

Nerve growth factor release by human synovial fibroblasts prior to and following exposure to tumor necrosis factor- α , interleukin-1 β and cholecystokinin-8: The possible role of NGF in the inflammatory response

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Abstract

Objective

Aim of this study was to investigate the synthesis, release and effects of nerve growth factor (NGF) in human synovial cells isolated from synovial tissue specimen from healthy and osteoarthritis (OA) patients.

Methods

Human synovial fibroblasts cultures were established starting from tissues from healthy and osteoarthritis patients. NGF protein levels in the culture medium, NGFmRNA and high-affinity NGF receptor (Tyrosine kinase A: TrkA) expression in the cells were evaluated in basal conditions and after stimulation with pro-inflammatory cytokines or with the neuropeptide cholecystokinin-8 (CCK-8). The effect of NGF supplement to culture medium on cell proliferation, TrkA expression, and tumour necrosis factor-alpha (TNF- α) and inducible-nitric oxide synthase (iNOS) production was investigated.

Results

Under basal conditions human synovial cells produce and release NGF. Both interleukin-1-beta (IL-1 β) and TNF- α , but not CCK-8 promote NGF synthesis and release from OA cells. TrkA NGF receptors are also expressed in both normal and OA synovial cells. NGF, but not IL-1 β , TNF- α and CCK-8, enhances the expression of TrkA in isolated synovial cells. NGF down-regulates IL-1 β -induced TNF- α and iNOS production by OA synovial fibroblasts.

Conclusions

NGF is produced and released and TrkA receptors are expressed in synovial inflammation. Overexpression of NGF in inflamed joints might be involved in the modulation rather than in the induction of the joint inflammatory response.

Key words

Neurotrophins, TrkA, NGF, inflammation, tissue remodelling, arthritis, cytokines.

This study was supported by grant NR. 1-2001-877 by Juvenile Diabetes Research Foundation International, and by CARISBO Foundation, Italy.

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Received on March 10, 2003; accepted in revised form on July 1, 2003.

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Introduction

In vitro and *in vivo* studies have produced a substantial body of knowledge on nerve growth factor (NGF) involvement in inflammatory responses (1, 2). Basal levels of NGF are significantly enhanced in a dose-dependent manner in cells exposed to interleukin 1 beta (IL-1) and tumour necrosis factor alpha (TNF-) two pro-inflammatory cytokines, suggesting that this molecule might participate in inflammatory response (3, 4). This hypothesis seems to be in line with the evidence that inflammatory responses is associated with an elevated local concentration of NGF (5).

Indeed, studies demonstrating that NGF is overexpressed by inflammatory cells and in inflammatory areas led to divergent interpretation ranging from the conclusion that NGF contribute and/or exacerbate the pathological manifestation of inflammatory disorder (6, 7) to the suggestion that NGF plays a role as anti-inflammatory agent (2,8). Studies on animal model carried out in our laboratory and in collaboration with other groups indicated that NGF exerts anti-inflammatory action (8-10). The hypothesis of the anti-inflammatory role of NGF is supported by evidence that joint-induced inflammation is enhanced after inhibition of endogenous NGF (9) and that the topical application of NGF promotes healing in human ocular inflammation (8). We and others have previously shown that synovial fluid and tissue from inflamed joint of human and animal model of arthritis, are characterised by an elevated concentration of NGF (11-13) and that the increase of NGF is associated with presence of inflammatory cytokines, particularly IL-1 and TNF- (14) and with an increased local expression of NGF high-affinity receptor [Tyrosine-kinase A, TrkA; (15)]. Thus it has been hypothesized that the elevated level of NGF in inflamed joint may have a role in anti-inflammatory response and be implicated in promoting tissue repair and remodelling rather than in triggering the inflammatory response (2, 9). Better knowledge of the regulation of NGF at the cellular level and the effect of NGF on synovial

fibroblasts might provide important insights to identify the mechanisms implicated in these events.

