مرض إنتفاخ المفاصل الروماتويدى والمجموعة الأخرى مكونة من 10 أشخاص أصحاء، في نفس المرحلة السنة ولاعبان من هذا المرض أو أي أمراض أخرى ظاهرة. وقد تم أخذ عينات الدم وعينات السائل اللزلي من كل شخص مشارك في البحث وفصل المصل والبلازما وحفظ العينات عند درجة - 20 درجة مئوية حتى تم قياس الآثى:

1- البيبتيد المتعلق بجين الكالسيتونين في البلازما والسائل اللزلي بواسطة طريقة جزيئية التفاعل الأنسابية.
2- الجلوكوز أمينوجليكان في المصل والسائل اللزلي بواسطة طريقة قياس الآثار.

وقد أوضح نتائج هذه الدراسة زيادة مستوى كل من البيبتيد المتعلق بجين الكالسيتونين والجلوكوز أمينوجليكان في السائل اللزلي لمرضى إنتفاخ المفاصل الروماتويدية مقارنة بالأشخاص

Vol. 30, No. 1 & 2 Jan. & April, 2000
الأصحاء. كما تبين وجود علاقة إيجابية ذات دلالة إحصائية بين مستوى كل من البيبتيد المتعلق بجين الكالسيترون والجليكوز أمينو جلبيكان في السائل الزالالي. ودراسة هذه النتائج أمكن استدلال على أهمية قياس مستوى كل من البيبتيد المتعلق بجين الكالسيترون والجليكوز أمينو جلبيكان في السائل الزالالي لهؤلاء المرضى واعتبارهما من الدلالات المميزة في تشخيص هذا المرض.