Osteoarthritis and Cartilage
Journal of the OsteoArthritis Research Society International

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Tumor necrosis factor alpha can contribute to focal loss of cartilage in osteoarthritis

C. I. Westacott, A. F. Barakat, L. Wood, M. J. Perry*, P. Neison, I. Bisbinas†, L. Armstrong‡,
A. B. Millar‡ and C. J. Elson
*Departments of Pathology and Microbiology, *Rheumatology, †Orthopaedic Surgery and ‡Medicine,
University of Bristol, Bristol, U.K.*

Summary

Objective: To evaluate the potential for tumor necrosis factor alpha (TNFα)-induced focal loss of cartilage in osteoarthritic (OA) knee joints.

Design: Fresh cartilage from specified regions of OA joints was immunostained for TNF-receptor (R) bearing chondrocytes. Cartilage explants from the same regions were cultured with or without small amounts of TNFα and cumulative GAG release into supernatants measured. Concentrations of TNFα, p55 and p75 soluble (s) TNF-R in supernatants from cultured OA and non-arthritic (NA) synovium were measured by ELISA.

Results: TNF-R bearing chondrocytes were identified in OA cartilage; more specimens contained p55 TNF-R- than p75 TNF-R-bearing chondrocytes and differences in TNF-R distribution were apparent in cartilage from different regions of the same knees. TNFα at 5, 1, 0.5 and 0.25 ng/ml (but not 0.1 ng/ml) significantly increased glycosaminoglycans (GAG) release from cartilage explants in a dose-dependent manner. Variation in susceptibility to TNFα was observed in explants from different sites. TNFα and p75 sTNF-R, but not p55 sTNF-R, concentrations were significantly higher in OA, as compared with NA, supernatants. A significant correlation between TNFα and p75 sTNF-R measurements was apparent only in NA supernatants.

Conclusions: Variations in chondrocyte TNF-R expression occur in OA cartilage *in vivo*. TNFα at concentrations produced by OA synovium *in vitro*, can degrade cartilage matrix. In most OA supernatants sTNF-R concentrations were insufficient to abrogate the effects of TNFα. Thus conditions exist in some OA knees for TNFα to contribute to focal loss of cartilage. © 2000 OsteoArthritis Research Society International

Key words: Osteoarthritis, Cartilage, Tumor necrosis factor-alpha (TNFα), Soluble tumor necrosis factor-receptors (sTNF-R).

Introduction

Osteoarthritis (OA), the most common cause of disability in the elderly, is characterized by focal loss of cartilage. However, why cartilage is lost from specific regions of the joint in knee OA remains unclear. It is now generally accepted that loss of cartilage matrix results from production of aggrecanase and other enzymes by chondrocytes in response to catabolic cytokines produced within the joint. For example, a strong correlation exists between specific cleavage of aggrecan core protein at the Glu^{373}-Ala^{374} bond and the release of aggrecan catabolites in response to interleukin (IL-1) and tumor necrosis factor (TNF)α[1] and fragments bearing this epitope are found in synovial fluids from OA patients.[2] In addition, IL1β and TNFα are present in OA synovial fluids[3] and concentrations of both cytokines are higher in supernatants from cultured OA synovium

than those from NA individuals.[4] Furthermore, OA synovial fluids[5] and OA synovium supernatants can stimulate GAG loss from explants (Westacott, unpublished observations). However, cartilage is lost from specific regions of OA knees. Thus if the cartilage catabolic cytokines IL-1β and TNFα cause focal loss of cartilage then other factors must contribute. It is known that explants from OA cartilage are more susceptible to the effects of IL-1[6] and TNFα[7] than similar explants from non-arthritic (NA) cartilage and that receptors for both cytokines are enhanced on OA chondrocytes.[8,9] However, for enhanced receptor expression to contribute to focal loss of cartilage, receptors for such cytokines must be focused on chondrocytes in specific regions of the knee joint. Moreover, susceptibility to the effects of the cytokine, as measured by GAG release from cartilage, must also be enhanced in those regions. Experiments designed to test these predictions revealed regional differences in cartilage susceptibility to TNFα which were closely related to expression of the biologically active p55 TNF receptor (R) in both OA and NA cartilage.[7] Thus focal loss of cartilage will occur in the presence of sufficient cytokine only in those regions which are susceptible to its effects.

However, the above conclusions can be challenged on a number of grounds. Firstly, chondrocyte TNF-R expression was evaluated on isolated chondrocytes *in vitro* and the question therefore arises as to whether these measurements reflect the situation *in vivo.* Secondly, the

Corresponding author: C. I. Westacott, PhD, Department of Pathology and Microbiology, University of Bristol, School of Medical Sciences, University Walk, Bristol, BS8 1TD, U.K. Tel: 44 117 9288605; Fax: 44 117 9287896; E-mail: carole.westacott@bristol.ac.uk
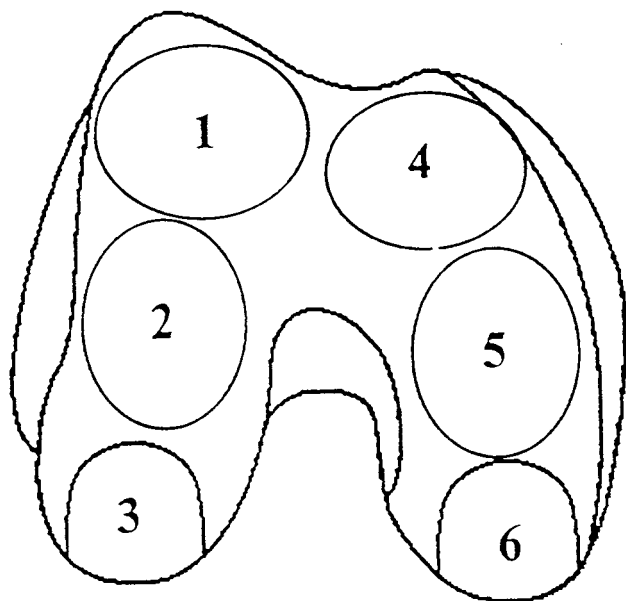
Fig. 1. Diagram of the right knee joint showing the different anatomical regions from which cartilage was obtained. Regions 1, 2 and 3 were located on the lateral femoral condyle, regions 4, 5 and 6 were located on the medial femoral condyle. Regions 1 and 4 were located at the anterior and regions 3 and 6 at the posterior aspects of the knee joint.

