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Role of Articular Cartilage and Chondrocyte Changes and Mononuclear Apoptosis in the Pathogenesis and Strategy of Therapy in Polyarticular Juvenile Rheumatoid Arthritis

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The objective of this research work is to study the changes in articular cartilage and chondrocytes in juvenile rheumatoid arthritis (JRA) and the implication of mononuclear cells apoptosis, proinflammatory cytokines, keratan sulfate (KS) and Transforming Growth Factor B (TGFB) in the pathogenesis of the joints changes. The study included 42 children diagnosed as polyarticular JRA, according to the criteria of the American College of Rheumatology and 50 normal children as control. The following investigations were carried out: histopathology of articular cartilage specimens and chondrocyte culture, assay of IL-1 α , IL-1 β , TNF- α , IL-1R, TNF- α p55, TNF- α p75 in synovial fluid (SF) and peripheral blood serum (ser), estimation of concentration of keratan sulfate (KS), transforming growth factor B (TGFB), CD95 (sFas) and assessment of apoptotic index of mononuclear cells, in the synovial fluid and peripheral blood (PB). The results could be summarized as follows: morphological and immunohistochemical changes in the matrix and chondrocytes, increased levels of proinflammatory cytokines and KS and decreased CD95, (sFas), TGFB and apoptotic index of mononuclear cells, in synovial fluid and peripheral blood. The changes are more significant in SF. In conclusion, the delayed mononuclear cell apoptosis and increased secretion of proinflammatory cytokines lead to the morphological and immunohistochemical changes in articular cartilage and chondrocytes, high KS and low TGFB. So far, we can recommend to add to the strategy of therapy, anti-proinflammatory cytokines and injections of TGFB for amelioration of the disease.

Key words: Articular cartilage, chondrocyte, apoptosis, juvenile rheumatoid arthritis

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Introduction

Juvenile rheumatoid arthritis (JRA) is an autoimmune disease characterized by infiltration of lymphocytes and macrophages into the synovium, hyperplasia of the synovial lining and destruction of cartilage and bone (Perlman *et al.*, 2001). Macrophage-like and fibroblast-like synoviocytes comprise the normal synovial lining, which consists of 1-2 cell layers but increases to as much as 10 or more cell layers in active JRA (Firestein, 1996). Synovial macrophages express high levels of collagenase, proinflammatory cytokines, colony-stimulating factor, inhibitory protein 1 α and chemoattractant protein 1, which are mediators of inflammation and joint destruction (Feldmann *et al.*, 1996).

Cytokines such as interleukin-1 β (IL-1 β) and tumour necrosis factor α (TNF α) play a central role in this process because they stimulate the production of proinflammatory mediators and inhibit matrix synthesis (Alaaeddine *et al.*, 2001). Death of articular chondrocytes leading to hypocellular cartilage may be an intrinsic death program caused by extrinsic factors as cytokines (Kuhn and Lotz, 2001). The mechanism of dysregulated hyperplasia of the invasive mononuclears and fibroblasts has been proposed to be due to imbalance between growth and apoptosis (Zhang *et al.*, 2000). One of the important factors regulating cell death in lymphocytes is the cell surface Fas (CD95), a member of TNF receptor family (Scaffidi *et al.*, 1998). Stimulation of Fas receptor on activated lymphocytes can induce apoptosis (Lee and Shactre, 2001). In autoimmune diseases the mononuclears accumulate and are attracted from the general circulation and congregate within the joint.

Erosion of articular cartilage is associated with degradation and release of matrix proteoglycans (PG). It was reported that keratan sulfate (KS) which is a component of PG is capable of diffusion into the systemic circulation. On the other hand, transforming growth factor B (TGFB) which is an intriguing modulatory factor counteracts interleukin 1 (IL-1) on cartilage through down regulation of IL-1 receptors (Gouze *et al.*, 2001).

The present work was planned to study the changes in articular cartilage and chondrocytes in polyarticular JRA and the implication of mononuclear cells apoptosis, proinflammatory cytokines, KS and TGFB in the pathogenesis of the joint changes, especially that this approach has not been published before in JRA.

Materials and Methods

The study included 42 children diagnosed as polyarticular juvenile rheumatoid arthritis and 50 normal children as control.

The patients

The range of age 4-16 years with mean 8.8 ± 4.16 years, 20 boys and 22 girls. The patients were collected from the Department of Rheumatology, the Main University Hospital and the Children University Hospital at Mansoura, Egypt. The patients fulfilled the diagnostic criteria for polyarticular JRA of the American College of Rheumatology (Brewer *et al.*, 1977). A detailed history and clinical examination was completed for every individual. Patients were examined and assessed by the same observers for clinical parameters of joint inflammation. The summed 28-joint

index score described by Prevoo *et al.* (1993) and slightly modified by Mangge and Chanenstein (1998) was used. All patients has active disease defined by the presence of arthritis, CRP and Westergren ESR levels.

The control group

Fifty healthy children with matched age and sex (mean age 10.3 ± 3.6 ys, 22 males and 28 females). The children were hospitalized for surgery of factures, with no history or joint disease. Permission was taken from the parEnts for drawing extrablood and aspiration of synovial fluid for all subjects and specimen from the articular cartilage of the affected knee for the patients and from only 10 out of the 50 normal non-rheumatoid subjects.

The following investigations were carried out:

Resected fragments of loose articular cartilage were used for histopathology of articular cartilage specimen and chondrocytes culture.

