Effect of cytokines on hyaluronan synthase activity and response to oxidative stress by fibroblasts

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ABSTRACT

Cytokines such as tumour necrosis factor-α (TNFα), interferon-γ (IFNγ), and transforming growth factor-β (TGF1β) modulate hyaluronan synthase (*HAS*) gene expression and protein activity. The aim of this research is to evaluate the response of *HAS* gene expression and the related protein synthesis in fibroblasts after treatment with TNFα, IFNγ and TGF1β and to assess the potential protective effect of increased hyaluronan (HA) synthesis during oxidative stress. In this study, gene expression, protein synthesis, hyaluronan content, cell death, lactate dehydrogenase (LDH) activity, membrane lipid peroxidation and endogenous antioxidant depletion are determined for HAS1, HAS2 and HAS3. Messenger RNA (mRNA) expression and protein formation of the three *HAS* genes is modulated using different cytokines and various doses and correlated with increased HA synthesis. Protection of fibroblasts from injury induced by exposure to reactive oxygen species was significantly increased by TGF1β and was associated with increased gene expression and protein formation of HAS1 and HAS2 enzymes synthesising high-molecular-weight HA. It is proposed that specific HAS enzyme activity and HA molecular weight specificity is involved in the protective mechanism.

KEY WORDS: Hyaluronan synthases.
Antioxidants.
Cytokines.
Oxidative stress.
Fibroblasts.

Introduction

Previous findings suggest that the generation of free radicals and other reactive oxygen species (ROS) may play multiple roles in physiological and pathological states in biological organisms.[1] Reactive oxygen species are thought to be involved in a number of widely occurring human pathologies. They are produced in aerobic mammalian cells physiologically and the main source is localised in the mitochondria, although several enzymes may also generate free radicals.

Glycosaminoglycans (GAGs) are linear polysaccharides built from repeated disaccharide units composed of hexosamine and, with the exception of keratan sulphate, hexuronic acid. With the exception of hyaluronan (HA), GAGs are sulphated to differing degrees and linked covalently to protein to give proteoglycans (PGs). Hyaluronan is a fundamental constituent of the extracellular matrix (ECM) and plays an essential part in regulating the biological dynamic status of cell migration, proliferation, adhesion, development and differentiation.[2]

It is reported that high levels of GAGs are found after free radical injury.[3] As GAGs possess antioxidant activity, the addition of HA and other GAGs are able to protect cells from oxidative stress. Positive outcomes have been obtained using both commercial HA and purified plasma GAGs of human origin.[4–6]

Hyaluronan plays several roles in the activation and modulation of the inflammatory response, including the antioxidant scavenging of ROS derived from polymorphonuclear leucocytes (PMNs) and other sources.[5,7–10] Hyaluronan and other GAG structures, such as chondroitin sulphates and heparan sulphate, are seen commonly in human plasma.[11] A marked increase in plasma HA levels has been observed in many diseases, especially those involving free-radical damage.[12–17] This excess production of GAGs could be primed by cells in an attempt to reduce free-radical injury, a biological cellular response that seeks to reduce the damage produced by oxidative stress.

Hyaluronan is synthesised on the inner surface of the plasma membrane by three related isoenzymes, HAS1, HAS2 and HAS3.[18] Each isoform is capable of synthesising HA molecules of a given size and exhibits different kinetic properties plus specific cell-type characteristics.[19] It has recently been reported that many cytokines (e.g., interleukins, transforming growth factor-β [TGF1β], tumour necrosis factor-α [TNFα], interferon-γ [IFNγ]) are able to modulate HAS transcription in cell cultures.[20–23]

Mononuclear cells are important regulators of fibroblast proliferation and collagen biosynthesis. Mononuclear cells that predominate in inflammatory cell infiltrate are also noted after many injuries and in many fibrotic disorders. This finding led to speculation that mononuclear cells may also be important regulators of GAG biosynthesis. Support for this hypothesis has already come from past studies showing that supernatants from activated mononuclear cells stimulate fibroblast GAG biosynthesis.[24–25] The same concept may be extended to growth factors, as it is well known that