concentration of TNFα (10 ng/ml) used for the cartilage explant experiments[7] was higher than that detected in OA synovial fluids (0.02–1.2 ng/ml)[3] or measured in supernatants from cultured OA synovium (0.02–5 ng/ml).[4] Finally, although TNFα may be present in OA joints at concentrations able to stimulate cartilage matrix degradation, its effects may be inhibited by soluble TNF-R (sTNF-R), which are known to be present in OA synovial fluid.[10] The purpose of this work was therefore to (a) determine if TNF-R are present on chondrocytes *in vivo,* and if so, ascertain whether chondrocytes expressing TNF-R are distributed evenly throughout the cartilage (b) determine the effects on cartilage matrix degradation of TNFα at the concentrations likely to be present within the OA joint and (c) to measure sTNF-R and TNFα concentrations in supernatants from cultured OA and NA synovium to determine whether sufficient inhibitory activity to negate the effects of TNFα can be produced within the joint.

Materials and methods

TISSUE PREPARATION

Full depth slices of cartilage were removed less than 2 hours post-operatively from six specific weight-bearing and non-weightbearing areas on the femoral condyles (Fig. 1) of patients who fulfilled the ACR criteria for OA undergoing surgery for total joint replacement. Details of joint damage were recorded diagrammatically. Synovium was obtained from the same OA joints. NA cartilage, and synovium when available, was obtained from patients undergoing hemiarthroplasty following subcapital fracture of the femoral neck. Cartilage and synovium specimens were washed copiously in phosphate buffered saline (PBS) to remove debris prior to processing for specific experiments. Insufficient cartilage was available to perform immunocyto-

chemistry and tissue culture experiments on specimens from the same donor. Cartilage from separate patient groups was therefore used for each set of experiments.

IMMUNOCYTOCHEMISTRY

Specimens of cartilage cut from specific regions of femoral condyles (N=6, mean±SD age 73.6±6.2 years) or from the superior pole of femoral heads (N=3, mean age 76.7±21.6 years) were frozen in Cryomatrix (Shandon) on a chuck suspended in liquid nitrogen for 2–3 minutes. Serial 8-μm sections were cut and laid onto slides previously coated with Vectabond (Vector) to assist tissue adherence. Cryostat sections of cartilage or synovium were washed in Tris/HCl buffer, pH 7.4 (1 min 4°C) to remove soluble receptors, then fixed in acetone (10 min, RT). Sections were washed in PBS (5 min, RT) and blocked (30 min, RT) with PBS containing 20% normal goat serum then followed by a further wash in PBS (10 min, RT). Sections were incubated with mouse monoclonal antibodies to p55 and p75 TNF-R (kind gift of Dr M. Brockhaus, Hoffman La Roche, Basel) or IgG$_1$ isotype control (Becton Dickinson). Unbound antibody was removed between this and subsequent steps by washing in PBS (10 min, RT). Antibody binding was detected by incubation (30 min, RT) with goat-anti-mouse IgG$_1$ linked to biotin and the signal amplified by incubation (30 min, RT) with ExtraAvidin-alkaline phosphatase conjugate (Sigma). Sections were incubated for up to 15 min with Fast Red insoluble alkaline phosphate substrate with levamisole (Sigma) and the reaction stopped by flooding the slides with distilled water. Comparison was made between sections stained with specific monoclonal antibodies and sections from the same tissue stained with isotype control. Cartilage samples exhibiting chondrocyte TNF-R expression when examined microscopically (×400) were scored as follows: +++, over 10 positive cells/field; ++, 5–10 positive cell/field; +, up to five positive cells/field. Photomicrographs of cartilage were taken using Fujichrome 64 tungsten film which was uprated one stop in the E6 (colour) process, to increase contrast and colour saturation.

CULTURE OF CARTILAGE WITH TNFα

Semicircular biopsies were cut in full depth cartilage using a 3-mm biopsy punch and experiments performed as described by Webb *et al.* (1997).[7] Briefly, biopsies were made from cartilage taken from each of six individual sites on femoral condyles. Four or more replicate biopsies were selected at random from each of the six biopsy pools obtained from each knee; each biopsy was cut in two. One half of each biopsy was cultured in RPMI 1640 containing 5% heat inactivated normal human AB serum alone (control), the other half of the biopsy pair was cultured in tissue culture medium containing either 5, 1, 0.5, 0.25 or 0.1 ng TNFα. The cartilage degrading capacity of each of the five different concentrations of TNFα, was tested on biopsies from specific regions of knee joints from four different patients (N=20, mean age 67.8±9.2 years, range 50–86). Biopsy replicates cultured with 5–0.25 ng TNFα were incubated (37°C, 5% CO$_2$) for 14 days, those cultured with 0.1 ng TNFα were incubated for 28 days, and the medium changed every two days. Culture supernatants from each time point were collected and stored at −20°C prior to measurement of glycosaminoglycans (GAG) content by

Table I
p55 TNF-R expression on chondrocytes in OA cartilage specimens obtained from six different regions on the femoral condyles of six different knee joints

Patient	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6	Total
A	ND	++	–	–	ND	ND	1/3
B	ND	+	–	–	ND	–	1/4
C	+	–	–	ND	ND	–	1/4
D	+	++	ND	+	ND	–	3/5
E	ND	–	–	–	ND	–	0/4
F	+++	–	–	–	ND	+	2/5
Total	3/3	3/6	0/5	1/5	0/0	1/5	

(+) denotes degree of positivity, (–) denotes negative, ND (not done) denotes regions for which insufficient full depth cartilage was available for processing.

colorimetric method.[11] The GAG content of the supernatants were adjusted for dry weight of biopsy and expressed as $\mu g/mg$ cartilage. Results were expressed as percentage increase in GAG release: % increase=Σ([Test-Control]/Control)$\times$100.

