ELEVATED LEVELS OF CORTICOTROPHIN-RELEASING FACTOR BINDING PROTEIN IN THE BLOOD OF PATIENTS SUFFERING FROM ARTHRITIS AND SEPTICAEMIA AND THE PRESENCE OF NOVEL LIGANDS IN SYNOVIAL FLUID

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SUMMARY

In view of the reported inflammatory effects of corticotrophin-releasing factor (CRF) and the associated regulatory elements in the gene of its binding protein (BP), we postulate that both BP as well as novel BP-ligands other than CRF may be involved in inflammatory disease. We have investigated BP in the blood of patients with arthritis and septicaemia, and have attempted to identify CRF and other BP-ligands in synovial fluid. The BP was found to be significantly elevated in the blood of patients with rheumatoid arthritis and septicemia. There was less BP-ligand and CRF in synovial fluid from patients with rheumatoid arthritis than from those with osteo- or psoriatic arthritis. There was at least 10-fold more BP-ligand than CRF in the fluid of all three groups of patients. A small amount of immunoreactive human (h)CRF, eluting in the expected position of CRF-41, was detected after high-pressure liquid chromatography of arthritic synovial fluid; however, the bulk of material with BP-ligand binding activity eluted earlier, suggesting that synovial fluid contained novel peptides that interacted with the BP. These results would suggest that the BP and its ligands could play an endocrine immunomodulatory role in inflammatory disease.

KEY WORDS: CRF, CRFBP, BP-ligand immunoreactivity, Blood, Synovial fluid, Arthritis, Septicaemia.

The 41 residue peptide corticotrophin-releasing factor (CRF) was originally isolated and characterized from the hypothalamus because of its well-established role in the control of corticotrophin release from the pituitary gland in response to stress [1]. It has subsequently been found at low concentration in tissues such as stomach and duodenum [2] and pancreas [3]. One rich source, which in pregnant women gives rise to peripheral plasma levels of CRF [4] which rival the concentrations found in the blood of the hypothalamic portal system during stress [5], is the placenta [6, 7]. The lack of activation of the pituitary adrenal axis by these high concentrations of CRF in the blood of pregnant women can now be explained by a specific plasma binding protein [8-10] which is secreted by the liver [11] in sufficient amounts to be able to sequester all the adrenocorticotropic (ACTH) releasing activity of the placental CRF in the blood [12] throughout most of gestation, apart from in the last few weeks when the levels of the protein begin to fall [13]. This negative correlation between blood levels of human (h)CRF and its binding protein (BP) appears to be due to the specific removal of a BP-CRF complex from the circulation, as bolus injection of hCRF into males and non-pregnant individuals resulted in a rapid reduction of BP concentrations which was too quick to be explained by the half-life of the protein [14]. This rapid and specific uptake appears to operate even in individuals who would never experience significant concentrations of hCRF in blood during their lifetime, and suggested that there may be other as yet undiscovered ligands (and physiology) which would interact with the BP in males and females in the non-pregnant state. This mechanism also appears to be independent of the pituitary adrenal axis as ovine CRF (oCRF), which is more potent than hCRF in releasing ACTH in the human by virtue of its low affinity for hBP, has no effect on plasma levels of the BP when given as a bolus dose [14].

There have been a number of reports suggesting that CRF may be involved in the immune response, with 'CRF' receptors being identified on peripheral lymphocytes and monocytes [15, 16] which appear to be linked to IL-6 production [17]. The presence of CRF mRNA and immunoreactive CRF has also been reported in peripheral blood leucocytes [18] and rat thymus [19]. More recently, CRF has been demonstrated in inflammatory sites of experimental animal models [20], in the synovium from adjuvant-induced arthritis in rats [21], and in synovial tissue and fluid taken from patients suffering from osteo- and rheumatoid arthritis [22].

Examination of the 5' promoter region of the BP gene has revealed enhancer elements such as NF-κB which would suggest that the product of this gene protein could be upregulated in the acute-phase response to inflammatory situations [23]. In a recent preliminary study using a competitive BP-ligand assay, we have also identified non-CRF ligands which react with recombinant hBP in sheep brain and a human liver tumour [24]. These observations have prompted the present study of plasma BP in inflammatory...
conditions such as arthritis and septicaemia, and the re-examination of the CRF-like material [employing a specific two-site hCRF immunoradiometric assay (IRMA) and the competitive BP ligand assay] in synovial fluid from patients suffering from arthritis in an attempt to understand the interrelationships between these factors in these conditions and help reveal the true identity of 'peripheral or immune CRF' and its mode of action.