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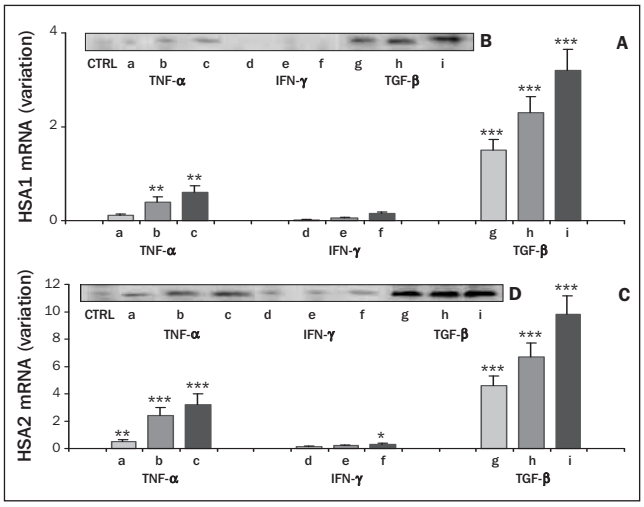

Fig. 1. Effect of cytokine treatment on fibroblast HAS1 (A) and HAS2 (C) mRNA expression and related protein production (B, D). At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β each at 1, 2 and 4 ng/mL. After 5-h incubation, fibroblasts were recovered for HAS1 and HAS2 evaluation. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and i) TGF-1β (4 ng/mL). Values are mean±SD of seven experiments and are expressed as the variation with respect to the controls (A and B) and Western blot analysis (C and D) for the HAS1 and HAS2 protein levels ($^{*}P<0.05$, $^{**}P<0.005$ and $^{***}P<0.001$ *vs*. control).

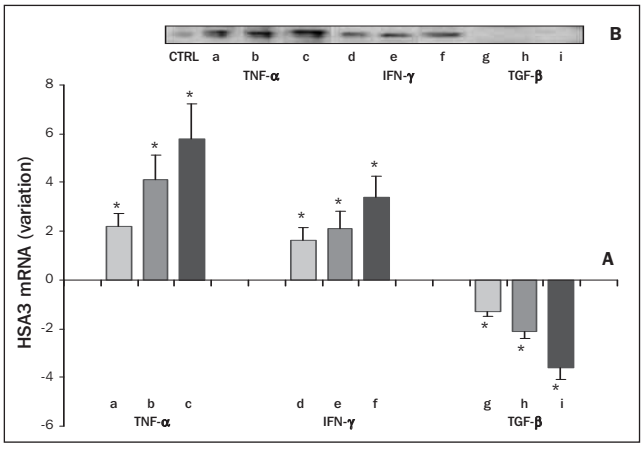

Fig. 2. Effect of cytokine treatment on fibroblast HAS3 mRNA expression (A) and related protein production (B). At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β each at 1, 2 and 4 ng/mL. After 5-h incubation, fibroblasts were recovered for HAS3 evaluation. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and i) TGF-1β (4 ng/mL). Values are the mean±SD of seven experiments and are expressed as the variation with respect to the control (A) and Western blot analysis (B) for the HAS3 protein levels ($^{*}P<0.001$ *vs*. CTRL).

their levels increase during cell development and differentiation.[26]

Activated mononuclear cells secrete IFNs,[27] TNF and lymphotoxin.[28] Interferon-γ and IFNβ regulate fibroblast growth and collagen biosynthesis.[27] Tumour necrosis factor and lymphotoxin have a wide range of cytoregulatory effects, including an ability to modulate fibroblast proliferation.[27–28] Recently, these results have been clarified by various studies, as these cytokines may modulate HA production by inducing HA synthases.[19–21,23,29,30] In analogous experiments using platelet-derived growth factor-BB (PDGF-BB) and TGF1β),[22,31,32] endogenous HA levels were correlated with *HAS* gene expression. However, the manner in which the three HAS were induced differed and was specific to cytokine and cell type.[20,23,29–32]

The aim of this study is to evaluate the activity of HA synthases and the variation in HA levels in normal and stressed fibroblasts after TNFα, IFNγ and TGF-1β treatment. The protective effects of the increased endogenous HA concentration obtained using cytokine stimulation in fibroblasts undergoing oxidative stress is also investigated, and the size of the newly synthesised HA chains is measured.