SYNOVIUM CULTURE

Specimens of synovium from NA (N=15, mean age 82.7±10.5, range 59–97 years) and OA subjects (N=37, mean age 66.7±9.4, range 47–90 years) were chopped finely and cultured for 24 hours essentially as described by Webb *et al.* (1998).[12] Supernatants were harvested, filtered (40 μm, Falcon), centrifuged (3500 rpm, 15 min) to remove debris and stored in aliquots at $-70°C$.

MEASUREMENT OF TNFα IN SYNOVIUM SUPERNATANTS

TNFα concentrations in serial dilutions of synovium supernatants were measured by ELISA as described by Westacott *et al.* (1997)[13] with the following modification to the detection step. After removal of the test supernatants and diluted standards, wells were washed three times with PBS containing 0.5% Tween (PBS/Tween). Polyclonal sheep anti-human TNFα (H91, NIBSC, Potters Bar, Herts) was added to all wells and the plate incubated (1 h, RT). Wells were further washed three time with PBS/Tween, and rabbit anti-sheep-biotin (Chemicon, Harrow, Middx) added before incubation for a further 1 hour at room temperature. After further washing (×3) with PBS/Tween, streptavidin-HRP (Amersham, Little Chalfont, Bucks) was added to all wells and incubation continued (30 min, RT). Wells were again washed three times prior to addition of peroxidase substrate, tetramethylbenzide (Sigma, 60 mg/ml in sodium acetate/citric acid buffer, pH 5.5), with further incubation for 30 minutes in the dark. The reaction was stopped by addition of 10% H_2SO_4 and absorbance read at 450 nm. As the dilution curve of supernatants was not linear when compared with dilutions of standard TNFα preparation, all results were subjected to PROBIT analysis.[14]

MEASUREMENT OF STNF-R IN SYNOVIUM SUPERNATANTS

The concentrations of both p55 and p75 sTNF-R were measured in supernatants by ELISA. Non-crossreacting antibodies, 5R13 for p55 sTNF-R and 7R10 for p75 sTNF-R, were kindly provided by Dr Sue Stephens (Celltech Therapeutics, Slough, U.K.) and the assays performed as described by Armstrong *et al.* (1999).[15]

STATISTICAL ANALYSIS

The significance of the difference in GAG release from cartilage biopsies cultured in the presence of TNFα as compared with biopsies cultured in medium alone was assessed by Student's *t*-test. The significance of the difference in the concentrations of TNFα, p55 sTNF-R and p75 sTNF-R in NA as compared with OA synovium supernatants and between p55 and p75 sTNF-R measurements within groups was assessed by *t*-test with Welch's correction for non-equal variance on log-transformed values. Correlations were sought between TNFα and p55 or p75 sTNF-R measurements and between both sTNF-R measurements within groups using Pearsons product moment correlation.

Results

VISUALIZATION OF TNF-R IN CARTILAGE

To determine if TNF-R is expressed on chondrocytes and whether the degree of expression varies in different regions of the knee joint *in vivo*, freshly processed cartilage was stained for both p55 and p75 TNF-R. In all, 24 specimens from six different knee joints were examined, eight of which (33.3%) stained positive for p55 TNF-R expression as shown in Table I. Differences in staining were apparent in cartilage from different regions within the same knee joint. For example, cartilage from patient A showed positive staining for p55 TNF-R on chondrocytes in region 2, whereas cartilage from regions 3 and 4 were negative. Similarly, cartilage from patient D showed positive staining for p55 TNF-R in regions 1, 2 and 4, whereas cartilage from region 6 was negative. In addition, differences in staining were also apparent in the same regions in different joints. For example, cartilage from patients C, D and F, contained p55 TNF-R positive chondrocytes in region 1, although only in cartilage from patient D were p55 TNF-R bearing chondrocytes detected in region 2. Typically, chondrocytes staining for p55 TNF-R were distributed throughout the depth of the tissue; the majority of positive staining chondrocytes were located in the superficial zone (Fig. 2A). Chondrocytes staining for p55 TNF-R were frequently seen in chondrones in cartilage near severe osteoarthritic lesions (Fig. 2B). By comparison, Table II shows only 3/24 (12.5%) sections from the same preparations stained positive for p75 TNF-R (Fig. 2C). Distribution of p75 TNF-R-positive cells through the tissue was similar to that of p55 TNF-R-positive cells, although the staining was less intense. Interestingly, staining for the two receptor
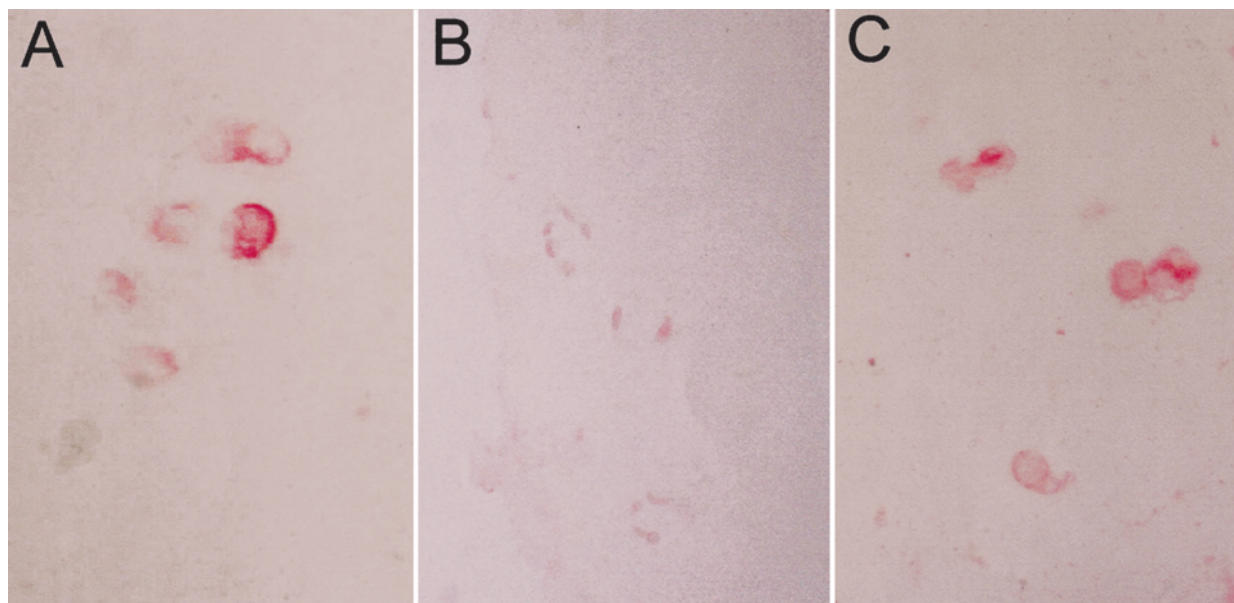
Fig. 2. Visualization of TNF-R on chondrocytes in cartilage from femoral condyles of OA joints. (A) chondrocytes staining for p55 TNF-R near the articulating region of OA cartilage (B) chondrocytes staining for p55 TNF-R in chondrones near severe osteoarthritic lesions (C) chondrocytes staining for p75 TNF-R in OA cartilage.

