

Increased expression of human type IIa secretory phospholipase A₂ antigen in arthritic synovium

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Abstract

Objective—To determine the localisation and level of expression of human type IIa secretory phospholipase A₂ (sPLA₂) in the synovium of rheumatoid arthritis (RA), osteoarthritis (OA), and non-arthritic (NA) patients and to examine the relation between sPLA₂ and histological features of inflammation.

Methods—Immunoperoxidase staining using the anti-sPLA₂ monoclonal antibody 9C1 was performed on frozen sections of knee synovium of 10 RA, 10 OA, and 10 NA patients. sPLA₂ positive cells were scored on a scale of 0–3 in 10 fields of a representative tissue section from each case. Double labelling immunofluorescence confocal microscopy with antibodies to CD14 or CD45 and 9C1 was used to determine cell type specificity. Inflammation was assessed by semiquantitative scoring of lining layer thickness and mononuclear cell infiltrates (MC) and a cumulative inflammation score, generated by summing the two parameters. Scores in each group were compared using non-parametric statistical analysis.

Results—sPLA₂ was localised to endothelium (EC), vascular smooth muscle (VSM), and mast cells (M) in all tissue sections. In RA and OA sections, staining was seen in both macrophage-like and fibroblast-like cells in the synovial lining layer (LL) and subsynovial lining layer (SLL). Perineural cells stained positively. Subintimal lymphoid aggregates (LA) were negative in all sections. The RA group showed significantly greater staining in extravascular synovial tissue (median 3.6, range 1.5–6.0) than the OA (median 1.95, range 0–5.3) or NA (median 0, range 0–5.9) groups ($p < 0.05$). LL staining was significantly higher in RA than both OA and NA sections ($p < 0.05$). The OA group showed a trend to higher staining scores than the NA group that did not reach significance. There was a significant correlation between the sPLA₂ staining score and inflammation score within the RA patient group ($p < 0.05$).

Conclusions—The synovium is a site of increased expression of sPLA₂ antigen in both RA and OA relative to NA. Its presence in both fibroblast and macrophage-like cells in the LL and SLL of synovial tissue in RA and OA, but not NA, indicates that the enzyme is specifically induced in these regions in both con-

ditions with expression in the LL being particularly characteristic of RA. The widespread expression of sPLA₂ in synovium suggests it is likely to play a significant part in synovial pathology

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The phospholipase A₂ family of enzymes catalyse the hydrolysis of cellular phospholipids at the *sn*-2 position to release lysophospholipids and free fatty acids. These products are precursors of the proinflammatory lipid mediators prostaglandins, thromboxanes, leukotrienes, and platelet activating factor. Cytokine mediated upregulation of this enzyme activity, together with other enzymes in the eicosanoid pathway, has been proposed as one mechanism by which eicosanoid mediated amplification of inflammatory responses occurs.^{1,2}

There are three major subclasses of mammalian phospholipase A₂.³ Calcium independent isoforms have been identified in several tissues including heart, lung, kidney, brain, and pancreatic islets.⁴ A higher molecular weight (85 kDa) calcium dependent cytosolic enzyme is present in all cell types so far examined and is implicated in arachidonic acid metabolism under physiological conditions.⁵ This enzyme is activated by both post-translational modification and transcriptional upregulation in response to stimulation of cells by the cytokines interleukin 1 β (IL1 β) and tumour necrosis factor α (TNF α), or by agents that increase intracellular calcium and by some growth factors. The low molecular weight secreted forms (about 14 kDa) comprise a family of four structurally related calcium dependent enzymes.^{1,2,6,7} The type I enzyme is secreted from pancreatic tissue. The type IIa enzyme was first characterised as a component of synovial fluid from patients with rheumatoid arthritis (RA)⁸ and has been identified in association with several inflammatory conditions in addition to arthritis, including sepsis and psoriasis.^{9,10} In normal physiological conditions, this enzyme is stored in granules of platelets, resident and circulating macrophages, neutrophils, and mast cells and is localised to secretory cells in the gut.² The remaining secretory isoforms are less well characterised, but have been cloned and are predominantly expressed in heart tissue and testis, respectively. The isoform predominantly expressed in heart is implicated in eicosanoid production in a murine macrophage cell line P388D₁.¹¹ Recently, a third novel sPLA₂ enzyme has been cloned from human fetal lung.¹²

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The involvement of type-IIa secretory PLA₂ (sPLA₂) in RA and other arthritides is well documented. High levels of sPLA₂ activity have been found in the synovial fluid of RA and osteoarthritis (OA) patients and sPLA₂ activity in the synovial fluid correlated with sPLA₂ concentration.¹³ In RA, serum activities of sPLA₂ are increased in patients with active disease relative to normal subjects.¹⁴ It has been reported that enzyme activity correlates with the severity of disease,^{15, 16} however the correlation is not seen consistently.¹⁷ This PLA₂ activity has been purified and characterised from rheumatoid synovial fluid and the cDNA and gene have been isolated.⁸ Injection of human recombinant sPLA₂ into rabbit joints results in hyperplasia of the synovial lining, neutrophil infiltration and an increase in prostaglandin E₂ (PGE₂) concentrations in synovial lavage fluid.¹⁸ Also, administration of recombinant human sPLA₂ exacerbates inflammation in the rat air pouch model of synovitis.¹⁹

The sources of sPLA₂ in the inflamed joint are not completely defined. It has been suggested that sPLA₂ found in synovial fluid may originate from chondrocytes as the enzyme has been localised to the chondrocytes but not previously to synovial tissue,²⁰⁻²² and cultured chondrocytes constitutively synthesise and release sPLA₂.²³ However, *in vitro* cell culture experiments have also shown that IL1 β and TNF α can activate sPLA₂ gene expression not only in the chondrocyte, but also in fibroblast, smooth muscle cell, and endothelial cell cultures.² Consequently, we examined the immunohistological expression of sPLA₂ in RA, OA, and non-arthritic (NA) synovium and its relation to histological inflammation.