In a previous study,[23] we looked at the effects of concomitant treatment with cytokines and oxidants on HAS messenger RNA (mRNA) expression, as well as HA concentration after treatment. Tumour necrosis factor-α, IFNγ and interleukin (IL)-1β were used at concentrations of 5, 10 and 20 ng/mL. In the present study we use lower concentrations of the cytokines, again using TNFα, IFNγ and TGF1β (instead of IL-1β), and a longer incubation period of 5 h rather than 3 h. The reason for selecting TGF1β was suggested by previously reported data.[20,22,31,32] The use of lower cytokine concentrations is prompted by the need to reduce other possible physiological effects due to this treatment.

Materials and methods

Materials

Recombinant human TNFα, IFNγ and TGF1β obtained from *Escherichia coli* were purchased from Peprotech (Rocky Hill, NJ, USA). Goat anti-HAS1, HAS2 and HAS3 polyclonal antibodies and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypsin-EDTA solution and phosphate-buffered saline (PBS) were obtained from Gibco BRL (Grand Island, NY, USA). All cell culture plastics were obtained from Falcon (Oxnard, CA, USA). Ascorbic acid, iron (II) sulphate, sucrose, ethylenediaminetetraacetic acid (EDTA), potassium phosphate, butylated hydroxytoluene (BHT), dichloromethane (DCM), trypan blue, RNase, proteinase K, protease inhibitor cocktail, sodium dodecyl sulphate (SDS) and all other general laboratory chemicals were obtained from Sigma-Aldrich (Milan, Italy).

Cell culture

Normal human dermal fibroblasts (type DPK-SKDF-H) were obtained from Dominion Pharmakine (Bizkaia, Spain). Fibroblasts were cultured in 75 cm^2 plastic flasks containing DMEM supplemented with 10% FBS, L-glutamine (2 mmol/L) and penicillin/streptomycin (100 units/mL, 100 µg/mL) and incubated at 37°C in humidified air with 5% CO_2.

Induction of oxidative stress

Fibroblasts were cultured in six-well culture plates at a density of 1.3 x 10^5 cells/well. Twelve hours after plating

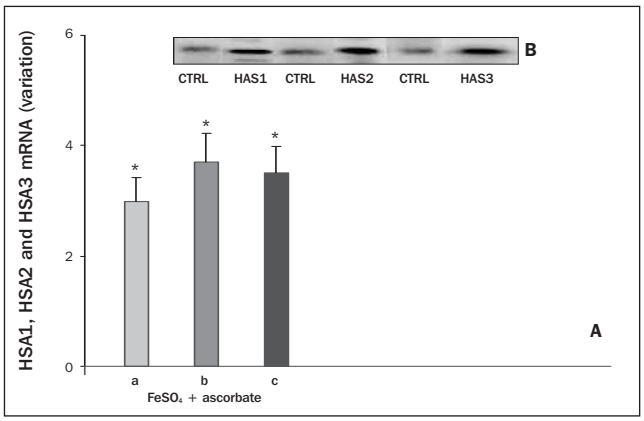

Fig. 3. Effect of $FeSO_4$ plus ascorbate on fibroblast HAS1, HAS2 and HAS3 mRNA expression (A) and related protein production (B). At time 0 fibroblasts were exposed to $FeSO_4$ plus ascorbate and incubated for 5 h. The cells were recovered for HAS mRNA expression. a) HAS1, b) HAS2, c) HAS3. Values are mean±SD of seven experiments and are expressed as the variation with respect to the control (A) and Western blot analysis (B) for HAS1, HAS2 and HAS3 protein levels ($^{*}P<0.001$ *vs*. control).