To address these questions, we used human synovial fibroblasts obtained from healthy and osteoarthritis (OA) patients. Dose-response studies were carried out to investigate the effect of pro-inflammatory cytokines IL-1 and TNF- and of the neuropeptide cholecystokinin-8 (CCK-8), which are both known to induce NGF synthesis and release (3,4, 16). We then evaluated the effect of NGF and of the above cited NGF-inducers on TrkA expression by healthy and OA synovial fibroblasts. The functional role of the NGF synthesis and release by synovial fibroblasts was investigated comparing the action of NGF and pro-inflammatory cytokines on cells proliferation and studying the production and release of inflammatory molecules – namely TNF- and inducible-nitric oxide synthase (iNOS) – by healthy and OA cells under basal conditions and after stimulation with IL-1 .

Materials and methods

Chemicals

Since NGF is a highly-conserved molecule throughout species, for these studies we used murine NGF 2.5 S, purified following the method of Bocchini and Angeletti (17). Human recombinant IL-1 and TNF- were purchased from Chemicon International (Prodotti Gianni, Milano, Italy). Sulphated and unsulphated cholecystokinin-8 (CCK-8) were purchased from Sigma Aldrich (Sigma-Aldrich S.R.L., Milan, Italy).

Cell cultures

Synovial cells were isolated from synovial tissue specimens collected during total hip prosthesis from 3 osteoarthritis patients and during arthroscopy for a late meniscal lesion from 5 healthy patients, as described by Moreschini and co-workers (18). Cells were maintained at 37°C in an humidified atmosphere of 95% air and 5% CO₂ and cultured in RPMI containing 20% heat inactivated foetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 U/ml penicillin and 10 µg/ml streptomycin. All culture cells were shown to be positive

for fibroblast specific marker ASO2 (19). No expression of the specific macrophage marker CD31 (20) has been detected in synovial culture, thus excluding possible contaminations of synovial fibroblasts by synovial macrophage. All experiments were performed with cells between the 3d and 5th passages seeded into 24-well plates. When the cells reached confluence the culture media was replaced with serum-free media for 24 hours to render the cells quiescent. For the experiments cell were stimulated with IL-1 (0.1-10 ng/ml), TNF- (0.01-1 ng/ml), CCK-8 (10^{-6} to 10^{-8} M) and/or NGF (10 and 100 ng/ml) dissolved in RPMI containing 2% FBS unless otherwise indicated (see below). All of the substances and doses were tested in five different wells of the multi-well plates ($n = 5$) unless otherwise indicated.

NGF assay

The levels of NGF were measured in cell culture media by a highly sensitive two-site enzyme-linked immunosorbent assay (ELISA) which recognises human and murine NGF and does not crossreact with brain-derived neurotrophic factor, as previously described (21).

RT-PCR-ELISA

The relative variations in NGF mRNA levels among the experimental groups were evaluated in cultured synovial fibroblasts using the semi-quantitative RT-PCR Elisa protocol described by Tirassa and co-workers (22). The Glyceraldehyde-3-Phosphate-Dehydrogenase (GAPDH) mRNA level was used to normalise for the relative differences in sample size, the integrity of the individual RNA and variations in reverse transcription efficiency. For methodological details and the primer/probe sequences see Tirassa *et al.* (22).

Immunocytochemical analysis of high affinity NGF receptor

Normal and arthritic cells, either unstimulated or stimulated for 48 hours with IL-1 (10 ng/ml) or TNF- (1 ng/ml), or NGF (100 ng/ml) or CCK-8 (10^{-6} M) were fixed with 4% paraformaldehyde dissolved in 0.1% phosphate buffer (pH 7.4) after washing in

PBS solution. The cells were centrifuged on special slides, and processed for immunofluorescence. The specific antiserum rabbit anti-human TrkA antibody (2 µg/ml, Santa Cruz, CA, USA) which does not cross-react with TrkB or TrkC was used. The specific binding of the primary antibody was detected using a fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (200 µg/ml Chemicon International Inc. CA, USA). Briefly, for immunofluorescence slides were pre-incubated with 10% BSA and 10% normal goat serum in PBS solution containing 0.1% saponin for 2 hours and then incubated overnight at 4°C with primary antibody. To assess the specificity in control slides the first antibody was replaced by purified rabbit IgG. After washing with PBS 0.1% saponin, the slides were incubated for 3 hours at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit IgG. Sections were examined under a fluorescence microscope.