Assay of IL-1 α , IL-1 β , TNF- α , IL-1R, TNF α -R55 and TNF- α R75, for their mRNA and serum levels in the synovial fluid and peripheral blood.

Estimation of the concentration of keratan sulphate in the synovial fluid and peripheral blood.

Estimation of the concentration of Transforming Growth Facor-B (ng ml⁻¹) in the synovial fluid and peripheral blood.

Estimation of CD95 (sFas) in the synovial fluid and peripheral blood.

Apoptosis of the mononuclear cells in the synovial fluid and peripheral blood.

Histopathology of cartilage and chondrocytes, before and after culture

A standard arthroscopic examination of the knee was performed through anterolateral and anteromedial portals using Starz wide angle arthroscope. Articular cartilage was resected using a 3.5 mm. straight or curved basket forceps. Three litres of saline were then run through the knee (Insall, 1993). Chondrocytes were isolated from cartilage by sequential enzymatic digestion and cultured in Eagle's minimum essential medium with Earl's salts, 100 units ml⁻¹ penicilline, 100 μ g ml⁻¹ streptomycine, 2 ml glutamine and 10% fetal calf serum. Chondrocytes were then incubated for a further 6 hours with IL-1 β (10 units ml⁻¹), TGFB and NSAIDS (50 μ M ml⁻¹) (Attur, 1998).

For immunohistochemical analysis, cartilage explants were cultured for 10 days in complete medium. The cartilage explants were then fixed in 2% formaldehyde, embedded in paraffin and cut into sections. A conventional peroxidase staining technique for monoclonal antibodies was used (Johansen *et al.*, 2001).

Cytokine assays

Measurement of serum levels of IL-1 α , IL-1 β and TNF α , IL-1R, TNF α -p55, TNF α -p75: Commercially available ELISA Kits (Research and Diagnostics Systems U.K) specific for IL-1 α , IL-1 β , TNF α IL-1R, TNF- α p55 and TNF α -p75 were used (Yamamura *et al.*, 1993).

Detection of cytokine-mRNA

Detection of IL-1 α IL-1 β and TNF α messenger RNA (mRNA) was carried as follows: Isolation of peripheral blood mononuclear cells (PBMC) 10-20 ml of peripheral venous blood was collected into sterile pyrogen-free disposable syringes anticoagulated with endotoxin-free heparin (10 U ml⁻¹). PBMC were separated by standard density gradient centrifugation (Ficol Hi Paque method).

RT-PCR

Total RNA was extracted from freshly isolated unstimulated PBMC using an acid phenol-chloroform extraction method. Quantification of total RNA was performed spectrophotometrically. Cytokine cDNA of PBMC was obtained by reverse transcriptase (RT) then amplified by polymerase chain reaction (PCR) using specific primers. The following commercially available (Clontech Laboratories, Inc, Palo Alto, USA) oligonucleotide 5' and 3' and primer sequences were used:

IL-1 α :

Sense CACCCTCTGTCACCTGCTCAACA
Antisense GGTTCTCGCTGCCTCAAGACTC

IL-1 β :

Sense GAATTCTGCAGTCCCAGCTCTGTGCAA.
Antisense GAATTCCACAGTCATATCCACAATAG.

TNF α :

Sense GAGTGACAAGCCTGTAGCCCATGTTGTAGCA
Antisense GCAATGATCCCAAAGTAGACCTGCCAGATC.

PCR was carried out in a Thermal Cycler (Perkin Elmer Cetus) for 30 cycles of denaturation (96°C, 1 min), annealing (60°C, 45s) and extension (72°C, 1min). The amplified gene products were separated by electrophoresis on 2% agarose gel and visualized by UV light illumination using ethidium bromide staining. The amount of cytokine PCR products was graded as follows: - = no band detected, in the undiluted cDNA: sample; + = band detected in the undiluted cDNA sample but not in the 1:5 diluted cDNA; ++ = band detected in the 1:5 diluted cDNA but not in the 1:25 diluted cDNA. (Chomeznski and Sacchi, 1987, Becker *et al.*, 1996)

Estimation of Keratan Sulfate (KS)

Concentration in the synovial fluid and serum

Keratan sulfate epitope was measured with an ELISA - inhibition assay as described by Hauselmann *et al.* (1992).

Estimation of Transforming Growth Factor B Concentration in the synovial fluid and serum

Transforming growth factor- β (TGFB) was estimated by ELISA-inhibition assay (Van Beuningen *et al.*, 1994).

Detection of apoptotic mononuclear cells in synovial fluid and peripheral blood

Detection of apoptotic cells by giemsa stain, Acridine Orange stain (AO), Propidium iodide stain (PI) and DNA fragmentation

Lymphocyte separation

Cells were obtained from whole EDTA-blood using lymphocyte-separating solution (Ficoll-Paque; Pharmacia, Uppsala, Sweden) by the method of Boyum (1968). Lymphocytes were washed twice with sterile PBS pH 7.6.

Culture of lymphocyte

Cells were suspended at 107/ml in RPMI 1640 (GIBCO) containing 25 mM glucose 20 mM glutamine, 92 µg gentamycin 20 µg ml⁻¹ (Walkersville MD) and 25 mM HEPES buffer (pH 7.3: GIBCO). Cells were suspended in sterile PBS (0.05 MPO4 + 0.15M NaCl, pH7.2). The cell suspension were divided into 4 aliquots to measure the apoptosis at zero time (base time), then 24, 48 and 72 h, cells were incubated at 37°C in 5% carbon dioxide. After incubation, cells were centrifuged (200xg 5min), resuspended in ice-cold PBS and analyzed on a Coulter counter.