Table II
p75 TNF-R expression on chondrocytes in OA cartilage specimens obtained from six different regions on the femoral condyles of six different knee joints

Patient	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6	Total
A	ND	–	+	+	ND	ND	2/3
B	ND	–	–	+	ND	–	1/4
C	–	–	–	ND	ND	–	0/4
D	–	–	ND	–	ND	–	0/4
E	ND	–	–	–	ND	–	0/4
F	–	–	–	–	ND	–	0/5
Total	0/3	0/6	1/5	2/5	0/0	0/5	

(+) denotes degree of positivity, (–) denotes negative, ND (not done) denotes regions for which insufficient full depth cartilage was available for processing.

types appeared to be mutually exclusive: those specimens that were positive for p55 were negative for p75 TNF-R, and vice versa. By contrast, none of the sections obtained from NA femoral head cartilage stained positive for either receptor type.

SUSCEPTIBILITY OF CARTILAGE TO LOW CONCENTRATIONS OF TNFα

To determine the effects on cartilage of TNFα at the concentrations likely to be produced by tissue within the knee joint (0.1–5 ng), replicate biopsies of OA cartilage from specific regions of the femoral condyles were incubated in the presence of cytokine for a minimum of 14 days and GAG release into supernatants measured. Differences in cumulative GAG release from biopsies incubated in the presence of TNFα as compared with GAG released from control biopsies incubated in medium alone were assessed. The results for each of the four experiments performed at each cytokine concentration are summarized in Table III. Regions where TNFα-induced GAG release into the medium was significantly higher than controls were considered to be susceptible to the effects of the cytokine and termed positive (+), those where insignificant amounts of GAG were released were termed negative (–). Variation was observed within the same knee joint in susceptibility of cartilage to the effects of 5 ng TNFα. For example, cartilage from regions 1, and 6 from patient 1 were susceptible to the effects of 5 ng TNFα whereas cartilage from regions 2 and 3 were not. Similarly, for patient 2, only cartilage from regions 1, 3 and 5, but not regions 2 and 6, were susceptible to the effects of 5 ng TNFα. In addition, differences were also seen at the same site in different knees. Although cartilage from region 3 was tested with 5 ng TNFα for all four patients, only biopsies from patient 1 were susceptible to the cytokine. Similarly, only in patient 4 was cartilage from region 2 susceptible to the effects of 5 ng TNFα. Comparable variation in susceptibility of different regions within the same knee and in the same region in different knees were apparent in cartilage incubated in the presence of 1, 0.5 and 0.25 ng TNFα. Figure 3 shows a typical experiment from each group illustrating the variation within and between knees in susceptibility to the effects of cytokine. Interestingly, the total number of regions responding to TNFα decreased with cytokine concentration. For

Table III
Summary of explant experiments on cartilage obtained from specific anatomical locations on the femoral condyles of 20 OA knee joints. Biopsies, in replicates of four or more, were incubated for 14 or 28 days in the presence of 5, 1, 0.5, 0.25, 0.1 ng/ml TNFα, and GAG release into supernatants measured. Comparison was made between GAG release from biopsies incubated in the presence of TNFα and that released from biopsies incubated in medium alone

TNFα (ng/ml)	Patient	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6	Positive/Total*
5.0	1	+	−	−	ND	ND	+	2/4
	2	+	−	+	ND	+	−	3/5
	3	−	−	−	+	−	−	1/6
	4	−	+	−	ND	−	+	2/5
								8/20 (40%)
1.0	5	−	−	−	−	−	−	0/6
	6	+	−	−	ND	ND	+	2/4
	7	−	+	−	+	ND	ND	2/4
	8	ND	−	−	−	ND	−	0/4
								4/18 (22%)
0.5	9	+	−	−	−	−	−	1/6
	10	ND	−	+	+	−	ND	2/4
	11	−	−	−	ND	+	−	1/5
	12	ND	−	−	ND	−	−	0/4
								4/19 (21%)
0.25	13	−	−	−	−	ND	−	0/5
	14	+	−	+	−	ND	−	2/5
	15	ND	−	−	−	ND	−	0/4
	16	−	−	−	ND	ND	−	0/4
								2/18 (11%)
0.1	17	−	−	−	−	ND	−	0/5
	18	ND	−	−	−	ND	−	0/4
	19	−	−	−	ND	−	ND	0/4
	20	−	−	−	−	ND	ND	0/4
								0/17 (0%)

(+) denotes regions where TNFα-induced GAG release was significantly greater ($P<0.05$) than corresponding control; (−) denotes regions where TNFα-induced GAG release was insignificant. *Denotes the number of regions responding to TNFα/total number of regions examined. ND (not done) denotes regions for which insufficient cartilage was available to make replicate biopsies.

example, whilst 40% of all regions tested were susceptible to the effects of 5 ng TNFα, only 11% were susceptible to 0.25 ng TNFα. None of the biopsies from any of the knees were susceptible to the effects of 0.1 ng TNFα, despite extension of the incubation period to 28 days. When divided into region of origin, more biopsies from the anterior (1 and 4) and posterior (3 and 6) regions of the femoral condyles were susceptible to the effects of TNFα than those from the central regions (2 and 5). Overall, there was no significant difference in responsiveness in biopsies originating from the lateral (10/45, 22%), as compared to the remaining medial (8/37, 22%), regions of femoral condyles.