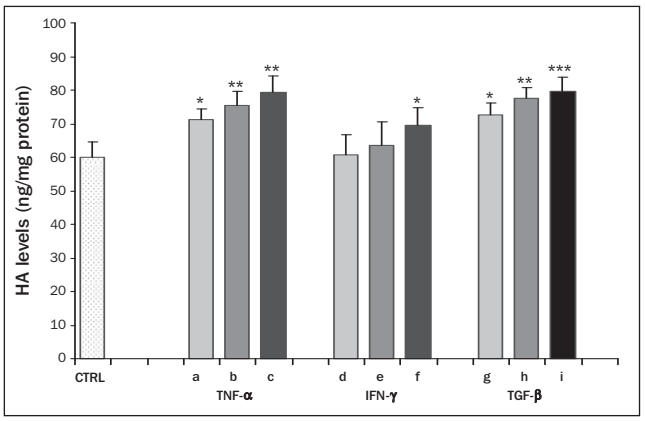

Fig. 4. Effect of cytokine treatment on fibroblast HA production. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL) incubated for 48 h. Then fibroblasts were recovered for HA level quantitation. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and i) TGF-1β (4 ng/mL). Values are mean±SD of seven experiments ($^{*}P<0.05$, $^{**}P<0.005$ and $^{***}P<0.001$ *vs*. control).

(time 0), when cells were firmly attached to the substratum (about 1 x 10^5 cells/well), the culture medium was replaced with 2 mL fresh medium containing TNFα or IFNγ or TGF1β, each at concentrations of 1, 2 and 4 ng/mL. After 5-h incubation, fibroblasts were recovered for HA synthase evaluation.

Another set of plates was treated with the three cytokines, each at the three concentrations above, and incubated for 48 h. Fibroblasts were recovered for HA quantitation and the analysis of the other biochemical parameters.

A further set of plates was treated with the three cytokines and 24-h later oxidative stress was induced in the cells by the addition of 10 µL 200 µmol/L $FeSO_4$ to each well (final concentration 1 µmol/L). After 15 min, 10 µL 100 mmol/L ascorbic acid was added for free radical production.[33] Then, 48-h later, the cells were subjected to morphological and biochemical evaluation.

Finally, a separate set of plates was exposed to $FeSO_4$ plus ascorbate and incubated for 5 h in order to evaluate the effect of oxidative stress on HAS mRNA expression.

RNA isolation, cDNA synthesis and real-time quantitative PCR amplification

For real-time reverse-PCR (RealTime PCR system, Mod. 7500, Applied Biosystems, USA) analysis of HAS1, HAS2 and HAS3, total RNA was isolated from 4–5 x 10^6 cells using the Omnizol reagent Kit (Euroclone, West York, UK). The first strand of complementary DNA (cDNA) was synthesised from 1 µg total RNA using a high-capacity cDNA archive kit (Applied Biosystems, USA). β-actin mRNA was used as an endogenous control to allow the relative quantification of HAS mRNAs.[34]

The real-time PCR method was performed with ready-to-use assays (Assays on Demand, Applied Biosystems) for both target and endogenous controls. The amplified PCR products were quantified by measuring the HAS and β-actin mRNA calculated cycle thresholds (C_T). The C_T values were plotted against log input RNA concentration in serially diluted total RNA of fibroblast samples and used to generate standard curves for all mRNAs analysed.

The amount of specific mRNA in samples was calculated from the standard curve and normalised with the β-actin mRNA. After normalisation, the mean normal fibroblast target levels provided the calibrator (one per sample) and the results were expressed as normalised difference relative to the normal controls (relative expression levels).

Western blot assay of HAS1, HAS2 and HAS3 proteins

For sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting, the cells were washed (x2) in ice-cold PBS and subsequently dissolved in SDS sample buffer (62.5 mmol/L Tris/HCl, [pH 6.8], 2% [w/v] SDS, 10% glycerol, 50 mmol/L dithiothreitol, 0.01% [w/v] bromophenol blue). Whole cell protein extracts (10–25 µL/well) were separated on a mini gel (10%). The proteins were blotted on to polyvinylidene difluoride membranes (Amersham Biosciences) using a semi-dry apparatus (Bio-Rad). The blots were flushed with double-distilled H_2O, dipped into methanol and then dried for 20 min.

