Elevated synovial tissue concentration of the common acute lymphoblastic leukaemia antigen (CALLA)-associated neutral endopeptidase (3.4.24.11) in human chronic arthritis

S. P. SREEDHARAN,* E. J. GOETZL*† & B. MALFROY‡ *Departments of *Medicine and †Microbiology & Immunology, University of California Medical Center, San Francisco, California and ‡Alkermes Inc., Cambridge, Massachusetts, U.S.A.*

Accepted for publication 11 May 1990

SUMMARY

The cell-surface neutral endopeptidase 3.4.24.11 (NEP) activity of the common acute lymphoblastic leukaemia antigen (CALLA) cleaves diverse peptide mediators at specific sites and it has been postulated that it regulates immune responses. The concentration of NEP was quantified in detergent extracts of synovial tissues by the percentage hydrolysis of [^{3}H-D-Ala]-Leu enkephalin/hr/100 mg of tissue. The synovial tissue concentration of NEP was higher in all patients with rheumatoid arthritis ($n=7$; group mean $\pm$ SD $= 29{\cdot}4 \pm 20{\cdot}2\%$), and was higher with degenerative joint disease ($n=6$ of 8; group mean $\pm$ SD $= 11{\cdot}9 \pm 10{\cdot}4\%$) than with traumatic arthropathy ($n=3$; $1{\cdot}1 \pm 0{\cdot}7\%$). The lack of direct relationship between synovial tissue NEP concentration and leukocytic infiltration suggests that the cellular source of NEP may be synoviocytes or fibroblasts, and that NEP may have distinctive pathogenetic roles in human arthritis.

Neutral endopeptidase 3.4.24.11 (NEP) is a 94,000 molecular weight (MW) integral membrane protein with zinc metallopeptidase activity, which cleaves peptide bonds amino-terminal to hydrophobic amino acids (Malfroy *et al.*, 1988; Shipp *et al.*, 1989; Jongeneel *et al.*, 1989; Erdös & Skidgel, 1989). NEP activity is greatest for small peptide substrates, such as the neuropeptides substance P, endorphins and enkephalins, for which it was first designated enkephalinase (Malfroy *et al.*, 1989). The highest concentrations of NEP are in the brain, kidneys, epithelia of intestines and lungs, fibroblasts, neutrophils and very immature lymphocytes (Erdös & Skidgel, 1989; Malfroy *et al.*, 1989). NEP is identical to the common acute lymphoblastic leukaemia antigen (CALLA) found on lymphoid malignancies of immature phenotypes, lymphocyte progenitors in foetal liver, bone marrow and thymus, and some cultured lines of pre-B cells (Shipp *et al.*, 1989; Jongeneel *et al.*, 1989).

The first functional role of NEP to be established was regulation of the vasoactive smooth muscle contractile and secretion-stimulating activities of neuropeptides, which are susceptible to rapid cleavage into inactive fragments. More recent results have suggested critical participation of NEP in the neutrophil chemotactic effects of *N*-formyl-oligopeptides (Painter *et al.*, 1988), peptide antigen processing and recognition by lymphocytes (Puri & Factorovich, 1988), and degradation of some cytokines (Pierart *et al.*, 1988). Thus the present findings of strikingly elevated levels of NEP in synovial tissues of patients with chronic arthritis imply an involvement in one or more of the elements of pathogenesis of synovitis and joint destruction.

Correspondence: Dr E. J. Goetzl, Division of Allergy and Immunology, U-426, University of California Medical Center, San Francisco, CA 94143-0724, U.S.A.

Large pieces of synovial tissue were obtained at surgery from patients with traumatic arthropathy (TA), due to internal derangements of joint structures ($n=3$), degenerative joint disease (DJD) ($n=8$) or rheumatoid arthritis (RA) ($n=7$), in the course of operative repairs and restorations (Table 1). The diagnoses in the patients studied were established by a classical clinical course, definitive joint X-rays, positive rheumatoid factor test in all patients with RA, and confirmatory synovial pathology. All patients were taking some form of analgesic or anti-inflammatory drug, but remission-inducing agents such as gold, penicillamine and cytostatic drugs were not being used, or had been discontinued at least 3 months before surgery. Three to nine synovial surface fragments, ranging from 17 mg to 60 mg, of tissue were taken from each surgical sample, frozen in liquid nitrogen and stored at -70°. Routine histological sections of the same synovial tissues were examined for inflammation, graded on a qualitative scale, and assigned scores of: 0, no inflammation; +, mild diffuse infiltration with neutrophils and/or mononuclear leucocytes; ++, intense leucocytic infiltration with lymphoid nodules, synovial hyperplasia of three or more layers, and/or vascular involvement. Of the three patients with DJD and ++ inflammation, tissue of only one showed any lymphoid nodules and none had vascular involvement (Table 1). In contrast, synovial tissues from five of six patients with RA and ++ inflammation contained lymphoid nodules and had vascular involvment. The common features in all samples with