Cell proliferation assay

The cells, resuspended at a concentration of 2.5×10^5 cells/ml in DMEM medium containing 5% FCS, antibiotics and fresh glutamine, were added in 100 µl/well aliquots in a 96-well sterile plate and left in the cell incubator at 37°C and 5% CO₂. After 24 h the cells had attained about 70-80% confluence and were ready to undergo the viability test by MTT assay (23). After 24 hours incubation with NGF (10-100 ng/ml), IL-1 (0.1 – 10 ng/ml) or TNF- (0.01 – 1 ng/ml), 10 µl of PBS-diluted MTT (5 mg/ml) was added to each well and the plate was incubated for 4 hours. Acid-isopropanol (0.04 N HCl in isopropanol, 100 µl/well) was added. The plates were maintained at room temperature for 5 min and then read on a microtiter plate reader (Multiskan MCC/340, Labsystem, Finland) at A570/690 nm.

TNFα assay

TNF activity in the culture media of synovial fibroblasts stimulated with IL-1 (1 ng/ml) and/or NGF (10 ng/ml) was determined by a cytotoxicity assay using the TNF-sensitive WEHI 164

clone 13 cell line as described (23). The amount of biologically active TNF- in the test samples was calculated on the basis of a human recombinant TNF-standard curve.

Western blotting analysis

For western blotting analysis of iNOS, cells stimulated with IL-1 (1 ng/ml) and/or NGF (10 ng/ml) were homogenized in sample buffer (0.01 M TRIS-HCl buffer pH 7.6, containing 0.25 M sucrose, 0.1 M NaCl, 1 mM EDTA, and 1 mM PMSF) at 4°C. After 8,000 g centrifugation for 10 min, the supernatants were used for western blotting. Samples (30 µg total protein) were dissolved with loading buffer (0.1 M TRIS-HCl buffer (pH 6.8) containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 12.5% SDS-PAGE, and electrophoretically transferred to PVDF membrane for 3 hr.

The membranes were incubated for 40 min at room temperature with blocking buffer (5% non-fat dry milk, 10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20). Membranes were washed three times for 10 min each at room temperature in TTBS (10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20) followed by an incubation for 1 hour at room temperature with rabbit anti iNOS (Cayman Chemical, Ann Arbor, MI, USA) (24) or with rabbit anti -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed three times for 10 min each at room temperature in TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signalling, Beverly, Mass., USA) as the secondary antibody. The blots were developed with ECL (Amersham Bioscience) as the chromophore. The optical density of the -actin bands was used as an internal control for differences in sample loading.

Statistical evaluation

NGF ELISA and NGFmRNA RT-PCR-ELISA data were evaluated by analysis of variance (ANOVA). Post-hoc comparisons within logical sets of means were performed using the Tukey's test. TNF- bioassay data were evaluated

by means of the unpaired t-test. A $p < 0.05$ was considered statistically significant. All the statistical evaluations were performed using the StatView package for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA) and data expressed as mean \pm S.E.M.

Results

Production and release of NGF by synovial fibroblasts

As shown in Table I, NGF levels in culture medium of OA human synovial fibroblasts were three-fold higher than in the medium from control cells. To identify potential biological mediators involved in the regulation of NGF production and release, synovial fibroblasts were cultured in the presence of different concentrations of molecules

Table I. NGF levels and NGFmRNA expression in synovial fibroblasts from healthy and osteoarthritis patients.