Assessment of cell viability: At time zero and then at subsequent times, cells were removed from culture and counted on a haemocytometer. Cell viability was determined by trypan blue dye exclusion test, one volume of trypan blue (0.4% GIBCO) was added to 5 volumes of cells at room temperature for 5 minutes.

Detection of apoptotic cells

Propidium iodide stain

Cells were stained with Hoechst 33342 and propidium iodide (PI) and visualized using fluorescence microscopy as described by Lee and Shacter (1998). A minimum of 200 cells were counted per sample and were classified as follows:

- Live cells (normal nuclei, blue chromatin with organized structure)
- Membrane intact apoptotic cells (bright blue chromatin that is highly condensed, marginated, or fragmented)
- Membrane permeable apoptotic cells (bright red chromatin, highly condensed or fragmented)

Giemsa stain

At time zero and at subsequent times, cells were removed from each culture, fixed in methanol, harvested on slides and slides were stained with May Grunwald Giemsa and examined by oil immersion light microscope (Wyllie and Morris, 1985). For assessment of the percentage of cells showing morphology of apoptosis 500 cells/slide were examined for each case at different times (0,24,48 and 72 h). Lymphocyte were considered apoptotic if they exhibited the highly characteristic morphological features of chromatin aggregation, nuclear pyknosis and cytoplasmic vaculation. The apoptotic Lymphocyte percentage at different times was calculated.

Acridine orange stain (AO)

One drop of cell suspension was added to one AO solution ($10 \mu\text{g ml}^{-1}$ in PBS), mixed gently on a slide and immediately examined with an olympus HB-2 microscope with fluorescence attachment. Green fluorescence was detected between 500 and 535nm cells exhibit bright fluorescent condensed nuclei (intact or fragmented) were interpreted as apoptotic cells and expressed as a percentage of the total number viable cells, which exhibited a green, diffusely stained intact nucleus (Kumagai *et al.*, 1995)

Assessment of apoptotic index

For this analysis in each case the mean number of monocytic cells per field was registered in 15 randomly chosen fields. The apoptotic bodies were then counted in a greater number of fields and expressed as percentages monocytic cells per case (Del Vecchio *et al.*, 1991).

Measurement of sFas

The levels of sFas were determined by the following enzyme linked immunosorbent assay (ELISA) using two anti-human Fas monoclonal antibodies, DX-2 (IgG1) and DX-3 (IgG2a), that were generated as described previously by Cifon *et al.* (1994). These antibodies react with distinct epitopes in the extracellular domain. Anti-Fas antibody (DX-3; $10 \mu\text{g ml}^{-1}$ in 0.01 m phosphate buffered saline, pH 7.2 (PBS), was coated onto 96 well microtiter plates (Sumililon multiplate MS-8496 F, Sumitomo Co, Ltd., Japan) and incubated at 37°C for 3 h. Blocking performed with 1:2 dilution with PBS of Block Ace (Dai Nihon Seiyaku Co, Ltd., Japan) at 37°C for 2 h. Then sera were added and incubated at room temperature for 1 h. After washing five times with 0,05% Tween/PBS, $10 \mu\text{g ml}^{-1}$ biotin conjugated anti-Fas antibody (DX-2) in 0.05% tween/PBS containing 5% mouse serum was added and incubated at room temperature for 1 h. After washing five times, 1:150 diluted ABC solution (Vectastain ABC kit Elite pK-6100, Vector Laboratories Inc., Burlingame, CA) was added and incubated at room temperature for 1h. After washing five more times, O-phenyldiamine (OPD) and 0,03% H₂O₂ in citrate buffer (pH 5.0) were added and incubated at room temperature for 30 min. OD490 was measured on an automated plate reader (Model 3550-UV Microplate Reader, Bio-Red, Hercules, CA) and the levels of sFas were determined by comparison to the standard curve obtained using recombinant sFas (19, 9.5, 4.75, 2.37, 1.18, 0.59, 0.29 and 0.14 ng ml^{-1}). The lower limit of detection was 0.59 ng ml^{-1} (Tokans *et al.*, 1996).

Results

Histopathology before and after culture

The morphologic appearance of normal chondrocytes maintained on tissue culture medium, shows average sized cells, with large dark nucleus and normal cytoplasmic to nucleus ratio (Fig. 1 and 2). On the other hand, the morphologic appearance of the chondrocytes taken from patients with JRA and maintained on tissue culture medium show pale nucleus, marked decrease of cytoplasm to nucleus ratio and decrease of cells size (Fig. 3). After treatment of these chondrocytes in the culture medium with nonsteroidal anti inflammatory drugs (NSAIDS) $50 \mu\text{M ml}^{-1}$ and TGF β for a further 6 hours the chondrocytes showed improvement of the above mentioned appearance and became near normal (Fig. 4).

Light microscopy of normal cartilage explants cultured for 7 days showed that the surface is smooth, large number of chondrocytes are embedded in the cartilage matrix surrounded by metachromatic material. The cartilage explants from patients with JRA and cultured for 7 days showed reduction in stainable proteoglycans, fibrillation, fissuring, chondrocytes necrosis (ghosting), sometimes clonning with loss of cartilage and matrix is depleted of metachromatic material. On the other hand articular cartilage explants cultured for seven days with treatment using NSA1DS (50 $\mu\text{M ml}^{-1}$) showed attempts of repair, focal proliferation surrounded by intense metachromatic material indicating increased proteoglycan (Fig. 5-8).