Table 1. Characteristics of the patients

Patient	Diagnosis	Age	Duration of disease	Medications	Synovial inflammation
1	TA	41	6 months	An	0
2	TA	50	2 months	An	0
3	TA	52	30 months	NSAID	0
4	DJD	76	16 years	An	+
5	DJD	68	10 years	NSAID	++
6	DJD	69	15 years	An	+
7	DJD	68	13 years	NSAID	+
8	DJD	72	5 years	An	+
9	DJD	74	20 years	An, Pr	+
10	DJD	70	9 years	NSAID	++
11	DJD	71	6 years	NSAID, Pr	++
12	RA	58	13 years	Pr	+
13	RA	49	19 years	NSAID	++
14	RA	61	17 years	NSAID, Pr	++
15	RA	47	4 years	NSAID	++
16	RA	55	7 years	NSAID, Cl	++
17	RA	62	21 years	NSAID, Pr	++
18	RA	53	15 years	NSAID, Pr	++

TA, traumatic arthropathy; DJD, degenerative joint disease; RA, rheumatoid arthritis.

An, analgesic; NSAID, non-steroidal anti-inflammatory drug; Pr, prednisone; Cl, hydroxychloroquine.

++ inflammation were synovial cell hyperplasia and marked leucocytic infiltration.

Each tissue fragment was suspended in 1 ml of 50 mM HEPES containing 1% (v:v) Triton X-100 (pH 7·2) and sonified at 200 watts for 2 min at 4°. Whole sonicates were diluted 10-fold in 50 mM HEPES without detergent and aliquots were incubated in duplicate with 20 mM [^{3}H-D-Ala]-Leu enkephalin for 60 min at 37°, followed by resolution and quantification of cleaved and intact substrate, as described elsewhere (Llorens *et al.*, 1982). The mean percentage of substrate hydrolysed was calculated, as an index of NEP activity, and expressed as a value per 100 mg of synovial tissue. In some assays, the suppression of NEP activity by 1, 10 and 100 nM DL-thiorphan (Peninsula Laboratories Inc., San Carlos, CA), a selective NEP inhibitor (Malfroy *et al.*, 1989), was determined with additional duplicate sets of samples. The significance of differences between group mean values for NEP activity was evaluated by a standard two-sample *t*-test.

NEP was reliably quantified in extracts of synovial tissue with the radioisotopic synthetic Leu enkephalin assay. With only two exceptions in more than 150 determinations, each duplicate value of NEP activity in the extract of a tissue sample was within 10% of the mean level. For replicate samples of synovial tissue from one patient with DJD and two with RA, NEP activities measured in fresh tissue showed mean values statistically indistinguishable from those determined with tissue which had been frozen and later thawed before assay. Striking differences were observed between the concentrations of NEP in synovial tissues of patients with chronic arthritis and those with reactions to trauma (Fig. 1). The group mean value for NEP concentration in synovial tissue of patients with RA was significantly higher ($P < 0{\cdot}05$) than that of patients with TA, and the individual levels in RA all were higher than any in TA. The group mean concentration of NEP in RA also was substantially

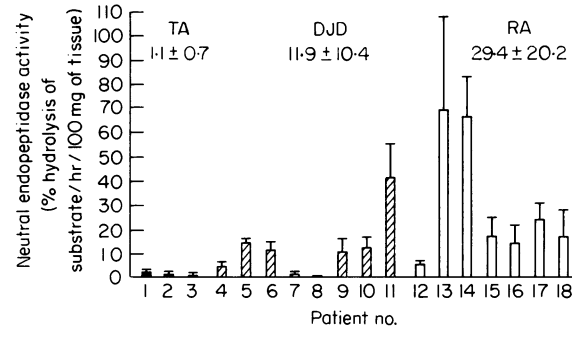

Figure 1. Synovial tissue concentrations of neutral endopeptidase 3.4.24.11. The number for each patient corresponds to that assigned in Table 1. Each bar and bracket depicts the mean and SD of the results of duplicate determinations of neutral endopeptidase 3.4.24.11 activity in three separate samples of synovial tissue from each patient, except for Patients 1–3 and 13 and 14, for whom two and four samples, respectively, were analysed. TA (■); DJD (▩); RA (□). Group mean values ± SD are presented under the patient group headings.

higher than in DJD ($P = 0{\cdot}05$), but the values overlap. While concentrations of NEP in synovial tissue samples of many patients with DJD were clearly elevated above any in TA, the group mean values were not significantly different. The identity of NEP was confirmed by applying the selective inhibitor DL-thiorphan. Ten nM DL-thiorphan significantly suppressed the activity of NEP, from 14·9 ± 2·9% to 1·4 ± 0·6% hydrolysis/hr/100 mg of tissue, in representative extracts of DJD tissues ($n = 3$), and from 45·9 ± 15·4% to 3·3 ± 1·7% in RA tissues ($n = 3$).