Methods

PATIENTS AND SYNOVIAL TISSUE BIOPSIES

Biopsy specimens and relevant clinical information from 10 RA, 10 OA, and 10 NA patients were selected at random for this study from the St Vincent's Hospital synovial tissue collection with the approval of the St Vincent's Hospital ethics committee. Patients came from St Vincent's Rheumatology clinics and diagnoses were made according to the American College of Rheumatology Criteria.²⁴ The RA patient group had a mean (SD) age of 44.6 (12.5) years, M/F ratio of 1/9, and disease duration of 7-12 years with the exception of patient 5 (table 1) whose duration was two years. All RA patients were taking disease modifying anti-rheumatic drugs (DMARDs) and non-steroidal anti-inflammatory drugs (NSAIDs). Three patients (3, 6, and 9) from the RA group were taking prednisone (7.5-12.5 mg dose). The OA patient group had a mean (SD) age of 70.1 (6.7) years and M/F ratio of 2/8. The NA patient group comprised patients with meniscal injury undergoing exploratory arthroscopic surgery for knee pain with no macroscopic arthritic change at arthroscopic surgery (mean age 38.4 (13.1) years; M/F = 6/4). Specimens were obtained from symptomatic knee joints undergoing surgical procedures (standard arthroscopy, mini-arthroscopy or knee joint replacement sur-

gery). Specimens not containing both lining and sublining layer were excluded from the study. Tissue specimens were quickly placed in optimal cutting temperature compound (OCT, Miles, USA), frozen, and stored in liquid nitrogen until use.

ANTIBODIES AND REAGENTS

Anti-sPLA₂ murine monoclonal antibodies 9C1, 10B2, and 4A1 (all IgG₁) were produced in our laboratory¹⁴ and used at 0.6 μ g/ml. These antibodies recognise independent conformational epitopes on human type IIa sPLA₂ because they are each effective with each other when used as capture or detection antibodies in sandwich ELISA assays for sPLA₂ but do not recognise denatured sPLA₂ on western blots (unpublished data). An isotype matched murine monoclonal antibody 81193 (Bioquest Ltd, Sydney, Australia) that does not cross react with human tissue, was used as a negative control on adjacent sections at the same concentration as anti-sPLA₂ antibodies. In addition to morphological features, several cell type specific markers were used to confirm relevant cell types in synovial tissue. The lymphocyte marker, leucocyte common antigen (LCA, CD45) monoclonal antibody (Dakopatts, Denmark) was used at 7.6 μ g/ml. The macrophage marker (CD68, EBM11 clone) monoclonal antibody (Dakopatts, Denmark) was used at 7.6 μ g/ml. The smooth muscle cell marker, smooth muscle actin monoclonal antibody (ICN, Lisle, France) was used at a dilution of 1:1800, and the endothelial cell marker, rabbit antihuman Factor VIII polyclonal antibody (Hoechst-Behring, Germany) was used at 1:500 dilution according to the manufacturer's instructions. Immunofluorescence studies were conducted using a goat antimouse-rhodamine conjugated secondary antibody (Jackson Laboratories, West Grove, PA, USA) at 1:100 dilution to detect murine monoclonal antibodies. Cell-type specific primary antibodies, CD45-FITC or CD14-FITC and control antibodies, IgG-FITC or IgG2a-FITC (Coulter Immunotech, Marseille, France), were used at 2 μ g/ml. sPLA₂ was produced using a stably transfected Chinese hamster ovary cell line, made in our laboratory, expressing the Type IIa cDNA.⁸ The enzyme was purified to homogeneity by affinity chromatography using the sPLA specific murine monoclonal antibody 10B2. The identity and purity of the enzyme was confirmed by amino terminal sequence analysis, SDS-polyacrylamide gel electrophoresis, and amino acid analysis (data not shown).

IMMUNOHISTOCHEMICAL METHODS

In most cases, 7 μ m sections were cut, mounted on poly-L-lysine coated slides and air dried for one hour. Selected cases were also examined using 4 μ m sections. Sections were fixed at room temperature in acetone for five minutes and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 15 minutes. Immunohistochemical staining, other than that used for confocal microscopy, was performed using the

avidin-biotin complex (ABC) technique.²⁵ Sections were incubated with 1.5% goat serum for 10 minutes followed by primary antibodies for 30 minutes. Slides were washed thoroughly with TRIS-phosphate buffered saline (TRIS 5.4 mM, phosphate 10 mM, NaCl 9% pH7.6) and incubated for 20 minutes with horse antimouse secondary antibody (Vector Laboratories, Berlingame, CA, USA) at 1:200 dilution, and then for 30 minutes with the Elite ABC reagent (Vector Laboratories, Berlingame, CA, USA). After washing, the colour was developed by adding diaminobenzidine (2.25 mg/ml) (Fluka Chemica Buchs, Germany) for two minutes. Sections were counterstained with Mayer's haematoxylin. The optimal concentration for the anti-sPLA₂ antibody 9C1 was determined by conducting serial dilution experiments in the range 1:200–1:64 000, of a stock solution at 2.3 mg/ml (data not shown). Based on these studies, experiments were performed at 0.6 µg/ml. All dilutions were done in 50 mM TRIS-HCl pH7.6. To confirm that the anti-sPLA₂ monoclonal did not bind synovial tissue through non-antigen-specific mechanisms, sPLA₂ was used to block antibody binding as follows. Purified recombinant sPLA₂ (8 µg/ml) and anti-sPLA₂ antibody 9C1 (0.6 µg/ml) were incubated for one hour at room temperature before use on tissue sections. Consecutive sections of synovium from an RA patient were stained with sPLA₂ antibody 9C1 or sPLA₂ antibody 9C1 preincubated with purified recombinant sPLA₂. Strongly positive staining, particularly in blood vessels and synovial lining layer was seen in the anti-sPLA₂ stained sections and staining was significantly reduced by preincubation with recombinant