TNFα AND STNF-R MEASUREMENTS IN SYNOVIUM SUPERNATANTS

Production of TNFα by synovium may contribute to loss of cartilage in knee joints in regions where high numbers of p55 TNF-R are focused. However, TNF-R shed from the surface of cells and solubilized in the joint space may bind to TNFα and effectively block bioavailability of the cytokine at the target cell. To determine whether sufficient soluble inhibitor can be produced to negate the effect of any TNFα present within the joint, TNFα and both p55 and p75 sTNF-R were measured in supernatants from cultured synovium. The concentrations of TNFα measured in supernatants from cultured NA and OA synovium are recorded in Fig. 4. TNFα measurements were significantly higher ($P=0.0004$) in supernatants from OA synovium

(752.1 ± 1261.4 pg/ml, range 23–4575, $N=37$) than similar supernatants from NA synovium (102.7 ± 73.5 pg/ml, range 23–244, $N=15$). The concentrations of soluble p55 and p75 TNF-R measured in a subset of the same NA and OA supernatants are recorded in Fig. 5. In supernatants from NA synovium, measurements of p55 sTNF-R (1.38 ± 1 ng/ml, range 0.06–2.9, $N=15$) were significantly higher ($P=0.02$) than those for p75 sTNF-R (0.4 ± 0.18 ng/ml, range 0.16–0.88, $N=15$). By contrast, measurements of p55 sTNF-R in supernatants from OA synovium (1.51 ± 1.44 ng/ml, range 0.06–4.96, $N=24$) did not differ significantly from those of p75 sTNF-R (0.89 ± 0.43 ng/ml, range 0.14–2.29, $N=24$). When measurements of p55 sTNF-R concentrations in supernatants from NA synovium were compared with those from OA synovium, no significant statistical difference between groups was apparent. However, significantly more ($P=0.0001$) p75 sTNF-R was detected in supernatants from OA synovium than similar supernatants from NA synovium. To determine whether synovium production of cytokine and soluble inhibitors was concurrent, correlations were sought within groups between measurements of TNFα and those of p55 and p75 sTNF-R. In supernatants from NA synovium there was a significant association between the concentrations of TNFα measured and p75 sTNF-R ($r=0.77$, $P=0.0008$, $N=15$), although there was no significant association between the cytokine and p55 sTNF-R ($r=-0.18$, $P=0.51$, $N=15$). In supernatants from OA synovium, there was no significant relationship between measurements of TNFα and either
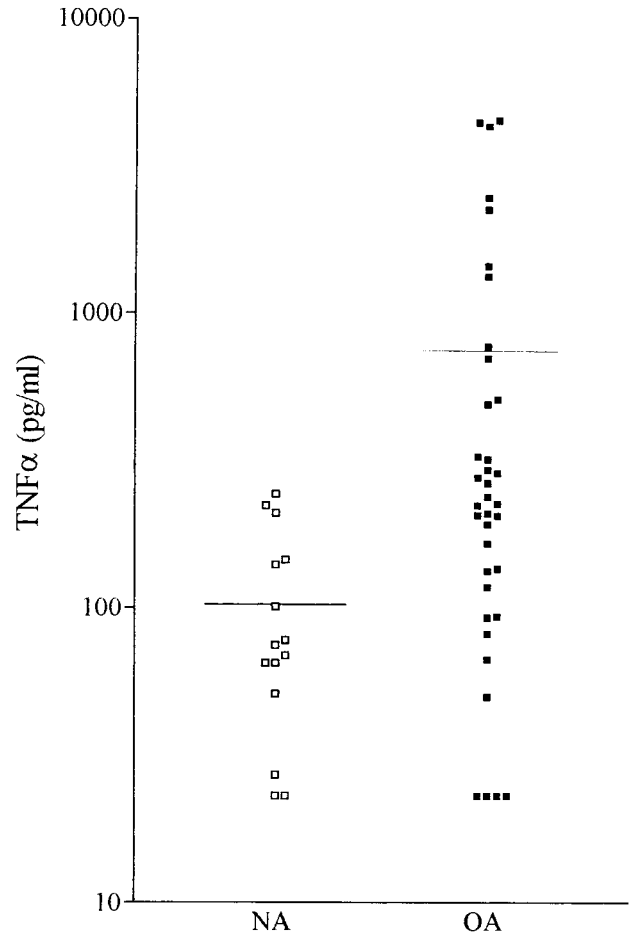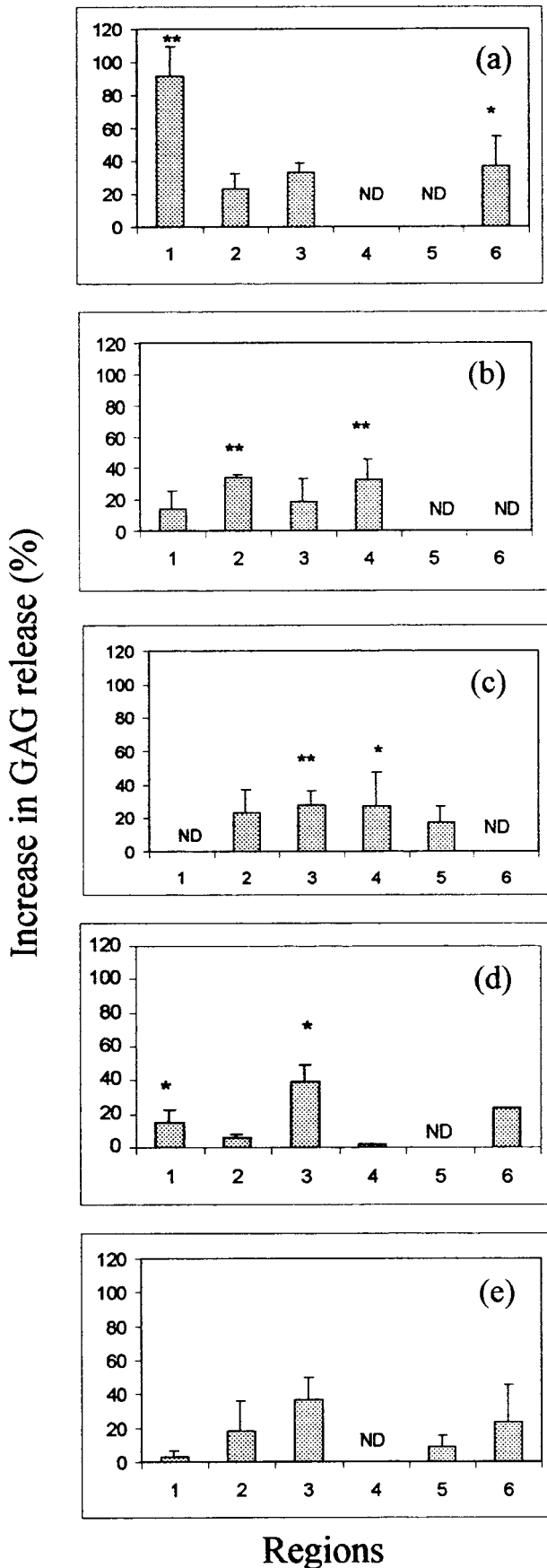
Fig. 4. TNFα concentrations in supernatants from 24 hour cultures of osteoarthritic (OA) and non-arthritic (NA) synovium.