MATERIALS AND METHODS

Synthetic human CRF

hCRF was generously supplied by Dr Jean Rivier, The Clayton Laboratories for Peptide Biology, Salk Institute, CA, USA.

Collection of samples

Blood was collected in EDTA from eight normal controls, four patients with polyarticular, HLA B27-negative psoriatic arthritis (PA), nine patients with osteoarthrosis of the knees (OA) and 45 patients with rheumatoid arthritis (RA) (ARA Criteria 1987 [25]). Blood was collected from a further eight patients who had an acute septicaemic illness. In all patients this was due to an acute bacterial pneumonia, usually pneumococcus. The blood was centrifuged and the plasma removed and stored at —20°C.

The synovial fluid was aspirated from the knees of patients with OA, PA and RA. This fluid was collected and frozen at —20°C in 30 ml aliquots in preservative-free heparin within 20 min of collection. All patients with OA, RA and PA were on NSAIDs. The patients with PA were on Salazopyrin in addition and the patients with RA were treated with i.m. gold (5), penicillamine (8), Salazopyrin (12) and methotrexate (20). The patients with PA and RA were deemed moderately to highly active on physician's assessment (JD) and CRP, the mean level for PA being 15 mg/l (6-36) and for RA 32 mg/l (9-89).

Binding protein immunoassay

This was carried out as described previously [13, 14] using purified recombinant BP (purified at alkaline pH) for radiolabel and standards [14], and a rabbit anti-binding protein antibody. Separation was achieved with sheep anti-rabbit (Fc region) antibody in the presence of 0.5% normal rabbit serum and 2% polyethylene glycol (PEG) 6000.

Immunoradiometric assay of CRF

This was essentially the same as that described previously [26], except that it used radiolabelled sheep IgG raised against the 36-41 NH2 C-terminal fragment of hCRF and a rabbit antiserum against the 1-20 N-terminal fragment of hCRF as the second site antibody. Separation was achieved by the addition of a precipitating sheep anti-rabbit IgG (Fc region) in the presence of 0.5% normal rabbit serum and 2% PEG 6000. Human CRF was used to prepare standards.

Assay of ligands which bind to CRF binding protein

This was carried out in 50 mM PO4 buffer (pH 7.4) containing 1% albumin and 0.1% NaN3 (w/v). BP (2.5 ng in 200 μl buffer) was incubated with 100 μl of increasing concentrations of either standard (hCRF) or unknowns overnight before the addition of 100 μl of 20 000 c.p.m. of radiolabelled CRF (25) and incubating for a further 3 h. Separation was achieved by the addition of a sheep anti-hBP antibody (100 μl diluted 1 in 1000) and incubating for 1 h, followed by 200 μl of a donkey anti-sheep precipitating antiserum mixture (antisemur diluted 1 in 10, normal sheep serum diluted 1 in 200, in 0.9% NaCl containing 2% PEG 6000). After centrifugation and aspiration of the supernatants, the radioactivity was counted in the pellets.

Extraction of synovial fluid

After thawing at room temperature, typically 100 ml of pooled synovial fluid were mixed with three volumes of methanol containing 1 mM N-ethyl maleimide, 1 mM phenyl methyl sulphonyl fluoride and 0.1% (w/v) trifluoroacetic acid. The precipitated material was removed by centrifugation at 3000 g at 4°C. The supernatant was extracted onto Sepak C8 cartridges (Waters) and eluted stepwise with 2 ml of increasing concentrations of acetonitrile (10% at each step) in 0.1% trifluoroacetic acid. For assay, 100 μg of Polypep, a digested collagen preparation (Sigma), were added as carrier to each of the fractions, which were then evaporated to dryness. Reconstitution in 200 μl of water containing phenol red indicated any residual acidity which could be corrected for by adding 10 μl of 2 M Tris base. The fractions were then diluted in assay buffer. When the Sepak extracts were to be further separated on high-pressure liquid chromatography (HPLC), they were dried down without the addition of Polypep.

High-pressure liquid chromatography

This was performed on a 0.4 x 15 cm column of Hypersil octadecysilysilicica (ODS) at a flow rate of 1 ml/min in 0.1% trifluoroacetic acid with a gradient of acetonitrile of 20-90% over 30 min. Before evaporation to dryness, 100 μg of Polypep were added to each fraction.

RESULTS

The plasma concentrations of binding protein as estimated in the radioimmunoassay are shown in Fig. 1. It can be seen that the blood of patients with RA and those with septicaemia contains elevated concentrations of BP compared to normal subjects. The greatest concentration was found in septicaemia. There was no significant difference between the concentration of BP in the blood of patients suffering from OA or PA with RA. In both assays, the bulk of activity from both groups of patients eluted in the 30-50% acetonitrile fractions.
with an ~10-fold greater amount of BP-ligand activity overall than CRF immunoreactivity. There was more hCRF immunoreactivity and BP-ligand activity found in the pool of fluid taken from OA patients than in that from PA patients.