sPLA₂ (data not shown). The same staining pattern was also seen with two other independently isolated murine monoclonal antibodies (10B2 and 4A1) against recombinant sPLA₂ (data not shown). This experiment was repeated several times on the same tissue specimen and specimens from other patients with equivalent results (data not shown).

sPLA₂ SCORING

All sections were blinded to the observer. Both intraobserver and interobserver variation was less than 10%. The number of sPLA₂ positively staining cells was estimated in 10 high powered fields (400 ×) chosen at random with the exception of one case (patient 1, table 1) in which only seven fields were assessable in the section. The 10 sections were randomised by moving the slides in the following directions, left to right, downward then right to left and so forth from top to bottom. To ensure the full section was adequately sampled, 0–4 fields were omitted between each field measured, depending on the size of tissue sample. In each high powered field, four histological features of synovium, endothelium (EC), vascular smooth muscle (VSM), subsynovial lining layer (SLL), and synovial lining layer (LL) were each scored separately based on the scoring system of Soden *et al.*²⁶ Each feature was scored on a scale from 0–3 to reflect the degree of specific staining as follows: 0, 0–5% positive staining; 1, 6–29%; 2, 30–59%; and 3, >60%. A mean score was derived for each case. All sections contained at least five fields in which the relevant histological feature was present with the exception of the synovial lining layer in which seven patients had less than five fields

Table 1 Semiquantitative analysis of sPLA₂ staining scores in synovial cell types and histologically scored inflammation

Patient number	sPLA ₂ staining score					Histological inflammation score		
	EC	VSM	SLL	LL	Cumulative	LLT	MC	Cumulative
Rheumatoid arthritis								
1		3	2.1	2.8	10.9	1	1	2
2	2.1	2.1	1.6	2.7	8.5	2	0.6	2.6
3	0.8	0.8	1	1.1	3.7	1	1	2
4	2.9	2.9	2.7	2.8	11.3	2	1	3
5	0.1	0.1	0.3	1.2	1.7	1	0.9	1.9
6	0.3	0.3	1	1.8	3.4	1	1.2	2.2
7	3	3	3	3	12	1	1.2	2.2
8	1.5	1.5	1.3	1.6	5.9	0	1.1	1.1
9	3	3	3	3	12	2	0.9	2.9
10	2.7	1.8	2.3	0	6.8	0	0	0
Osteoarthritis								
11	0.6	0.6	0	0	1.2	0	0.3	0.3
12	0.6	0.6	0	0	1.2	0	0.6	0.9
13	2	1.9	2.1	0.5	6.5	0	0	0
14	3	3	2.7	2.6	11.3	0	0	0
15	1.6	0.8	0.9	1.5	4.8	1	0	1
16	2.4	2.5	2	0	6.9	0	0.2	0.2
17	2.9	2.6	2.6	2.3	10.4	0	0.1	0.1
18	1.7	1.6	1.6	0.3	5.2	0	0.4	0.4
19	1.4	1.4	0.5	0	3.3	0	0.4	0.4
20	0.4	0.6	0.4	0	1.4	0	0	0
Non-arthritic								
21	0.3	0.3	0	0	0.6	0	0.1	0.1
22	0	0.5	0.6	0	1.1	0	0	0
23	0.2	0.2	0	0	0.4	0	0	0
24	1	1.2	0	0	2.2	0	0	0
25	2	1.0	1.7	0	4.7	1	0.3	1.3
26	2.5	2.4	0	0	4.9	0	0	0
27	0.4	0.2	0	0	0.6	0	0	0
28	0.9	0.1	0.6	0	1.6	0	0	0
29	1.3	1.0	0	0	2.3	0	0	0
30	2.9	2.9	2.9	3	11.7	0	0.1	0.1

EC, endothelium; VSM, vascular smooth muscle; SLL subsynovial lining layer; LL, synovial lining layer; LLT, synovial lining thickness; MC, mononuclear cell infiltrates. Scoring was performed as described in methods.