Subsequently, the blots were transferred to a blocking buffer solution (1× PBS, 0.1% Tween 20, 5% [w/v] non-fat dried milk) and incubated for 1 h. The membranes were then incubated with the specific diluted primary antibody in 5% bovine serum albumin, 1× PBS and 0.1% Tween 20 at 4°C overnight in a roller bottle.

After being washed in three stages in wash buffer (1× PBS, 0.1% Tween 20), the blots were incubated with the secondary polyclonal goat anti-rabbit antibody conjugated with peroxidase in TBS/Tween-20 buffer containing 5% non-fat dried milk. After 45 min of gentle shaking, the blots were washed (x5) in wash buffer and the proteins were made visible using a UV/visible transilluminator (EuroClone, Milano, Italy) and Kodak BioMax MR film.

Hyaluronan assay

Hyaluronan analysis was performed in the supernatant of 4–5 x 10^5 cell samples obtained 48 h after cytokine treatment. Hyaluronan levels were analysed as a product of HAS

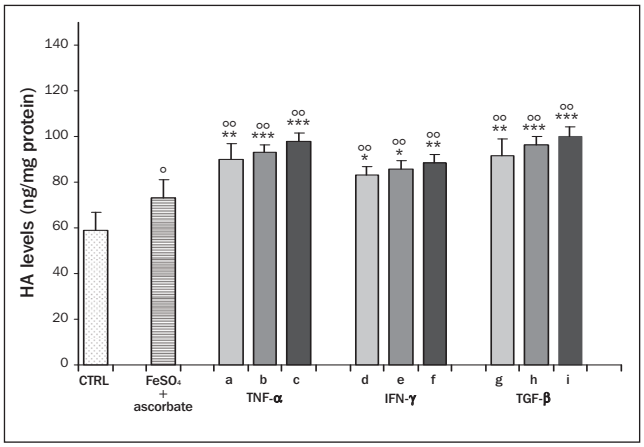

Fig. 5. Effect of cytokine treatment 24 h before oxidative stress induction on fibroblast HA levels. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL). After 24 h, oxidative stress was induced, and 48 h later cells were recovered for HA analysis. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and i) TGF-1β (4 ng/mL). Values are mean±SD of seven experiments ($^{*}P<0.05$, $^{**}P<0.005$ and $^{***}P<0.001$ *vs*. $FeSO_4$ + ascorbate. $^{\circ}P<0.05$ and $^{\circ\circ}P<0.001$ *vs*. control).

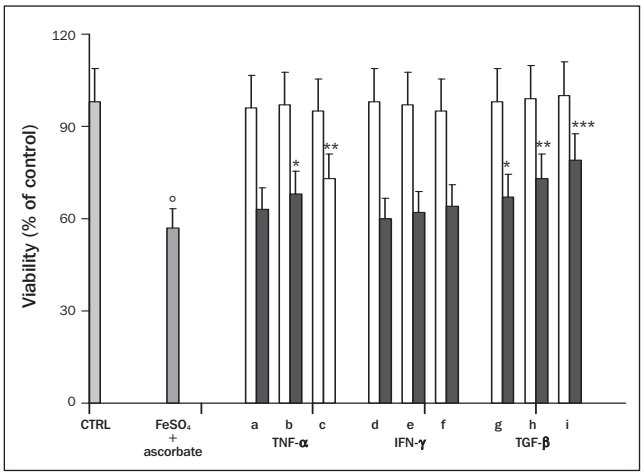

Fig. 6. Effect of cytokine treatment 24 h before oxidative stress induction on fibroblast survival. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL). After 24 h, oxidative stress was induced, and 48-h later cells were subjected to morphological evaluation. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and i) TGF-1β (4 ng/mL). White bars are related to cells treated with cytokines only, while black bars are related to cells treated with cytokines and then exposed to $FeSO_4$ + ascorbate. Values are mean±SD of seven experiments ($^{*}P<0.05$, $^{**}P<0.005$ and $^{***}P<0.001$ *vs*. $FeSO_4$ + ascorbate. $^{\circ}P<0.001$ *vs*. control).

activity. The assay was carried out using a specific enzyme-linked binding protein assay test kit (Corgenix, Cambridgeshire, UK).