p55 (r=0.06, *P*=0.77, *N*=22) or p75 (r=0.25, *P*=−0.6, *N*=22) sTNF-R. For example, the supernatant containing the highest amount of cytokine (2492 pg/ml) contained low amounts of both p55 sTNF-R and p75 sTNF-R (0.06 ng/ml and 0.2 ng/ml respectively), whilst another supernatant containing only 93 pg/ml TNFα, contained relatively high p55 and p75 sTNF-R measurements (3.19 ng/ml and 2.29 ng/ml respectively). On average, TNFα measure-ments were approximately seven-fold greater in super-natants from OA synovium as compared with those from NA synovium. By contrast, p75 sTNF-R measurements in OA supernatants were only two-fold higher.

Fig. 3. Typical examples of variation in percentage increase in GAG release from cartilage explants during 14 days culture in the presence of (a) 5 ng/ml, (b) 1 ng/ml, (c) 0.5 ng/ml, (d) 0.25 ng/ml TNFα. Incubation of explants in the presence of (e) 0.1 ng/ml) TNFα was extended to 28 days. All values are the mean±SD of at least 4 replicates. ND (not done) denotes regions where insuffi-cient cartilage was available. Significant increase in GAG release as compared with control is indicated by **P<0.01, *P<0.05 (paired *t* test). ND (not done) denotes regions for which insufficient cartilage was available to make replicate biopsies.
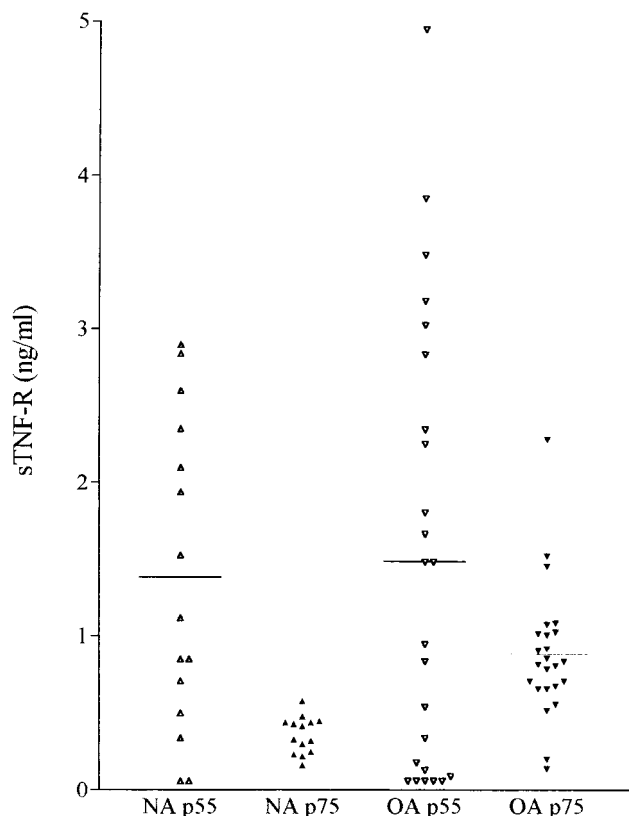
Fig. 5. Measurements of p55 and p75 sTNF-R in supernatants from 24 hour cultures of osteoarthritic (OA) and non-arthritic (NA) synovium.

Discussion

In previous work we suggested a role for TNFα in focal loss of cartilage in osteoarthritis. Here we examine further the criteria required for cytokine mediated cartilage damage to occur at specific locations on femoral condyles. Firstly, we determined whether the distribution of TNF-R expression on chondrocytes *in vivo* paralleled those *in vitro*. Here we show for the first time chondrocytes staining for TNF-R in cartilage obtained from specific regions of OA knee joints and flash-frozen less than 2 hours after surgery. Moreover, more preparations stained for p55, the major signal transducer of TNFα-induced cellular responses, than for p75 TNF-R. Previously we showed that cartilage from different sites within the same joint varied in its response to TNFα, as measured by GAG release, and that such variation was directly linked to p55 TNF-R on chondrocytes from the same region.[7] The results reported here therefore confirm the presence of such p55 TNF-R-bearing chondrocytes in fresh cartilage and further demonstrate that receptor expression and, by inference, susceptibility to the effects of TNFα, is not consistent in all regions of the joint at the same time.