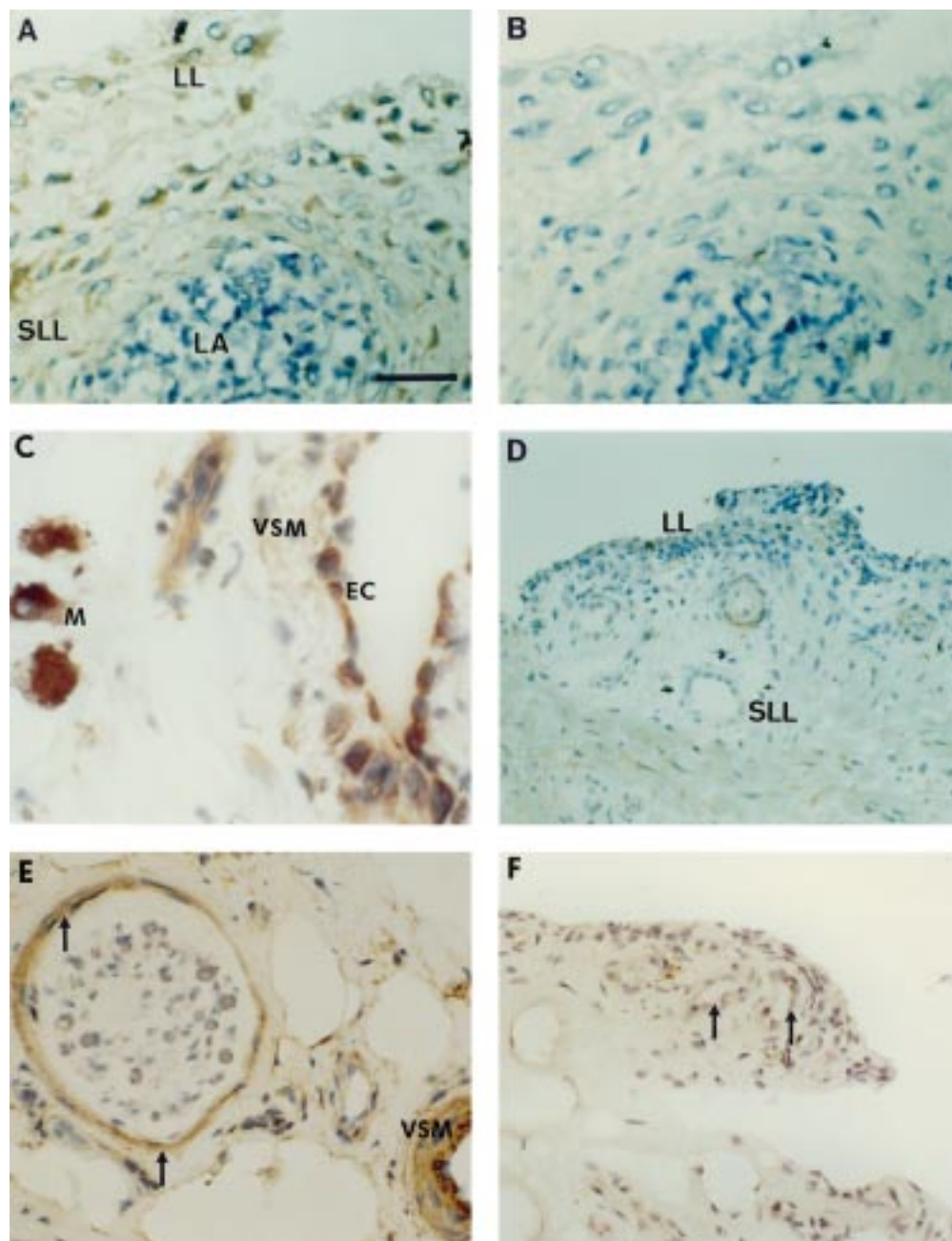


Figure 1 Immunolocalisation of sPLA₂ in synovial tissue from RA (A–C), OA (D–E), and NA (F) patients. (A) Patient no 7 (table 1); synovial lining layer cells (LL) and subsynovial lining layer cells (SLL) stained strongly. No staining was observed on subintimal lymphoid aggregates (LA). (B) Control antibody 81193 demonstrates no significant staining in an adjacent section to that shown in A. (C) Patient no 8 (table 1) strong positive staining is seen in endothelial cells (EC) and mast cells (M). Vascular smooth muscle cells (VSM) were not positively stained in this field. (D) Patient no 15 (table 1) the majority of cells in SLL and one vessel stained positively for sPLA₂, with no staining in the LL. (E) Patient no 15 (table 1) perineural cells (arrows) of a large nerve, and vascular smooth muscle cells (VSM) stained positively. (F) Patient no 24 (table 1) LL and SLL cells were not stained. Positive staining was observed in some endothelial cells (arrows). (Original magnification $\times 400$ for A–C, $\times 200$ for D–F). (Bar marker=25 μ m for A–C; 50 μ m for D–F).

containing lining layer. These were patients 1 (3), 10 (4), 16 (3), 23 (2), 25 (1), 26 (4), and 30 (1) parentheses show number of fields measured, table 1. Endothelial cells in any one vessel stained uniformly either positive or negative as did cells in VSM. Therefore, EC and VSM were scored as % positive blood vessels using the 0–3 scoring system described above. The median and the range for the blood vessel numbers for each patient group were RA=41 (27–55), OA=47 (10–70), and NA=43 (18–58). Cumulative staining scores were calcu-

lated by summing the mean score of these four parameters for each case. Cell types were identified by morphological criteria and antibody-based cell specific markers (described above) on adjacent sections. Mast cells were identified using toluidine blue.

INFLAMMATION SCORING

Intraobserver and interobserver variation in this scoring system was less than 10%. Synovial lining layer thickness was assessed in 10 high powered ($\times 400$) fields in most cases as

described above. Minimum and maximum lining layer thickness (LLT) was measured for each field and a mean thickness was derived from all measurements for each case. This mean thickness was assigned a score (0–3) according to Soden *et al*²⁰: 0–2 cells was scored 0; 3–4 cells was scored 1; 5–7 cells was scored 2; >7 cells was scored 3. Mononuclear cell infiltration (MC) was assessed using the scor-

ing system described above for sPLA₂ staining. Mononuclear cells were identified morphologically and cells in perivascular mononuclear cell aggregates were included in the analysis. Haematoxylin and eosin stains were performed on adjacent tissue sections and used to confirm morphology. A cumulative inflammation score was calculated for each case by summing the LLT score and the MC score.