	NGF (pg/ml)		NGFmRNA (OD450/690)	
	Healthy	OA	Healthy	OA
Unstimulated	11.9 \pm 2.6	35.2 \pm 2.6*	1.07 \pm 0.04	0.56 \pm 0.08*
IL-1 (ng/ml)				
0.1	16.7 \pm 4.6	113.9 \pm 11.4*	0.74 \pm 0.07	3.30 \pm 0.20*
1	30.8 \pm 11.5	111.1 \pm 11.4*	1.64 \pm 0.14	2.69 \pm 0.54
10	22.9 \pm 5.3	99.9 \pm 13.1*	1.17 \pm 0.20	4.17 \pm 0.13*
TNF- (ng/ml)				
0.01	10.5 \pm 1.9	25.0 \pm 7.0	1.81 \pm 0.81	2.31 \pm 0.44
0.1	12.0 \pm 2.2	27.6 \pm 7.0	1.65 \pm 0.36	1.96 \pm 0.38
1	21.5 \pm 3.7	43.7 \pm 6.8*	1.51 \pm 0.23	2.23 \pm 0.35

NGF and NGFmRNA expression by human synovial fibroblasts under basal conditions (unstimulated) and after stimulation with different doses of recombinant human IL-1 or recombinant human TNF-. NGF levels in the culture media are expressed as pg/ml. NGFmRNA data are expressed as normalized 450/690 nm optical density (O.D.450/690; see Materials and Methods section for details). Data are expressed as mean \pm S.E.M. * $p < 0.05$ when OA cells are compared with normal cells.