For TNFα-mediated cartilage damage to occur cytokine produced within the joint, and contained in synovial fluid, must have reasonable access to TNF-R-bearing chondrocytes. In normal human articular cartilage, chondrocytes in the superficial zone express greater numbers of high affinity binding sites for IL-1 and are therefore more sensitive to stimulation by IL-1 than chondrocytes in deeper zones.[16] If chondrocytes in the superficial zone are also more respon-

sive to TNFα, then it could be argued that in the OA cartilage used in these experiments, the zone containing most TNFα-sensitive chondrocytes has been lost. This suggestion is not borne out by the results. Whilst chondrocytes staining for p55 TNF-R were detected throughout the cartilage matrix, intensely staining chondrocytes were usually detected nearest the articulating surface of cartilage, whether it was fibrillated or not, as well as in chondrones in cartilage from regions with severe lesions. These observations are similar to those of Kammerman *et al.* (1996)[17] where chondrocytes staining intensely for p55 TNF-R were detected in fibrillated canine cartilage from which proteoglycan had been lost. Thus in human joints, the spatial location of p55 TNF-R bearing chondrocytes within matrix and differential expression within joints is likely to predispose cartilage from specific regions to cytokine-induced damage.

For TNFα to be associated with focal loss of cartilage *in vivo*, chondrocytes must be responsive to the amount of cytokine produced locally. Earlier work showed site-related differences in susceptibility to the effects of 10 ng/ml TNFα in explants from both OA and NA cartilage.[7] However, the amount of cytokine used was higher than that measured in OA synovial fluids (0.02–1.2 ng/ml)[3] or supernatants from cultured OA synovium (0.02–5.0 ng/ml) and certainly outside the range likely to be found in NA joints.[4] We next performed similar experiments using explants of OA cartilage from specific regions of different joints together with much less cytokine. Here we show GAG release to be significantly increased in supernatants from susceptible explants cultured in the presence of 5 ng, 1 ng, 0.5 ng or 0.25 ng TNFα. Variation in susceptibility was observed at different sites within the same knee joint which were similar to that observed with 10 ng TNFα.[7] In addition, differences in susceptibility were also seen at the same site in different knees. Overall there was no apparent difference in responsiveness to TNFα in biopsies originating from the lateral as compared to the medial femoral condyles or in macroscopically normal cartilage as compared with damaged cartilage. These observations suggest that by end-stage disease, as judged by the need for surgery, macroscopically normal OA cartilage also becomes susceptible to the effects of cytokines. Although less GAG was released from the explants in these experiments, the results are consistent with our earlier findings and demonstrate for the first time that TNFα at concentrations likely to be found in OA, and some NA, joints can indeed degrade cartilage. Interestingly, the number of specimens responding to TNFα decreased with concentration until none of the explants responded to 0.1 ng/ml cytokine.

It is now well documented that receptors for many cytokine species are shed from the surface of various cell types and act as soluble inhibitors by reducing availability of the cytokine. The extracellular portion of both p55 and p75 TNF-R are released by proteolytic cleavage at the membrane level and the resulting sTNF-R have been detected in the serum of normal individuals,[18] as well as in biological fluids from patients with a variety of clinical conditions.[10,19,20,15] It thus became important to determine whether in OA the balance between agonist and antagonist are such that the net effect of TNFα at the target cell is abrogated or significantly reduced. Since no detectable p55 or p75 sTNF-R were found in supernatants from cultured OA chondrocytes[9] it was considered unlikely that chondrocytes contribute significantly to synovial fluid sTNF-R concentrations. We therefore measured concurrently the concentrations of both sTNF-Rs and TNFα in OA and NA

synovium supernatants. In agreement with previous studies,[4] TNFα concentrations were significantly higher in supernatants from cultured OA synovium as compared with NA synovium. For sTNF-R, measurements of p55 were higher in OA supernatants, but not statistically so, whereas concentrations of p75 were significantly higher in OA as compared with NA supernatants. As incubation of synovial fibroblasts with exogenous TNFα results in shedding of p75 TNF-R from the cell surface,[21] correlations between the two parameters were expected. In NA supernatants there was indeed a significant relationship between p75 TNF-R and TNFα measurements which supported our prediction. As TNFα induces a time and dose dependent increase in the release of p75, but not p55 TNF-R, from the surface of monocytes *in vivo*,[22] our observations indicate the existence of a similar mechanism in NA synovial cells. By contrast, in OA supernatants, no significant association was found between measurements of the two molecules. Similarly, no correlation was found between measurements of TNFα and either sTNF-R in synovial fluids from patients with OA.[10] Previous work *in vivo*[20] and *in vitro*[23] showed that sTNF-R release occurs some hours after exposure to TNFα. Perhaps in a chronic condition such as OA, cells in the synovium may be exposed to relatively high concentrations of TNFα over a long period so that the mechanism by which binding of cytokine to its receptor results in shedding of sTNF-R becomes dysphasic or even attenuated with time.

Attempts to quantify the inhibitory capacity of sTNF-R using in-vitro experiments[20] suggest that 5 ng/ml p55 sTNF-R is required to reduce the bioactivity of 585 pg/ml TNFα by 66%, whereas 95% reduction of the same amount of cytokine was achieved only by 500 ng/ml p55 sTNF-R. By comparison, the same amount of p75 sTNF-R was inadequate to block the response. The data reported here show only one of 13 supernatants from OA synovium contained p55 sTNF-R in the region of 5 ng/ml, whereas none of the supernatants contained greater than 2.3 ng/ml p75 sTNF-R. In OA joints it therefore seems unlikely that inhibitory activity in the form of shed receptors is sufficient to bring about significant diminution of TNFα-mediated activities. Moreover, neither p55 sTNF-R nor p75 sTNF-R were found in supernatants from cultured OA chondrocytes[9] although TNFα was detected by immunocytochemistry in OA but not NA chondrocytes.[24] Consequently, the balance between TNFα and its soluble inhibitors is likely to be even more biased toward agonistic activity in the immediate extracellular environment of OA chondrocytes than in the joint fluid. In addition, cytokine:inhibitor complexes may serve as slow release reservoirs for bioactive TNFα effectively prolonging the circulation of cytokine within the joint.

Taken together, the results presented here provide further support for TNFα contributing to the process(es) leading to focal loss of cartilage in OA. It remains to be determined, however, just how TNF-R become upregulated on chondrocytes in cartilage from specific regions. The answer may lie in the differences in weight-bearing experienced by chondrocytes in cartilage from different regions of the joint. Loading has a profound effect on chondrocyte metabolism;[25] shear stress can alter IL-6 mRNA expression[26] and IL-1β and TNFα production is altered by mechanical stress.[27] If environmental changes of the type evoked by loading alter chondrocyte expression of TNF-R, then an explanation for focal loss of cartilage in OA would be identified.