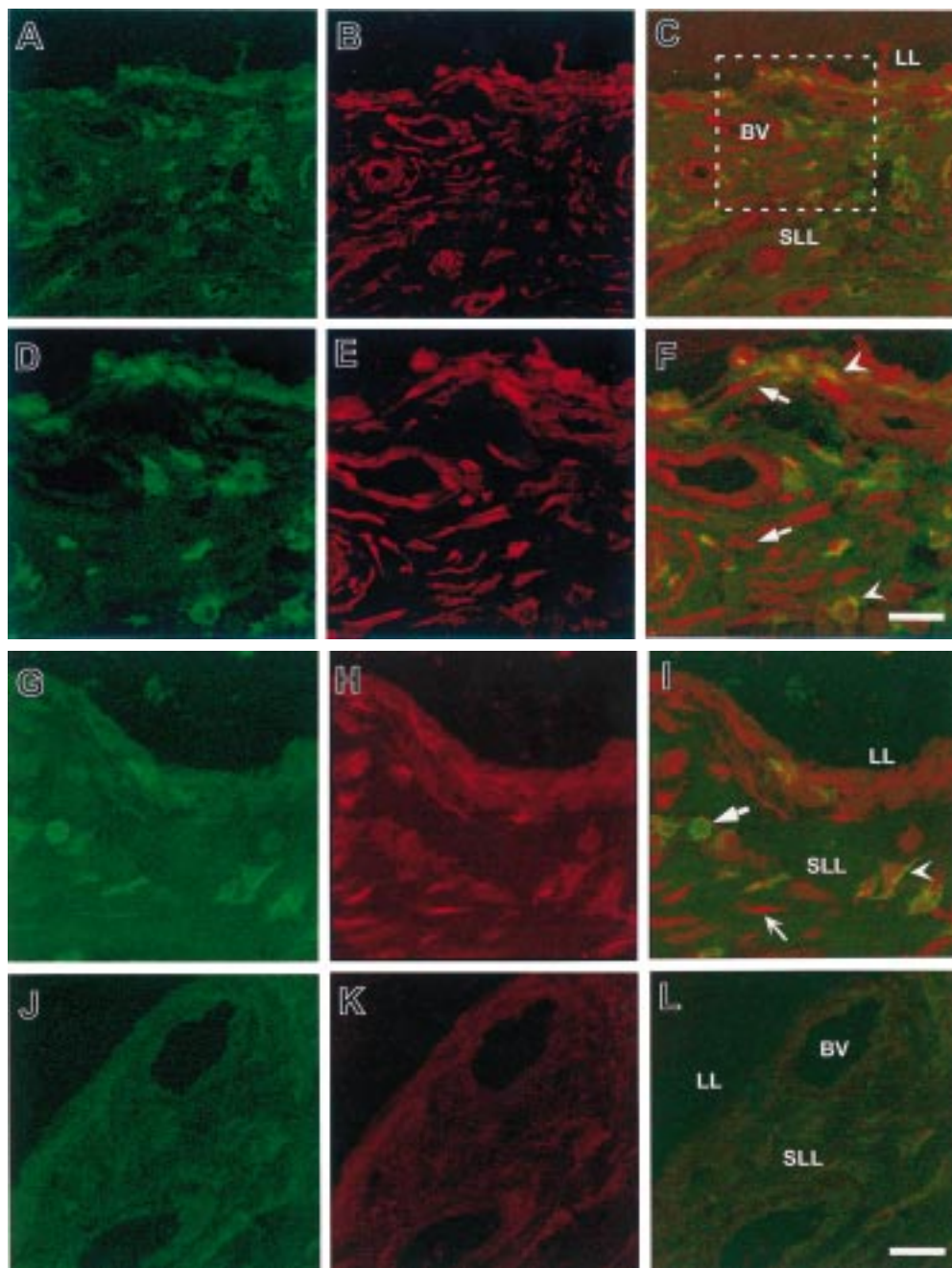


Figure 2 Double labelling immunofluorescence of rheumatoid synovial tissue for sPLA₂ and cell type specific markers imaged by confocal microscopy. In each group of three images, A–C, D–F, G–I, and J–L, FITC (A, D, G, J) and TRITC (B, E, H, K) channels are each presented separately followed by the two channels viewed simultaneously (C, F, I, L). Immunofluorescence staining was performed on synovial tissue with CD14-FITC and sPLA₂ detected with antimouse-rhodamine (A–F). CD14 and sPLA₂ staining is seen in the lining layer (LL) and sublining layer (SLL) with sPLA₂ also staining blood vessels (BV). D–F show a higher magnification (the region identified by the dotted lines in C). Both sPLA₂ positive, CD14 negative cells (indicated by arrows in F) and sPLA₂ positive, CD14 positive cells (indicated by arrowheads) were found in the LL and SLL. Rheumatoid synovium was also dual stained with CD45-FITC; and sPLA₂-antimouse-rhodamine (G–I). Positive staining was seen in both the LL and SLL for both markers. sPLA₂ positive, CD45 negative (thin arrow), sPLA₂ positive, CD45 positive (arrowhead) and CD45 positive, sPLA₂ negative cells (thick arrow) were identified. J–L shows a representative section from synovial tissue dual labelled with control antibodies IgG-FITC and murine monoclonal 81193 detected with antimouse-rhodamine. Space bars in F and L represent 40 μ m in A–C and 20 μ m in D–L.