The concentrations of NEP in synovial tissue are clearly elevated in relation to the chronic synovitis of RA and DJD compared with those detected in TA. However, there was no apparent relationship between individual values of NEP and the qualitatively assessed level of synovial tissue inflammation (Table 1, Fig. 1). Similarly, the synovial tissue concentration of NEP did not correlate with the duration of synovitis in DJD or RA, or with the type of medication (Table 1).

The finding of significantly elevated synovial tissue concentrations of NEP in all patients with RA and some with DJD raises questions as to the cellular sources and possible pathogenetic contributions of this plasma membrane metallopeptidase. The B and T lymphocytes, and macrophages, constituting the chronic inflammatory lesions of rheumatoid synovitis have no detectable NEP activity (Malfroy *et al.*, 1989), although it remains to be demonstrated whether responses to antigenic and mitogenic stimulation may evoke latent NEP activity. Thus the most likely cellular sources of the NEP in arthritis are fibroblasts and synoviocytes (Lorkowski *et al.*, 1987; Werb & Clark, 1989), which are each increased to different levels in tissues of patients with RA and DJD relative to those with TA.

A greater understanding of the potential roles of NEP in the many elements of arthritis will depend on the development of new techniques for *in situ* quantification of nucleic acid message, protein and catalytic activity specific for NEP during the course of disease. The results of studies of possible involvement of NEP in peptide antigen presentation and in the destruction of cytokines and peptide mediators may provide additional insights into mechanisms of the effects of NEP in arthritis.

ACKNOWLEDGMENTS

The study described here would not have been possible without the co-operation of Drs Harry B. Skinner and Franklin T. Hoaglund of the Department of Orthopedic Surgery (UCSF), who provided many of the tissue samples for study.

REFERENCES

Erdös E.G. & Skidgel R.A. (1989) Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. *FASEB J.* **3,** 145.

Jongeneel C.V., Quackenbush E.J., Ronco P., Verroust P., Carrell S. & Letarte M. (1989) Common acute lymphoblastic leukemia antigen expressed on leukemia and melanoma cell lines has neutral endopeptidase activity. *J. clin. Invest.* **83,** 713.

Llorens C., Malfroy B., Schwartz J.C., Gacel G., Rogues B.P., Roy J., Morgat J.L., Jaroy-Agid F. & Agid Y. (1982) Enkephalin dipeptidyl carboxypeptidase (enkephalinase) activity: selective radioassay, properties, and regional distribution in human brain. *J. Neurochem.* **39,** 1081.

Lorkowski G., Zijderhand-Bleekemolen J.E., Erdös E.G., Von Figura K. & Hasilik, A. (1987) Neutral endopeptidase-24.11 (enkephalinase). Biosynthesis and localization in human fibroblasts. *Biochem. J.* **248,** 345.

Malfroy B., Giros B., Gros C., Llorens-Cortes C. & Schwartz J.-C. (1989) Metabolism of enkephalins in the central nervous system. In: *Opioid Peptides* (eds J. I. Szekely and K. Ramabadran), Vol. IV, p. 27. CRC Press, Orlando, FL.

Malfroy B., Kuang W.-J., Seeburg P.H., Mason A.J. & Schofield P.R. (1988) Molecular cloning and amino acid sequence of human enkephalinase (neutral endopeptidase). *FEBS Let.* **229,** 206.

Painter R.G., Dukes R., Sullivan J., Carter R., Erdös E.G. & Johnson A.R. (1988) Function of neutral endopeptidase on the cell membrane of human neutrophils. *J. biol. Chem.* **263,** 9456.

Pierart M., Najdovski T., Appelboom T.E. & Deschodt-Lanckman M.M. (1988) Effect of human endopeptidase 24.11 ('enkephalinase') on IL-1-induced thymocyte proliferation activity. *J. Immunol.* **140,** 3808.

Puri J. & Factorovich Y. (1988) Selective inhibition of antigen presentation to cloned T cells by protease inhibitors. *J. Immunol.* **141,** 3313.

Shipp M.A., Vijayaraghavan J., Schmidt E.V., Masteller E.L., D'Adamio L., Hersch L.B. & Reinherz E.L. (1989) Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 ('enkephalinase'): direct evidence by cDNA transfection analysis. *Proc. natl. Acad. Sci. U.S.A.* **86,** 297.

Werb Z. & Clark E.J. (1989) Phorbol diesters regulate expression of the membrane neutral metalloendopeptidase (EC 3.4.24.11) in rabbit synovial fibroblasts and mammary epithelial cells. *J. biol. Chem.* **264,** 9111.