In brief, samples were added to a microtitre well together with primary antibody and incubated at room temperature (RT) for 1 h. After washing, a secondary antibody conjugated with peroxidase (HRP-conjugated) was added to the well and incubated at RT for 30 min. Subsequently, after adding a one-component substrate solution (3,3′,5,5′-tetramethylbenzydine plus hydrogen peroxide) and a further incubation period (RT for 30 min), the reaction was stopped with 0.36 N H_2SO_4 and absorbance (*A*) was read at 450 nm using a microplate reader (DASI, Rome, Italy). The concentration of HA in each sample was determined by interpolation from a standard curve ranging from zero to 800 ng/mL.

Cell viability assay

Forty-eight hours after cytokine treatment in normal fibroblasts and 24 h after $FeSO_4$ plus ascorbate exposure in stressed fibroblasts, cell viability was determined under a photozoom inverted microscope (Optech, Munchen, Germany) connected to a digital camera (Coolpix 4500, Tokyo, Japan). The number of viable cells was quantified using the trypan blue dye exclusion test from several randomly chosen areas of each well.[35]

Lactate dehydrogenase assay

Forty-eight hours after cytokine administration, the cell medium was collected, centrifuged at 10,000 x*g* for 10 min at 4°C and frozen at –80°C until assay. In order to estimate total lactate dehydrogenase (LDH), cells (4–5 x 10^6) were also collected in 500 µL PBS and sonicated in Triton X-100 after centrifugation at 500 x*g* for 5 min at 4°C. Lactate dehydrogenase evaluation was performed using a partly modified published method.[36]

In brief, 50 µL of thawed sample was mixed with 100 µL 2 mmol/L reduced nicotinamide adenine dinucleotide (NADH) and 850 µL 20 mmol/L phosphate buffer (pH 7.4). After mixing for 5 sec, identical aliquots (200 µL) of each sample were placed in 96-well plates at RT and the reaction was initiated by the addition of 20 µL 3.3 mmol/L sodium pyruvate. The rate of disappearance of NADH was measured at 340 nm using a plate reader (DAS). Percentage release was determined by dividing LDH activity in the medium by total LDH activity and multiplying by 100.

Lipid peroxidation

Measurement of malondialdehyde (MDA) was performed to estimate lipid peroxidation in fibroblast lysates obtained 48 h after cytokine treatment. Cells (4–5 x 10^6) were collected in 500 µL PBS containing 200 µmol/L butylated hydroxytoluene (BHT) and stored at –80°C until required. After thawing, samples were centrifuged at 500 x*g* for 5 min at 4°C and the cell pellet was resuspended and sonicated in 250 µL sterile H_2O. Lipid peroxidation was evaluated using a commercial kit (Bioxytech MDA-586, Oxis Research, Portland, OR, USA), in accordance with the manufacturer's instructions.

Briefly, 0.65 mL 10.3 mmol/L N-methyl-2-phenyl-indole in acetonitrile was added to 0.2 mL of the sonicated pellet. After vortex-mixing for 3–4 sec, the sample was acidified with 0.15 mL 37% HCl and incubated at 45°C for 60 min. The samples were cooled rapidly on ice and *A* measured at 586 nm. The concentration of MDA in cell lysates was determined against a standard curve and expressed as nmol/mg protein.