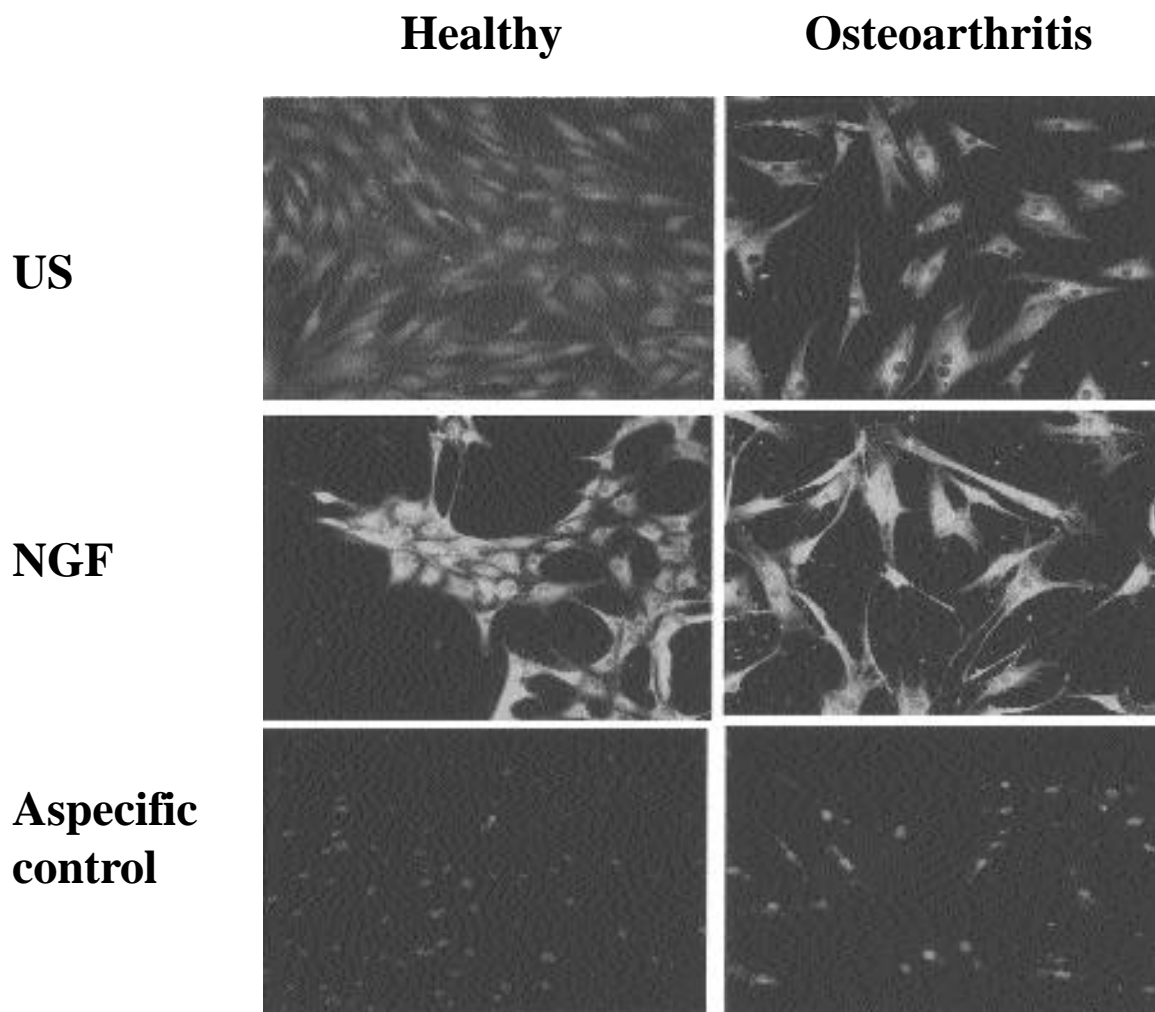


Fig. 1. Expression of NGF high-affinity receptor (TrkA) in synovial fibroblasts from healthy and OA patients. The fluorescent immunocytochemistry was performed under basal (unstimulated) conditions (US) and then after NGF was added to the culture medium (see M&M section for details). Incubation with non-specific rabbit serum was used as the internal control (Aspecific Control). Magnification: X400.

that are known to promote NGF synthesis such as IL-1, TNF- α or CCK-8.

Effect of IL-1 β , TNF- α or CCK-8 on NGF production by synovial fibroblasts

As reported in Table I, the dose-response study showed that IL-1 induced a significant increase in NGF release into the culture medium from arthritic cells at concentrations of 0.1 – 10 ng/ml. Likewise, a significant increase of NGF-mRNA was detected in arthritis synovial fibroblasts when IL-1 was added to the culture medium (Table I).

TNF- α (range 0.01–1 ng/ml) was able to stimulate release of NGF protein at the highest concentration used (Table I). No significant changes in NGF-mRNA were induced by TNF- α in both normal and arthritic synovial fibroblasts (Table I).

CCK-8 has been shown to stimulate NGF synthesis and be implicated in the joint inflammatory response (9). To further characterise the role of NGF, normal and OA synovial cells were exposed to different concentrations of CCK-8. No significant difference in NGF protein and NGF gene expression by normal and OA cells was observed (data not shown) in normal or OA cells exposed to CCK-8 at concentrations ranging from 10^{-6} to 10^{-8} M.

NGF-receptor expression by synovial fibroblasts

To explore whether synovial fibroblasts are receptive to the action of NGF, cells were fixed and immunostained for presence of TrkA. As indicated in Figure 1, immunoreactivity for TrkA was detected in both unstimulated (US) normal cells and, to a more marked extent, in US arthritic cells. IL-1, TNF- α and CCK-8 were unable to induce the expression of TrkA in normal and OA cells at the concentration used (data not shown). On the contrary, TrkA expression was enhanced after exposure of normal and OA synovial fibroblasts to NGF (Fig. 1).