By immunostaining, the cartilage specimens from patients with JRA showed identified IL-1 at the surface as well as in clefts, fissures and the matrix of the superficial zone of the cartilage. After treatment in culture with NSA1DS, IL-1 was not present (Fig. 9 and 10).

Immunohistochemical localization of TGFB in articular cartilage of the patients with JRA was done using anti-TGFB antibody. Without treatment there was negative stain (Fig. 11) and after invitro treatment with NSA1DS TGFB was positively stained in the cytoplasm of the chondrocytes (Fig. 12).

Cytokines

The results of the concentration of the proinflammatory cytokines IL-1 α ,IL-1 β , TNF α and receptors IL-R, TNF α -p55,TNF α -p75 (Table 1) and their mRNA expression (Table 2) in the synovial fluid and serum of the peripheral blood are similar. The concentrations of all the cytokines are highly significant increased in patients with JRA in both synovial fluid and serum compared to control (P < 0.01). Furthermore, the concentrations are significantly increased in synovial fluid compared to serum (P < 0.05).

Keratan sulphate

Table 3 presents the concentration of keratan sulphate in synovial fluid and peripheral blood of patients with JRA and control. The concentration is very highly significant in patients with JRA compared to control both in synovial fluid and peripheral blood (>0.001). The concentration is highly significant increased in synovial fluid compared to peripheral blood (<0.01).

Table 1: Concentration of the IL-1 α , IL-1 β , TNF- α and their receptors (P₉ ml⁻¹) in synovial fluid (SF) and serum (ser)

Cytokine	Synovial fluid		Serum		P-value			
	JRA (n=42)	Control (n=10)	JRA (n=42)	Control N=50)	P1	P2	P3	P4
IL-1 α	189.83 \pm 9.86	8.99 \pm 2.31	156.83 \pm 8.86	7.19 \pm 1.51	<0.001	<0.001	>0.05	>0.05
IL-1 β	78.21 \pm 8.55	14.26 \pm 2.47	30.62 \pm 2.99	16.26 \pm 1.45	<0.001	<0.05	<0.01	>0.05
IL-R	69.22 \pm 6.73	20.22 \pm 3.14	52.22 \pm 4.41	22.21 \pm 2.41	<0.01	<0.01	<0.05	>0.05
TNF- α	143.50 \pm 8.11	21.31 \pm 3.21	98.21 \pm 1.20	24.52 \pm 5.10	<0.001	<0.01	<0.05	>0.05
P55	59.39 \pm 3.19	4.82 \pm 1.55	44.39 \pm 3.84	4.90 \pm 2.55	<0.01	<0.01	<0.05	>0.05
P75	61.32 \pm 4.84	6.19 \pm 1.13	32.63 \pm 3.84	6.16 \pm 2.13	<0.001	<0.01	<0.01	>0.05

P1 = SF: JRA(vs) control, P2 = Ser: JRA(vs) control, P3 = JRA-SF (vs) JRA-SerE, P4 = Control - SF(vs) Control-Ser.

Table 2: mRNA expression of IL-1 α , IL-1 β , TNF α , IL-R, TNF α P55 and P75

Cytokine		Synovial fluid		Serum		P-value			
		JRA (n=42)	Control (n=10)	JRA (n=42)	Control (N=50)	P1	P2	P3	P4
IL-1 α	-	0.0	90.0	0.0	94.0	<0.001	<0.001	>0.05	>0.05
	+	0.0	10.0	0.0	6.0				
	++	4.5	0.0	4.5	0.0				
	+++	95.5	0.0	95.5	0.0				
IL-1 β	-	0.0	80.0	0.0	80.0	<0.001	<0.05	<0.01	>0.05
	+	0.0	20.0	0.0	20.0				
	++	100.0	0.0	100.0	0.0				
	+++	0.0	0.0	0.0	0.0				
TNF- α	-	0.0	10.0	0.0	94.0	<0.001	<0.01	<0.05	>0.05
	+	0.0	10.0	0.0	6.0				
	++	9.1	0.0	9.1	0.0				
	+++	90.0	0.0	90.9	0.0				
IL-R	-	0.0	90.0	0.0	90.0	<0.001	<0.01	<0.05	>0.05
	+	0.0	10.0	0.0	10.0				
	++	9.1	0.0	100.0	0.0				
	+++	90.0	0.0	0.0	0.0				
TNFP55	-	0.0	80.0	0.0	90.0	<0.01	<0.01	<0.05	>0.05
	+	0.0	20.0	0.0	90.0				
	++	100.0	0.0	100.0	0.0				
	+++	0.0	0.0	0.0	0.0				
TNFP75	-	0.0	90.0	0.0	90.0	<0.001	<0.01	<0.01	>0.05
	+	0.0	10.0	0.0	10.0				
	++	4.5	0.0	4.5	0.0				
	+++	95.5	0.0	95.5	0.0				

P1= SF: JRA(vs) control, P2 = Ser: JRA(vs) control, P3 = JRA-SF (vs) JRA-SerE, P4 = Control - SF(vs) Control-Ser.