Superoxide dismutase activity

Fibroblasts (4–5 x 10^6), obtained 48 h after cytokine

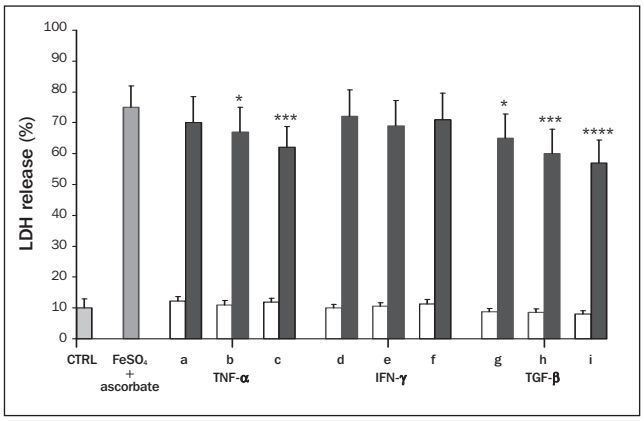

Fig. 7. Effect of cytokine treatment 24 h before oxidative stress induction on fibroblast LDH release. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL). After 24 h, oxidative stress was induced, and 48-h later culture cell medium was recovered for LDH evaluation. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and i) TGF-1β (4 ng/mL). White bars are related to cells treated with cytokines only, while black bars are related to cells treated with cytokines and then exposed to $FeSO_4$ + ascorbate. Values are mean±SD of seven experiments ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ and $^{****}P<0.001$ *vs*. $FeSO_4$ + ascorbate. $^{\circ}P<0.001$ *vs*. control).

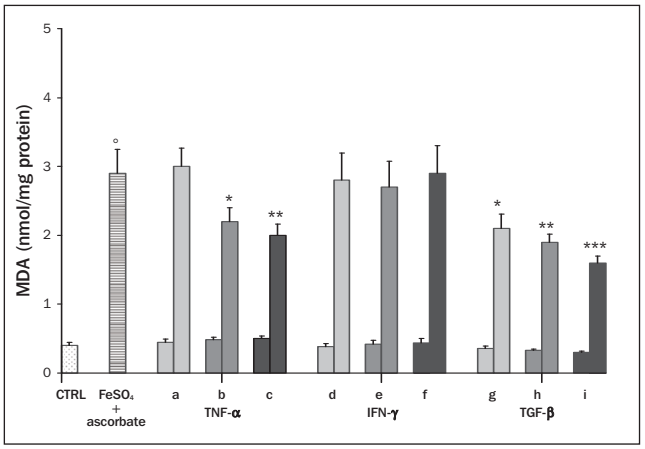

Fig. 8. Effect of cytokine treatment 24 h before oxidative stress induction on fibroblast MDA levels. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL). After 24 h, oxidative stress was induced, and 48-h later cells were recovered for MDA evaluation. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and. i) TGF-1β (4 ng/mL). White bars are related to cells treated with cytokines only, while black bars are related to cells treated with cytokines and then exposed to $FeSO_4$ + ascorbate. Values are mean±SD of seven experiments ($^{*}P<0.01$, $^{**}P<0.005$ and $^{***}P<0.001$ *vs*. $FeSO_4$ + ascorbate. $^{\circ}P<0.001$ *vs*. control).

treatment, were collected in 500 µL PBS and centrifuged at 1000 x*g* for 5 min at 4°C. The pellet was resuspended in 250 µL ice-cold 0.25 mol/L sucrose containing 1 mmol/L diethylenetriamine pentaacetic acid and then sonicated. After centrifugation at 6000 x*g* for 20 min at 4°C, the supernatant was collected and total superoxide dismutase (SOD) activity was determined at 505 nm using a commercial kit (Ransod, Randox Laboratories, Crumlin, UK).

In brief, 50 µL diluted samples (1:10 [v:v] with 0.01 mol/L potassium phosphate buffer [pH 7.0]) were mixed with 1.7 mL of a solution containing 0.05 mmol/L xantine and 0.025 mmol/L iodonitrotetrazolium chloride. After mixing for 5 sec, 250 µL xantine oxidase solution (80 units/L) was added. Initial *A* was read and the final *A* was read after a further 3 min. Superoxide dismutase values were expressed as units/mg protein.