Effect of IL-1 β /TNF- α and NGF on synovial fibroblasts survival

To investigate the possible functional

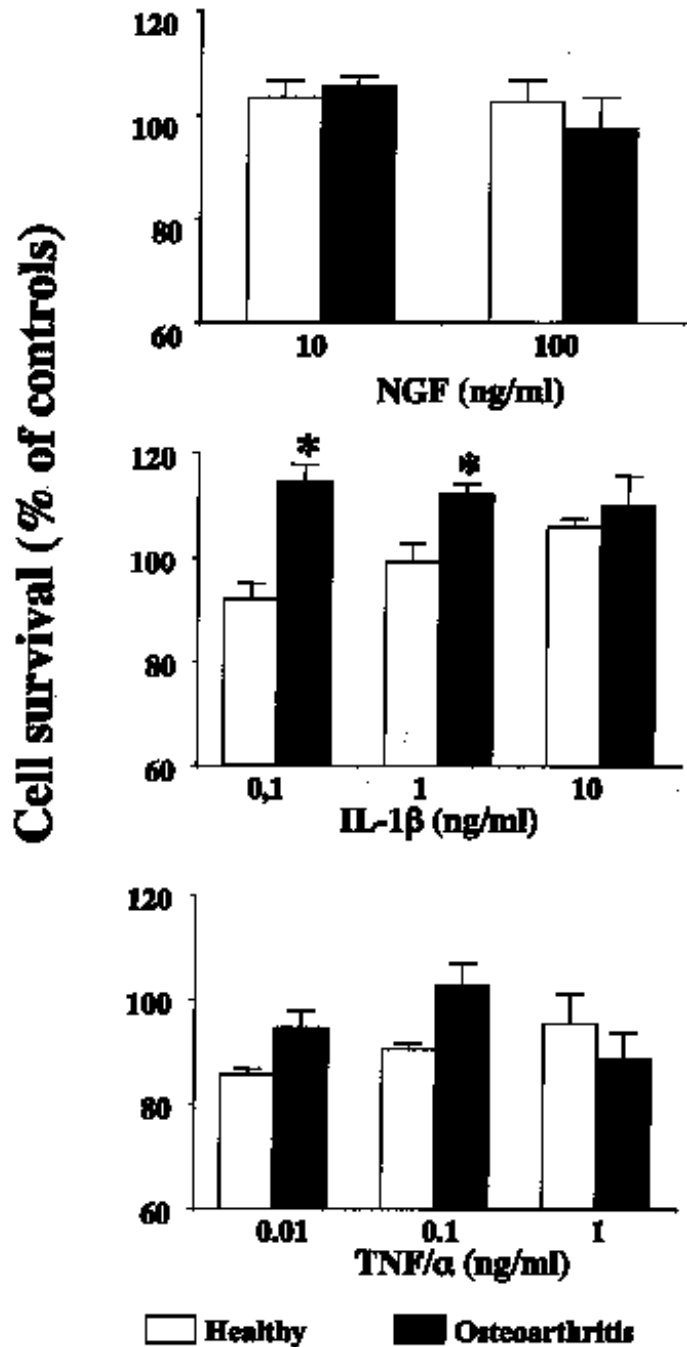


Fig. 2. Quantification of normal and OA cell survival after exposure to different doses of NGF, IL-1 or TNF- α . Data (mean \pm S.E.M) are presented as percent of controls. * p < 0.05 versus unstimulated (control) cells.

role of NGF, synovial fibroblasts were cultured in presence of different concentration of NGF, IL-1 or TNF- α . As illustrated in Figure 2A, NGF (10 and 100 ng/ml) treatment did not induce any significant variation in cell viability in either normal or OA synovial fibroblasts, suggesting that NGF at

these concentration neither stimulated cell proliferation nor promoted cell death. IL-1 (Fig. 2B) at the dose of 0.1 ng/ml induced a minor increase in cell viability when added to the medium of normal synovial fibroblasts, while the effect on OA cell viability was evident starting at a concentration