Table 3: Concentration of keratan sulphate (ng ml⁻¹) in synovial fluid and peripheral blood

	Synovial fluid	Peripheral blood	P-value
JRA	210 \pm 11.46	124 \pm 9.24	<0.01
Control	12.5 \pm 3.25	13.5 \pm 3.25	>0.05
P-value	<0.001	<0.001	

Table 4: Concentration of Transforming Growth Factor B in the synovial fluid and peripheral blood (ng ml⁻¹)

	Synovial fluid	Peripheral blood	P-value
JRA	31.14 \pm 2.51	33.9 \pm 3.12	>0.05
Control	81.5 \pm 3.11	87.2 \pm 4.11	>0.05
P-value	<0.01	<0.01	

Transforming growth factor B

Table 4 illustrates the concentration of transforming growth factor B in the synovial fluid and peripheral blood. The levels are highly significant lower in synovial fluid and peripheral blood of JRA compared to control (P<0.01). No significant difference is present between levels in the synovial fluid compared to peripheral blood (>0.05).

Table 5: Mean \pm SD of the concentration of CD95 (sFas) (ng ml^{-1}) and apoptotic index of synovial (Sf) and peripheral blood (PB) mononuclear cells after 72 h

Group	Number	CD95	P1 stain	Geimsa stain	AO stain
JRA-SF	42	10.15 \pm 2.41	11.54 \pm 3.06	12.36 \pm 4.24	13.62 \pm 3.11
JRA-PB	42	16.28 \pm 3.11	18.21 \pm 4.32	21.46 \pm 2.9	22.69 \pm 4.82
Control-SF	10	49.1 \pm 4.1	55.12 \pm 5.10	57.23 \pm 4.91	54.31 \pm 6.20
Control-PB	50	47.6 \pm 3.8	56.23 \pm 3.62	59.11 \pm 4.62	52.63 \pm 4.12
P1		<0.05	<0.05	<0.05	<0.05
P2		<0.001	<0.001	<0.001	<0.001
P3		<0.001	<0.001	<0.001	<0.001

P1: JRA-SF (vs) JRA-PB, P2 : JRA-SF (vs) Control -SF, P3 : JRA-PB (vs) Control -PB

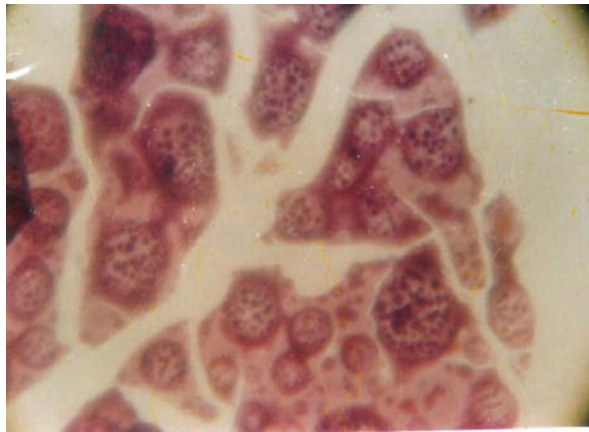


Fig. 1: A photomicrograph of chondrocytes of normal subject control in culture (Hx and E X 400)

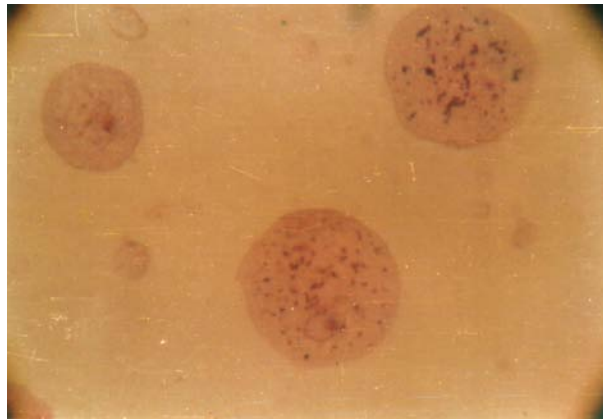


Fig. 2: A higher magnification of chondrocytes of normal subject showing average sized cells with large nucleus and normal cytoplasmic nucleus ratio (Hx and E X 1000)

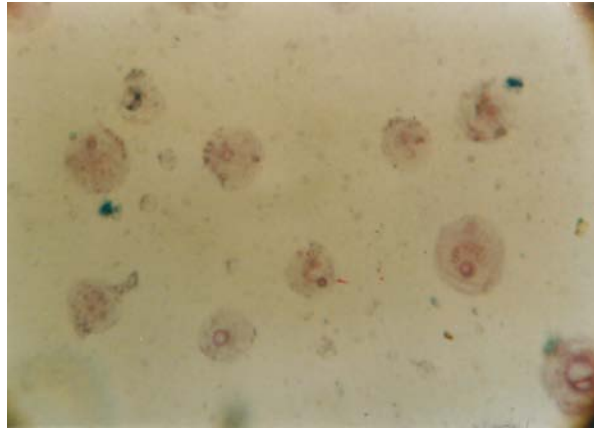


Fig. 3: A photomicrograph of chondrocytes of a patient with JRA showing decrease of cell's size and cytoplasmic to nucleus ratio (Hx and E 1000)

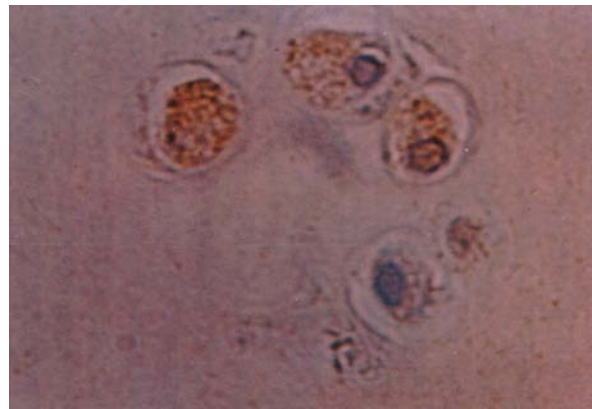


Fig. 4: A higher magnification of chondrocytes of JRA after treatment in culture with NSAIDs drugs showing average sized cells with large nucleus and normal cytoplasmic nucleus ratio similar to chondrocytes of Normal control. (Hx and E X 100)