IMMUNOFLUORESCENCE

Four μm frozen tissue sections were cut, air dried, and fixed in acetone for five minutes. Sections were blocked with 1.5% goat serum for 10 minutes, followed by adding the first primary antibody 9C1 (2 $\mu\text{g}/\text{ml}$) (or the same concentration of 81193 antibody to serve as a control) to tissue sections for 30 minutes. Sections were incubated in goat antimouse-rhodamine conjugate for 30 minutes, blocked with mouse serum at 1:50 dilution for 20 minutes followed by 1.5% goat serum for 10 minutes before addition of the second primary antibody: the leucocyte marker CD45-FITC, IgG-FITC, the macrophage marker CD14-FITC or IgG2a-FITC. Sections were washed thoroughly between each step, except after blocking with goat serum. Sections were then mounted in a 1% *p*-phenylenediamine (Sigma, USA) in PBS containing 90% glycerol and coverslips were sealed with nail polish. Slides were stored in the dark at -20°C before visualisation.

CONFOCAL MICROSCOPY

Confocal scanning microscopy was performed using a Leica TCS NT (v1.5.451) confocal system with an argon krypton laser and using a Plan Apochromatic 40×1.25 NA oil immersion lens. Optical sections were acquired with excitation at 488 and 568 nm using a double dichroic beam splitter, a 580 nm dichroic filter, and then an FITC band pass filter as barrier filter for the first channel and a 590 long pass filter for the second channel. Images were averaged and the FITC and rhodamine channels superimposed using the TCS-NT software package. Images were then exported into Canvas (v5.0.2) for presentation.

STATISTICAL ANALYSIS

Non-parametric statistical analysis was used in all comparisons. In each patient group, overall staining was determined by calculating the median and range of each parameter from one representative section for each of the 10 patients. The Mann-Whitney U test was used for comparison between patient groups. Correlations between sPLA₂ staining and inflammation scores were evaluated using the Kendall correlation test.

Results

LOCALISATION OF sPLA₂ IN SYNOVIUM

Serial sections of synovial tissue from each patient were stained for sPLA₂ or isotype matched control antibody. To identify individual cell types, using double labelling immunofluorescence confocal microscopy on selected sections was undertaken. Representative sections of synovium are shown in figure 1 for the RA (A–C), OA (D, E), and NA (F) patient groups. A representative control antibody stained section matching figure 1A is shown in figure 1B. Double labelling of representative sections from RA patients for sPLA₂ and the markers CD14 or CD45 is shown in figure 2. In RA patients, sPLA₂ staining was observed in the LL, SLL (figure 1A, 2B, 2E, 2H), VSM (figure 2B), EC and mast cells (fig-

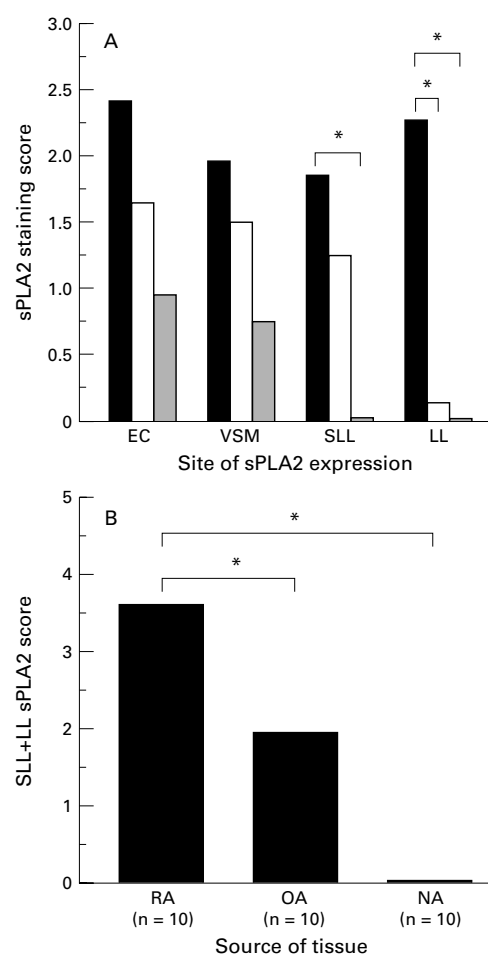


Figure 3 Semiquantitative analysis of sPLA₂ staining of synovial tissue from RA, OA, and NA patients. (A) Comparison of sPLA₂ staining by site in synovial tissue of the three patient groups. EC, endothelium; VSM, vascular smooth muscle; SLL, subsynovial lining layer; LL, synovial lining layer. sPLA₂ staining score is the median of the mean staining scores calculated from up to 10 fields per patient as described in Methods (table 1). RA group, (filled bars); OA group, (open bars); NA group (shaded bars). (B) Comparison of sPLA₂ staining in extravascular tissue of the three patient groups. The extravascular sPLA₂ score for each group is the median of the mean staining score for SLL and LL. Statistical analyses are as described in Methods. * Indicates significance of $p < 0.05$.

ure 1C). Perineural cells and nerve fibres also stained positively on RA sections (data not shown). sPLA₂ staining was also seen on extracellular matrix fibres in most RA cases (fig 2B, 2E, 2H). In RA sections, both CD14 positive and negative cells of the LL and SLL stained positively (fig 2C, F) although the intensity of sPLA₂ staining varied particularly in macrophage-like cells. This result was also confirmed by double labelling with CD45-FITC (fig 2I). In some sections (3 of 10) only the superficial LL cells stained positively (data not shown). No staining could be observed on subintimal lymphoid aggregates (LA) (fig 1A). The pattern of staining in the OA group was similar to the RA group with staining in SLL, EC, VSM, (fig 1D) perineural cells (fig 1E), and mast cells (data not shown). To confirm that staining seen in synovium was specific for sPLA₂, serial $4\mu\text{m}$ sections of selected RA cases were stained with each of three sPLA₂ specific antibodies, 9C1, 10B2 or 4A1. All