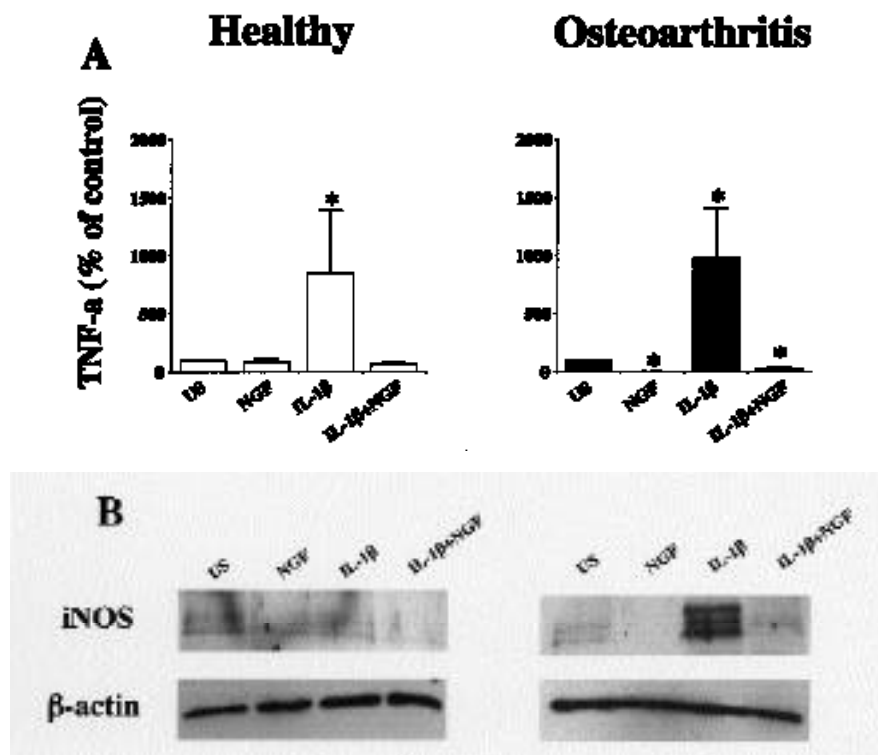


Fig. 3. TNF- α (A) and iNOS (B) expression in normal and OA synovial fibroblasts under basal conditions (US) and after stimulation with IL-1 β and/or NGF.

of 0.1 ng/ml and was more significant at a concentration of 1 ng/ml. As shown in Figure 2C, TNF- α decreased the viability of normal cells at the concentration of 0.01 ng/ml while it had no significant effect on arthritis synovial fibroblasts.

NGF-mediated modulation of TNF- α and iNOS

Treatment with NGF alone induced a decrease in the basal expression of TNF- α in arthritic but not in healthy cells (Fig. 3A). Both healthy and arthritic cells cultured in the presence of IL-1 β (1 ng/ml) released a statistically significant amount of TNF- α into the culture medium. This effect was downregulated in OA synovial fibroblasts if the cells were cultured in the presence of 10 ng/ml of NGF. As showed in Figure 3B, NGF reduced the basal expression of iNOS in both healthy and arthritic synovial fibroblasts. IL-1 β did not exert significant effects on iNOS expression in normal synovial fibroblasts, while it induced an overexpression of iNOS in arthritic cells. The effect of IL-1 β on the over-

expression of iNOS was markedly reduced (Fig. 3B) if arthritic cells were cultured in the presence of NGF.

Discussion

There are a large number of studies showing that NGF, a neuroimmune mediator, increases in many types of inflammation and can play a important role linking the response of the immune and nervous system during inflammation (5, 24). Findings published in recent years indicate that synovial cells and synovial fluid from joint tissue with inflammatory arthritis in humans and in animal models express elevated concentrations of NGF (11, 13). Our data show that OA synovial cells produce and release more NGF than normal synovial cells both under basal conditions and after stimulation with pro-inflammatory molecules. This finding extends to an acute inflammatory condition the concept of a role for NGF previously advocated in chronic joint inflammation (11, 14), in inflammatory joint diseases both in experimental models and in humans. However, the role played by NGF in

this inflammatory response is still unclear. Since inflammatory responses are commonly associated with a local increase in IL-1 and TNF- α (25) and since these pro-inflammatory cytokines are known to promote local NGF synthesis (12), we investigated whether these cytokines are implicated in NGF up-regulation by synovial fibroblasts. Our findings showed that under basal conditions, OA cells produce and release a greater amount of NGF than that produced by normal cells and that IL-1 more than TNF- α stimulate not only NGF protein, but also its mRNA expression by OA synovial cells, suggesting that NGF is locally produced. These findings are consistent with previous *in vivo* data indicating that the inflamed joint is characterised by elevated levels of NGF (1) and that resident cells produce NGF after stimulation with pro-inflammatory cytokines (12).