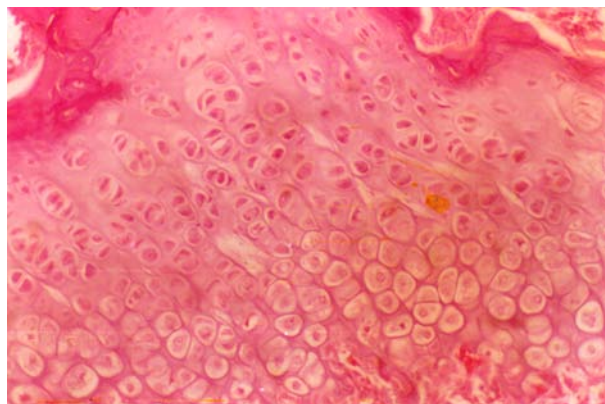


Fig. 5: A photomicrograph of joint cartilage of normal subject (control) (Hx and E X 100)

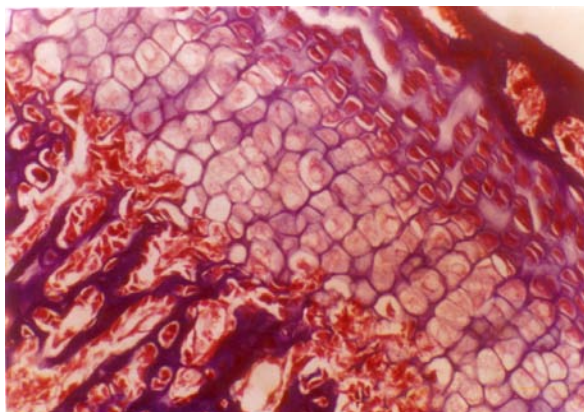


Fig. 6: A photomicrograph of joint of normal control showing collagen fibers in capsule and matrix (Massontrichrome X 100)

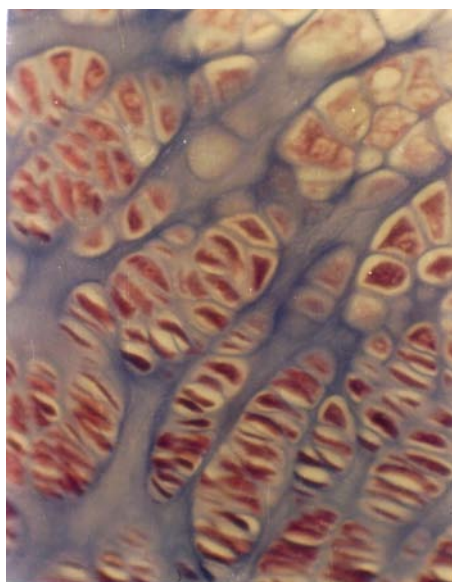


Fig. 7: A photomicrograph of joint of JRA showing marked decreased collagen fibers (Massontrichrome X 400)

CD95 and apoptosis

Table 5 shows the mean \pm SD of the concentration of CD95 and apoptotic index of mononuclear cells from the synovial fluid and peripheral blood after 72 hours culture. The results by the 3 stains are similar. The concentration of CD95 and apoptotic index of mononuclear cells are lower in JRA than control and significantly lower in synovial fluid than peripheral blood (<0.05).

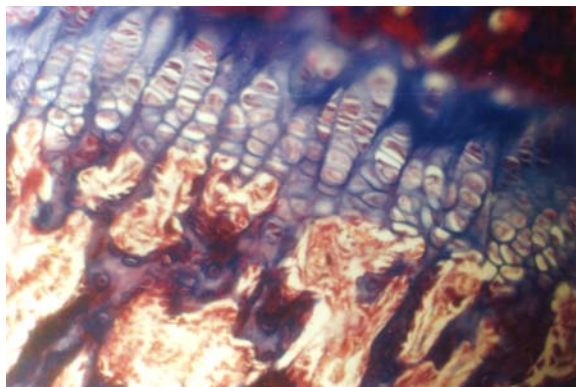


Fig. 8: A photomicrograph of joint of JRA treated with NSAIDs appears as normal (Massontrichrome X 400)

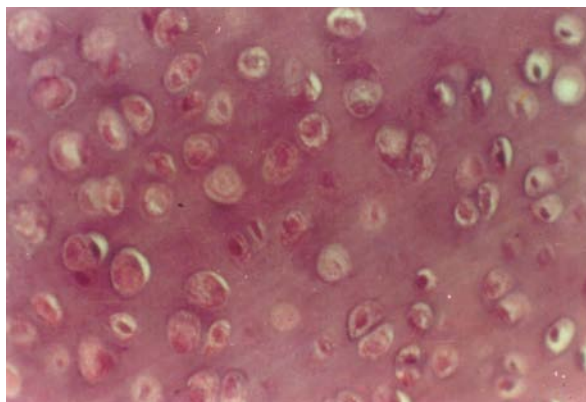


Fig. 9: A photomicrograph of cartilage of JRA patient in culture before treatment with NSAIDs showing expression of IL-1 at the cell surface of chondrocytes and matrix (PAS immunostain X 400)