References

1. Arner EC, Hughes CE, Decicco CP, Caterson B, Tortorella MD. Cytokine-induced cartilage proteoglycan degradation is mediated by aggrecanase. Osteoarthritis Cartilage 1998;6:214–28.
2. Fosang AJ, Last K, Maciewicz RA. Aggrecan is degraded by matrix metalloproteinases in human arthritis. J Clin Invest 1996;98:2292–9.
3. Westacott CI, Whicher JT, Barnes IC, Thompson D, Swan AJ, Dieppe PA. Synovial fluid concentration of five different cytokines in rheumatic diseases. Annals Rheum Dis 1990;49:676–81.
4. Elson CJ, Mortuza FY, Perry MJ, Warnock MG, Webb GR, Westacott CI. Cytokines and focal loss of cartilage in osteoarthritis. Brit J Rheum 1998;37:106–7.
5. Warnock MG. Mechanisms of cartilage degradation in osteoarthritis. PhD thesis, Bristol University, 1995.
6. Ismaiel SI, Atkins RM, Pearse MF, Dieppe PA, Elson CJ. Susceptibility of normal and arthritic human articular cartilage to degradative stimuli. Brit J Rheum 1992;31:369–73.
7. Webb GR, Westacott CI, Elson CJ. Chondrocyte tumor necrosis factor receptors and focal loss of cartilage in osteoarthritis. Osteoarthritis Cartilage 1997;5:427–37.
8. Martell-Pelletier J, McCollum R, DiBattista J, et al. The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes. Arthritis Rheum 1992;35:530–40.
9. Westacott CI, Atkins RM, Dieppe PA, Elson CJ. Tumor necrosis factor receptor expression on chondrocytes isolated from human articular cartilage. J Rheumatol 1994;21:1710–15.
10. Cope AP, Aderka D, Doherty M, Engelmann H, Gibbons D, Jones A, et al. Increased levels of soluble tumor necrosis factor receptors in the sera and synovial fluid of patients with rheumatic disease. Arthritis Rheum 1992;35:1160–9.
11. Farndale RW. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem Biophys Acta 1986;883:173–7.
12. Webb GR, Westacott CI, Elson CJ. Osteoarthritic synovial fluid and synovium supernatants upregulate tumor necrosis factor receptors on human articular chondrocytes. Osteoarthritis Cartilage 1998;6:167–76.
13. Westacott CI, Webb GR, Warnock MG, Sims JV, Elson CJ. Alteration of cartilage metabolism by cells from osteoarthritic bone. Arthritis Rheum 1997;40:1282–91.
14. Bailey M, Williams, NA, Wilson AD, Stokes CR. PROBIT: weighted regression analysis for estimation of biological activity. J Immunological Methods 1992;153:261–2.
15. Armstrong L, Foley NM, Millar AB. Inter-relationship between tumour necrosis factor alpha (TNFα) and TNF soluble receptors in pulmonary sarcoidosis. Thorax 1999;54:524–30.
16. Hauselmann HJ, Flechtenmacher J, Michal L, Thonar EJ-MA, Shinmei M, Kuettner KE, Aydelotte MB. The superficial layer of human articular cartilage is more susceptible to interleukin-1 induced damage than the deeper layers. Arthritis Rheum 1996;39:478–488.
17. Kammerman JR, Kincaid SA, Rumph PF, Baird DK, Visco DM. Tumor necrosis factor-α in canine osteo-

arthritis: immunolocalisation of TNFα, stromelysin and TNFα receptors in canine osteoarthritic cartilage. Osteoarthritis Cartilage 1996;4:23–34.

18. Aderka D, Engelmann H, Shemer-Avni Y, et al. Variation in serum levels of the soluble TNFa receptors among healthy individuals. Lymphokine and cytokine Research 1992;3:157–9.

19. Aderka D, Engelmann H, Hornik V, Skornick Y, Levo Y, Wallach D, Kustai G. Increased serum levels of soluble receptors for tumor necrosis factor in cancer patients. Cancer Research 1991;51:5602–7.

20. Van Zee KJ, Kohno T, Fischer E, Rock CS, Moldower LL, Lowry SF. Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor-a *in vitro* and *in vivo*. Proc Natl Acad Sci 1992;89:4845–9.

21. Alaaeddine N, DiBattista JA, Pelletier J-P, Cloutier J-M, Kiansa K, Dupois M, Martel-Pelletier J. Osteoarthritic synovial fibroblasts possess an increased level of tumor necrosis factor-receptor 55 (TNF-R55) that mediates biological activation by TNFα. J Rheumatol 1997;24:1985–94.

22. Joyce DA, Steer JH. Tumor necrosis factor alpha and interleukin-1 alpha stimulate late shedding of p75 TNFα receptors but not p55 TNFα receptors from human monocytes. J Interferon Cytokine Res 1995;15:947–54.

23. Aderka D, Sorkine P, Abu-Abid S, Lev D, Setton A, Cope AP, Wallach D, Klausner J. Shedding kinetics of soluble tumor necrosis factor (TNFα) receptors after systemic TNFα leaking during isolated limb perfusion. Relevance to the pathophysiology of septic shock. J Clin Invest 1998;101:650–9.

24. Moos V, Fickert S, Muller B, Weber U, Sieper J. Immunohistological analysis of cytokine expression in human osteoarthritic and healthy cartilage. J Rheumatol 1999;26:870–9.

25. Urban JPG, Hall AC, Gehl KA. Regulation of synthesis in articular chondrocytes by extracellular osmolality and ionic composition. J Cell Physiol 1993;154:262–70.

26. Mohtai M, Gupta MK, Donlan B, et al. Expression of interleukin-6 in osteoarthritic chondrocytes and effects of fluid-induced shear on this expression in normal human chondrocytes in vivo. J Bone Joint Surgery 1993;14:67–73.

27. Cameron ML, Frondoza CG, Holland C. Hungerford DSH Expression of proinflammatory IL-1 and TNFα by osteoarthritic chondrocytes is altered in response to mechanical stress. Trans Orthop Res Soc 1999;24:606.