Table 2 Correlations between sPLA₂ staining and histological inflammation score in rheumatoid arthritis patients

Variable 1	Variable 2	Correlation coefficient (r)	p Value
Cumulative sPLA ₂ score	Cumulative inflammation score	0.44	0.04
Cumulative sPLA ₂ score	LLT score	0.73	0.004
Cumulative sPLA ₂ score	MC score	0.12	0.323
(SLL+LL) sPLA ₂ score	Cumulative inflammation score	0.57	0.01
(SLL+LL) sPLA ₂ score	LLT score	0.79	0.002
(SLL+LL) sPLA ₂ score	MC score	0.26	0.156

three antibodies consistently showed similar staining patterns in the LL and SLL including endothelial cells and mast cells (data not shown). In both RA and OA sections, perinuclear sPLA₂ staining was seen. Staining was also observed on the surface of cells and in connective tissue (fig 2B). The majority of cases (9 of 10) in the NA group showed positive staining in both endothelium and vascular smooth muscle. However, only 4 of 10 sections showed positive staining in SLL. No staining was observed in the LL for the majority (9 of 10) of these cases (fig 2F). Mast cells generally stained positively. Tissue sections from one NA patient (patient 30, table 1) showed significant staining in all cell types examined although no obvious signs of inflammation (for example, synovial lining layer thickening) was observable in this patient.

COMPARATIVE STAINING BETWEEN RA, OA, AND NA GROUPS

Mean sPLA₂ staining scores in EC, VSM, SLL, and LL for each patient are shown in table 1 and median scores for each patient group are shown in figure 3. Staining in EC and VSM was highest in RA patients (median 2.4, range 0.1–3; median 1.95, range 0.1–3 respectively) relative to the OA (median 1.65, range 0.4–2.9; median 1.5, range 0.6–3) and NA groups (median 0.95, range 0–2.9; median 0.75, range 0.1–2.9), however, these differences were not significant (fig 3A). In contrast with vascular tissue, the staining scores in SLL and LL cells for the RA group (median 1.85; range 0.3–2.7 and median 2.25, range 0–22.8 respectively) were significantly greater than those in the NA group (median 0.00, range 0–2.9, $p < 0.05$; median 0.00, range 0–3.0, $p < 0.05$) (fig 3A). Staining scores in SLL and LL cells were reduced in the OA group (median 1.25, range 0–2.7; and median 0.15, range 0–2.6 respectively) compared with the RA group, however this reduction was statistically significant in the LL cells only ($p < 0.05$). The OA group showed greater sPLA₂ staining in LL and SLL compared with the NA however this was not significant (fig 3A).

Cumulative staining scores for each patient are shown in table 1. Significant differences in these scores between patient groups were seen for RA relative to NA ($p < 0.05$) only. Furthermore, when the contribution of staining in blood vessels (EC and VSM) was removed from these scores (data not shown), staining scores for extravascular tissue were significantly higher in the RA group (median 3.6, range 1.5–6.0) compared with both OA (median 1.95, range 0–5.3, $p < 0.05$) and NA (median 0.0, range 0–5.9, $p < 0.05$) groups (fig

3B). sPLA₂ staining was generally more intense in RA compared with OA and NA sections (data not shown). While the OA group shows a higher score relative to the NA group in this analysis the difference is not significant.

RELATION OF sPLA₂ STAINING TO HISTOLOGICAL INFLAMMATION

Two histological features of inflammation, lining layer thickness (LLT) and mononuclear cell infiltrates (MC), were scored separately for each section and a cumulative inflammation score calculated for each patient (table 1). LLT was significantly higher ($p < 0.05$) in the RA group (median 1, range 0–2) relative to the OA and NA groups, which had identical scores (median 0, range 0–1). MC scores were also significantly higher ($p < 0.05$) in the RA group (median 1.05, range 0–1.2) relative to the OA (median 0.15, range 0–0.6) and NA (median 0, range 0–0.3) groups. These trends are reflected in the cumulative scores (RA median 2.1, range 0–2.9 > OA median 0.35, range 0–0.9 > NA median 0, range 0–1.3) with the RA group showing significantly higher scores than both the OA and NA groups ($p < 0.05$). The difference between the OA group and NA group was not significant. When sPLA₂ staining and inflammation scores were compared using data from the RA patient group, significant correlations were observed as shown in table 2. Both cumulative sPLA₂ staining score and the extravascular staining score (SLL and LL) correlated positively with the cumulative inflammation score and LLT. No correlation was seen with MC. There was no significant correlation between sPLA₂ staining score and any inflammation score in the OA and NA groups (data not shown).

Discussion

In this study we have shown for the first time that type IIa sPLA₂ is present in synovial tissue of patients with RA and OA. The enzyme was localised throughout synovium in most RA patient tissue, particularly in blood vessels, fibroblast-like and macrophage-like cells of the synovial lining layer and subsynovial tissue, perineural cells, and mast cells. Lymphocyte aggregates consistently showed no staining. In the OA and NA patient groups the enzyme localised predominantly to blood vessel endothelium and vascular smooth muscle. These findings are in contrast with those reported by Nevalainen *et al* who found the enzyme localised to chondrocytes with no detectable sPLA₂ staining in RA synovium.^{20,21} Our study confirmed chondrocyte staining in those sections in which cartilage was present (data not shown). The difference in synovial staining

may reflect differences in the antibodies used in the two studies or the treatment of tissue sections before staining. In our hands we failed to see staining in formalin fixed RA synovial tissue. However, our monoclonal antibody did positively stain chondrocytes in formalin fixed tissue. Furthermore, staining of human jejunal mucosa showed an identical staining pattern (data not shown) to that observed by Nevalainen *et al*, where secretory granules in paneth cells stained positively.²⁰ These experiments suggest that formalin fixation masks or destroys sPLA₂ epitopes in synovial tissue but not in cartilage.