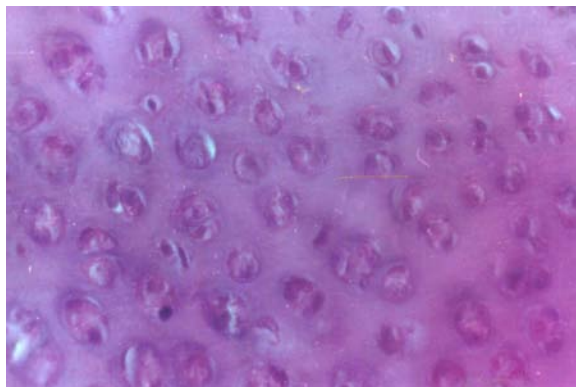


Fig. 10: A photomicrograph of the cartilage after treatment in culture with NSAIDs showing no IL-1 at the cell surface of chondrocytes and matrix (PAS immunostain X 400)

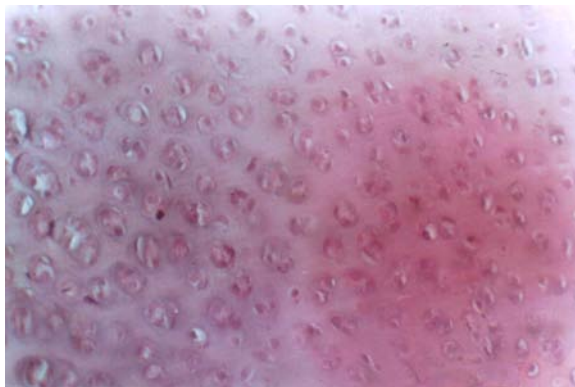


Fig. 11: A photomicrograph of cartilage of JRA in culture with anti TGFβ showing negative reaction (PAS X 400)



Fig. 12: A photomicrograph of cartilage of JRA in culture treated with NSAIDs and stained with anti TGFβ showing positive reaction (PAS X 400)

Discussion

Degradation of articular cartilage and inflammation of synovium characterize the pathogenesis of arthritis. Proinflammatory cytokines play a central role in the process (Alaaddine *et al.*, 2001). In the present study IL-1 α , IL-1 β , TNF α and their receptors IL-R, TNF-p55 and TNF-p75 are increased markedly in the synovial fluid and peripheral blood. The more increase in the synovial fluid which is due to homing of the mononuclear cells in the target tissue, secreting the proinflammatory cytokines, which stimulate proinflammatory mediators and proteases and inhibit extracellular matrix synthesis, thus leading to cartilage destruction (Le and Vilcek, 1987). IL-1 β and TNF α express the synthesis of proteoglycans, types II and IX collagen (Goldring *et al.*, 1988) and tissue inhibitor of metalloproteinases (Sandy *et al.*, 1992); increase the synthesis of matrix metalloproteinases (MMPS) and plasminogen activator (Compbell *et al.*, 1988) and induce the production of inducible nitric oxide synthase (Stadler *et al.*, 1991).

The combination of IL-1 α and the family IL-6 family cytokine oncostatin M (OSM) can synergize to promote cartilage collagen degradation (Cawston *et al.*, 1998). Several studies have implicated cytokine modulation of IL-1R levels on chondrocytes (Pronost *et al.*, 1995) and have proposed that an increase in the receptor of IL-1 levels could augment the IL-1 α response, leading to increased proteinase induction (Harvey *et al.*, 1993 and Rowan *et al.*, 2000). In the present study IL-1 α and IL-1R are markedly increased in synovial fluid of patients with JRA and in addition the immunohistochemical study of the chondrocytes and cartilage identified IL-1 at the surface as well as in clefts, fissures and the matrix of the superficial zone of the cartilage. In tissue culture, after treatment with NSAIDS, IL-1 was not present. Furthermore, the changes in the chondrocytes we found in the present study as pale nucleus, marked decrease of cytoplasm to nucleus ratio and decrease of cell size, were markedly improved after treatment in the culture with NSAIDS (50 μ M ml⁻¹) and TGFB.

Matrix metalloproteinases (MMPs) play key roles in mediating the degradation of connective tissues such as articular cartilage in joints (Billingham *et al.*, 1997). The erosions of articular cartilage are associated with degradation and release of matrix proteoglycans (PG), which in case of chronic arthritis can be detected in the synovial fluid of patients (Witter *et al.*, 1987) and experimental animal models (Ratcliffe *et al.*, 1988). These matrix components may be further processed by the synovial milieu (Greiling *et al.*, 1986). PG components and glycosaminoglycans such as keratan sulfate (KS) and hyaluronic acid are capable of diffusion into the systemic circulation (Goldberg *et al.*, 1988). It has been suggested that high serum levels of KS reflect high rates of cartilage turnover and indicate genetic susceptibility to develop chronic arthritis (Sweet *et al.*, 1988). In the present study an interesting finding is the high level of KS in peripheral blood and synovial fluid in patients with JRA compared to normal, which reflects the degradation and release of matrix proteoglycans. This goes in parallelism with the morphological changes of the articular cartilage we found in the present study. The higher concentration of KS in the synovial fluid compared to peripheral blood confirm that the release is from the articular cartilage of the joint.