Since the neuropeptide CCK-8 is able to promote NGF synthesis in the central and peripheral nervous systems (26, 27), we investigated whether this neuropeptide was also implicated in the regulation of NGF production by synovial cells. The result showed that CCK-8 has no effect on the NGF protein concentration and NGF gene expression. Why CCK-8 is able to induce NGF synthesis in the brain and peripheral tissues, but not in human synovial fibroblasts is not known. One possible explanation is that CCK receptors are not expressed by synovial fibroblasts. This hypothesis seems to be consistent with preliminary *in vivo* observations showing that these cells do not express CCK receptors (Manni *et al.* unpublished).

To investigate the possible functional correlation between NGF and NGF-receptor presence in human synovial fibroblasts, stimulated and unstimulated cells were immunostained for localisation of TrkA. Our studies showed that normal and OA synovial fibroblasts express TrkA, indicating that these cells are receptive to the action of NGF and that they may require this molecule for survival and functioning. The results also showed that IL-1 β and TNF- α stimulated NGF synthesis but

failed to enhance the expression of high-affinity NGF-receptor, while the addition of exogenous NGF to cultured synovial cells enhanced the expression of TrkA receptor. This differential effect indicates that the amount of endogenous NGF induced by IL-1 and TNF- might be not sufficient to stimulate NGF-receptor expression. The fact that the amount of NGF stimulated by these cytokines is significantly lower than 100 ng/ml, which is the amount of NGF added to cultured synovial fibroblasts to stimulate the upregulation of TrkA expression, supports this hypothesis. One important question raised by the present observations is the functional role of NGF synthesis and release by synovial cells under normal and pathological conditions and whether NGF plays a pro- or anti-inflammatory role in the pathogenesis of joint inflammation.

It has been shown that dysregulation of cell proliferation/death controlling mechanisms induced by pro-inflammatory cytokines might play a central role in the pathogenesis of arthritis (28). Our studies clearly showed that NGF fails and IL-1 promotes synovial cell proliferation. These results confirm previous findings indicating that IL-1 affect synovial cell viability by increasing the percentage of living cells (28) suggesting that NGF and IL-1 may mediate different actions on human synovial fibroblasts. Different effects were also observed after stimulation of normal and arthritic cells with IL-1 and/or NGF on TNF- and iNOS production and release. Indeed, exposure to NGF reduced and/or blocked the action of IL-1 on TNF- and iNOS, two well known inflammatory mediators (25). It is known that IL-1, TNF-

and iNOS play a crucial role in inflammatory arthritis (25, 29) and that IL-1 stimulates NGF synthesis in knee joint of animal models of arthritis (12). Our findings, obtained on isolated synovial cells, support the hypothesis that the effect of IL-1 on NGF synthesis and release is not associated with a pro-inflammatory response but most probably to other mechanisms. Previous studies showed that NGF exerts a protective action on autoimmune

inflammatory disorders of the CNS (30, 31), ocular surface (8) and isolated inflammatory cells (10). Our study clearly indicating that NGF is able to counteract the expression of pro-inflammatory markers by isolated human synovial fibroblasts and that it does not affect cell proliferation, implies that NGF does not promote joint inflammation. It also provide evidence supporting previous *in vitro* and *in vivo* findings suggesting that NGF, at least under certain conditions, exerts an anti-inflammatory action (8, 10, 30) and/or participates in the process of tissue repair and remodelling (32).

Acknowledgements

Authors would like to thank Dr. Daniela Merlo for help and suggestions about the Western Blotting analysis.

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