The cell types in which sPLA₂ is localised in synovium are consistent with cell culture studies.^{1,2} In RA, OA, and NA synovial fibroblasts in culture, sPLA₂ protein and mRNA is detectable in resting cells. Several reports indicate that sPLA₂ protein and mRNA are also inducible with IL1 β ,^{27,28} however induction is not observed in all studies.^{29,30} The detection of sPLA₂ protein in LL cells in RA synovium and not NA synovium is consistent with specific induction of sPLA₂ in RA cells. Our preliminary *in situ* hybridisation studies indicate that sPLA₂ mRNA is present in these cells in RA synovium (data not shown). The observed constitutive expression of sPLA₂ in RA fibroblasts in culture may result from the influence of specific culture conditions such as activation of gene expression by serum factors. In our hands sPLA₂ mRNA is detectable in RA fibroblasts up to 96 hours after serum starvation. However, serum starvation results in significant reduction of steady state sPLA₂ mRNA levels in these cells relative to control constitutively active genes (C Salom and K Scott, unpublished data). Murakami *et al*³¹ have shown that sPLA₂ activity in human umbilical vein endothelial cells was increased twofold to threefold on TNF stimulation. sPLA₂ has been detected immunohistochemically in both endothelial and vascular smooth muscle cells of the human placenta^{32,33} and mast cells have also been reported to store sPLA₂.^{34,35} Human macrophage cells also express sPLA₂ in culture.^{36,37}

Our finding that sPLA₂ is localised to the surface of cells and connective tissue between cells is consistent with cell culture studies. It is now well established that sPLA₂ binds to cell surfaces in culture via ionic interactions and can be displaced by polysulphated glycosaminoglycans such as heparin.^{31,38} Furthermore, membrane microvesicles have been identified in synovial sections by electron microscopy^{39,40} and have been isolated from synovial fluid.⁴¹ These microvesicles are a substrate for sPLA₂ and the extracellular localisation of the enzyme suggests that sPLA₂ may be active on microvesicles released from activated cells in synovium. Therefore, these vesicles may represent an alternative substrate for the type IIa enzyme.⁴¹

The perinuclear localisation of sPLA₂ in synovial cells is of interest because it is not clear whether the enzyme is active intracellularly. sPLA₂ is optimally active in the presence of millimolar concentrations of calcium. The enzyme has seven disulphide bridges and its

activity is sensitive to reducing agents. It has been suggested that in the reducing environment and low calcium concentration found within resting cells, the enzyme would be inactive. However, it has been reported that sPLA₂ has activity at the calcium concentrations (nanomolar–micromolar) commonly found in cells.⁴² Furthermore, the antibody used in this study does not recognise reduced sPLA₂ in western blots (unpublished data). The detection of sPLA₂ intracellularly with this antibody would suggest that the enzyme detected in these cells may not be reduced. Consequently, intracellular action of sPLA₂ in synovium cannot be ruled out.

We have demonstrated differences in sPLA₂ staining between RA, OA, and NA tissue and correlated sPLA₂ staining with a histological score of inflammation. Although the patient groups in our study were not age and sex matched, they did represent typical age and sex distributions for these diseases. Scores for sPLA₂ staining were highest in RA patients compared with OA and NA patients. The findings are in accordance with the increased levels of sPLA₂ found in the synovial fluid and serum⁴³ of RA and OA patients where the level of sPLA₂ expression is highest in RA patients followed by OA and NA patients. These findings are also consistent with the proposition that sPLA₂ is specifically induced by proinflammatory stimuli present in RA and to a lesser extent OA synovium. Induction is seen most significantly in the LL and SLL of RA and OA patients relative to NA patients and staining in vessels is seen more consistently in all three groups. Furthermore, the only site where staining scores were significantly higher in RA than OA was the lining layer. This finding is consistent with studies of other cytokine induced gene products such as cyclooxygenase-2⁴⁴ and inducible nitric oxide synthase.⁴⁵

Type IIa sPLA₂ has been implicated in the pathogenesis of RA, however a causative role for the enzyme has not been definitively shown. There are data showing an association between enzyme activities in synovial fluid and the onset and severity of disease¹⁵ although this correlation is controversial.¹⁷ The precise role of the enzyme in the pathogenesis of RA relative to other phospholipases A₂ is also not fully defined. Cell culture studies have clearly demonstrated that cytosolic PLA₂ is the major enzyme mediating cytokine induced prostanoid release in synovial fibroblasts.²⁸ However, externally added sPLA₂ can augment cytokine induced prostanoid synthesis in several cell types.^{1,2} Animal model studies with purified recombinant sPLA₂ suggest that the enzyme is proinflammatory,^{6,18} although overexpression of the sPLA₂ gene in transgenic mice does not cause arthritis.⁴⁶ The presence of the enzyme throughout synovium in patients with RA and to a lesser extent OA, none the less suggests that sPLA₂ may amplify directly and/or indirectly eicosanoid and lysophospholipid mediated inflammatory processes in synovial tissue in these diseases.

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