Attempts to shed light on the potential value of measuring the concentration of joint derived molecules in the blood of patients with rheumatoid arthritis (RA) have led to conflicting results. While the results of 3 studies of serum levels of antigenic keratan sulfate (Ag Ks) have shown that this marker is found at lower levels in patients with RA than in age-matched normal controls, a fourth study has indicated that it is increased in RA patients (Manicourt *et al.*, 1999).

TGFB is a multifunctional cytokine that plays an important role in the regulation of repair and regeneration following tissue injury (Border and Ruoslahti, 1992). It has been shown to induce the synthesis and secretion of extracellular matrix proteins such as transcan, fibronectin and collagens as well as proteoglycans (Haubeck *et al.*, 1995). The degradation of the extracellular matrix is blocked by TGFB which decreases the synthesis of proteases and induces the increased synthesis of protease inhibitors (Gunther *et al.*, 1994). It is an antagonist of proinflammatory cytokines, as IL-1 α , IL-1 β and TNF- α and has beneficial effect in inflammatory joint diseases (Haubeck *et al.*, 1995).

Cytokines and growth factors are actively involved in the pathogenesis of chronic inflammatory and degenerative joint diseases. In the present study the proinflammatory cytokines and their receptors were found to be significantly high in the synovial fluid and serum and more increased in the former. The results of the mRNA expression and the corresponding circulating IL-1 α , IL-1 β and TNF- α are in parallelism which indicate unaffected transcriptional or translational processes. Giovine *et al.* (1986) and Fava *et al.* (1989) found high levels of IL-1 β , TNF- α and IL-8 as well as TGF β 1 in the synovial joint diseases. Hafez *et al.* (2000) found high level of TNF- α in addition to IL-2, IL-4, IL-6, IL-10, IF γ IL-1R, TNF-p55 and TNF-p75 in the peripheral blood of patients with JRA.

The inhibitory effect of IL-1 β on chondrocyte proteoglycan synthesis is well established (Tyler, 1988). It is also involved in the degradation of proteoglycan and collagens (Gowen *et al.*, 1984, Arner and Pratter, 1989). TNF- α has similar but often weaker effects than those of IL-1 β (Dinarelli *et al.*, 1986). On the other hand, TNF- α was found to antagonize most of TGF β effects on gene expression (Verrecchia *et al.*, 2000). In addition, TGF β counteracts IL-1 effects on cartilage through down regulation of IL-1 receptors, up regulation of proteoglycan synthesis and release of enzyme inhibitors and IL-1 receptor antagonist (Van Beuningen *et al.*, 1994).

However, the higher concentration of cytokines in the synovial fluid compared to peripheral blood in our patients with JRA, indicate the role of activated T-cells in the pathophysiological events of the disease (Steiner *et al.*, 1999). JRA as an autoimmune disease can result from failure to remove autoimmune cells that arise during immune response to an antigen (Thompson, 1995). The migration of autoreactive lymphocytes and other leukocytes from the blood stream and homing into the target organ, which is the synovium and joint lead to aggregation of these cells and hence secretion of more cytokines leading to pathological effects on cartilage, chondrocytes and synovial membranes. This migration is controlled in part by selective expression and functional regulation of cell adhesion molecules on the cell surface and vascular endothelium cells. Furthermore, the high levels of the cytokines in the peripheral blood is also due to failure to remove the autoimmune cells from the circulation.

In the research, we found low apoptotic index of the mononuclear cells which can lead to their increased numbers and consecutively secretion of cytokines with high concentration and homing of cells to the joints. We studied the apoptosis of mononuclear cells from the peripheral blood by different methods of stains to endorse the assessment. McGahon *et al.* (1995) and Maruyama *et al.* (2000), reported that the order of apoptotic features such as morphological changes or nuclear condensation is not the same in different types of cells and several methods are needed. The results are the same with the different stains and revealed low counts of apoptotic mononuclear cells.

One of the important factors regulating cell death is the cell surface receptor CD95 (sFas), a member of TNF receptor family (Scaffidi *et al.*, 1998). Stimulation of Fas receptor on activated lymphocytes can induce apoptosis. The death-inducing signal complex is formed by interaction of FADD/MORT-1 activating caspase-8 and caspase-3 which proceed the process of apoptosis (Lee and Shactes, 2001). In the present study the low level of CD95 (sFas) explain the inhibition of apoptosis. However, the high level of TNF- α in our children with JRA can explain the decreased

sFas level. Zhang *et al.* (2000) reported that stimulation of the cells with TNF- α has been shown to generate 2 signals, one that initiates programmed cell death and another that leads to activation of the transcription factor KB (NF-kB), which induces inhibition of apoptosis and promotes the production of proinflammatory factors. TNF- α strongly activates NF-kB through a second class of adaptor protein TNFR-associated factors and this transcriptional factors regulate the expression of antiapoptotic gene products (Beg and Baltimore, 1996, Van Antwerp *et al.*, 1996), such as antiapoptotic members of the Bcl-2 family and the inhibitor of apoptosis c-1AP1 and c-1AP2. Andersen *et al.* (2000) postulated that there is synergistic effect of TNF- α , IL-1 β , IFN- γ in the functional inhibition and induction of cell death.

In conclusion, in patients with JRA there is delayed apoptosis of mononuclear cells due increased level of TNF- α and decreased CD95. This leads to migration of the cells to the joints which are the target organ, infiltration of the synovium and increase secretion of proinflammatory cytokines. This lead to the articular chondrocytes and matrix changes with increased concentration of KS and decreased TGFB which augment the joint changes. So far, we recommend to add to the strategy of therapy, anti-proinflammatory cytokines and injections of TGFB, for amelioration of the disease.

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