The results from Sepak C₁₈ extraction of three separate pools of synovial fluid taken from patients with RA are shown in Figs 4 and 5. As in Figs 2 and 3, the bulk of material detected in both assays eluted in the 30–50% acetonitrile fractions, but the overall amount of material in these patients is between 10- and 50-fold less than that found in the fluid taken from patients with either PA or OA. The relationship between the material detected in the CRF immunoassay and BP-ligand assay was the same as above, with at least 10-fold more material being found in the ligand assay.

When the material from the Sepak extraction of a pool of fluid collected from patients with RA was submitted to HPLC, there was evidence for at least three peaks of BP ligand activity, two of which were significantly less hydrophobic than hCRF and had negligible CRF immunoreactivity (Fig. 6). The peak of hCRF immunoreactivity eluted in its expected position.

**DISCUSSION**

Although CRF and its BP were originally discovered for their quite separate roles from the field covered by this journal, the present study would suggest that the BP and its ligands are involved in some way in the inflammatory response.

The increase in plasma levels of the BP in the blood of patients suffering with RA and septicaemia is reflected in the severity of systemic inflammation in these conditions. This is in agreement with the presence of enhancer elements in the 5' region of the BP gene [23]. The lack of increase in the level of BP in the plasma of patients with OA is also in agreement with the low level of systemic inflammation in this group.

A previous report of CRF in synovial fluid [22] has been confirmed in the present study using a specific two-site radioimmunoassay for hCRF to detect material in fractions resulting from HPLC of fluid extracts.

In contrast with the previous report [22], the levels of hCRF and (BP-ligand) we have found in synovial fluid taken from patients with OA were much higher than that from RA. One explanation of this discrepancy is the different type of immunoassay used in
achieve this level of specificity because fragments (and generally reacts with a single epitope, it is difficult to immunoassays [26]. In radioimmunoassays in which hCRF IRMA although an epitope for one but not bothalogues, e.g. oCRF and urotensin, do not register a
response [27]. In the same study, the alpha helical CRF (9-33) (ahel-CRF) antagonist was also shown to have a high affinity for hBP. This peptide has been shown to have some antagonist activity at pituitary receptors [28], but strong antagonist activity at peripheral ‘immune CRF’ receptors, e.g. during the stimulation of release of acute-phase response proteins by IL 1β [29]. In the latter study, the involvement of a peptide which was not identical immunologically to rat (r)hCRF was implicated by the lack of a result seen with anti-CRF antibodies, which in this case were ineffective in immunoneutralizing the CRF-like material released by IL 1β and thought to be responsible for the acute-phase response [29]. This is particularly relevant here as, in addition to a separate peptide, it would suggest that there is a separate peripheral receptor which has more in common with the BP spectrum of peptide ligand activity than with that of hCRF. Indeed, a CRF receptor has recently been identified in heart and skeletal muscle which has a high affinity for urotensin I and sauvinagin, and a low affinity for o-CRF [30]. The BP ligand material we have detected in human synovial fluid appears to be due to peptide(s) which have sufficient structural homology with hCRF to confer high affinity for the BP, but insufficient homology to confer immunoreactivity in the IRMA. This would suggest that they are separate peptides from a separate stem hormone precursor. In all synovial fluid samples examined, there appeared to be more non-CRF ligands that hCRF. Whether this is a true representation of the physiological situation at the time the sample was taken cannot be assessed at this juncture. It is possible than the non-CRF ligands are more stable than hCRF. This consideration would be of greater importance in synovial fluid taken from the joints of patients with RA, where there would be a much higher level of neutrophil invasion into the inflamed tissue and thus a greater chance of cleavage of labile peptides by proteolytic activity present in this situation. It might also explain the generally overall lower concentrations of peptides detected in this condition.

In conclusion, we have observed that the increase in concentration of blood CRF-BP appears to be coupled to the severity of systemic inflammation in arthritis. This is the only situation that we have found plasma BP to be elevated above normal and its increased sequestering potency may contribute to the defective hypothalamic response seen in these individuals [31]. As well as identifying the hCRF with a specific IRMA, we have also found non-CRF ligands for this BP in synovial fluid samples from each group of patients with arthritis. These observations would suggest that the BP...
and its ligands are involved in the genesis of inflammation and with the immune system, and hopefully once these ligands are isolated and characterized will help to further our understanding of peripheral CRF and their involvement in arthritis.

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REFERENCES