Reduced glutathione assessment

Fibroblasts (4–5 x 10^6) obtained 48 h after cytokine treatment were collected in 500 µL PBS and stored at –80°C. Biochemical analysis was performed using a specific colorimetric assay (Bioxytech GSH-400, OxisResearch). After thawing, cell samples were centrifuged at 2500 x*g* for 5 min at 4°C. The pellet was resuspended and sonicated in 500 µL 5% metaphosphoric acid (MPA) at 4°C. Each sample was then mixed and centrifuged at 3000 x*g* for 10 min at 4°C. A sample (0.2 mL) of each supernatant was added to 0.7 mL potassium phosphate buffer containing diethylenetriamine pentaacetic acid and lubrol in a polyethylene tube. After vortex-mixing, 50 µL 4-chloro-1-methyl-7-trifluoromethyl-quinolinum methylsulphate in HCl was added. The samples were vortex-mixed again and 50 µL 30% NaOH was added. After vortex-mixing, the samples were incubated in the dark for 10 min at 25°C. The *A* was then read at 400 nm. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of reduced glutathione (GSH). The amount of fibroblast GSH was expressed in nmol/mg protein.

Hyaluronan size analysis

The HA size was evaluated on agarose gel (1%, 1xTAE buffer, 30 V) using a previously published method.[37] Each sample (5 mL) was first dried in a vacuum drier (CentriVap Console, Labconco) and the pellet was resuspended with 10 µL buffered solution[37] and then loaded per lane. Defined DNA ladder (100 bp, Stratagene) and HA molecular weights (100 kDa, 500 kDa and 2000 kDa) were also run as standards. Bands were then stained with Stains-All dye (0.005% [w/v] in ethanol). After electrophoresis, the gel was removed and illuminated under a digital camera (Coolpix 4500) in a darkroom. Data were acquired and processed by the computer using a specific data acquisition program (Nikon View 5).

Protein analysis

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad, Richmond, CA, USA) and a published method.[38] Bovine serum albumin was used as the standard.

Statistical analysis

Data are expressed as mean±SD of at least seven experiments for each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Statistical significance was set at $P<0.05$.

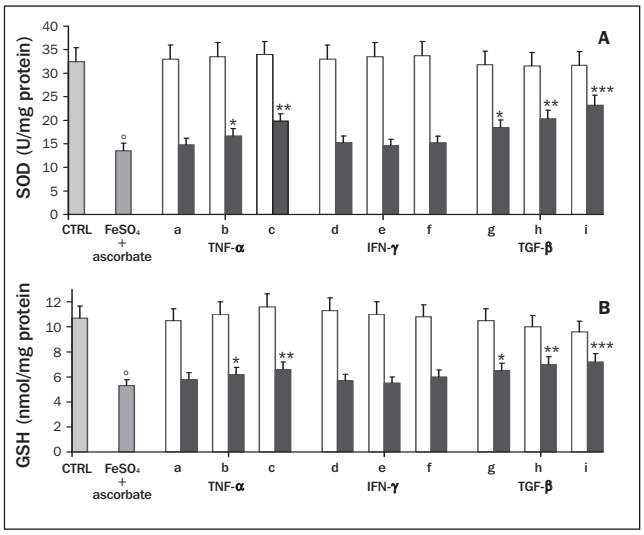

Fig. 9. Effect of cytokine treatment 24 h before oxidative stress induction on fibroblast SOD (A) and GSH (B) depletion. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL). After 24 h, oxidative stress was induced, and 48-h later cells were recovered for SOD and GSH evaluation. a) TNF-α (1 ng/mL), b) TNF-α (2 ng/mL), c) TNF-α (4 ng/mL), d) IFN-γ (1 ng/mL), e) IFN-γ (2 ng/mL), f) IFN-γ (4 ng/mL), g) TGF-1β (1 ng/mL), h) TGF-1β (2 ng/mL) and i) TGF-1β (4 ng/mL). White bars are related to cells treated with cytokines only, while black bars are related to cells treated with cytokines and then exposed to $FeSO_4$ + ascorbate. Values are mean±SD of seven experiments ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$ *vs*. $FeSO_4$ + ascorbate. $^{\circ}P<0.001$ *vs*. control).

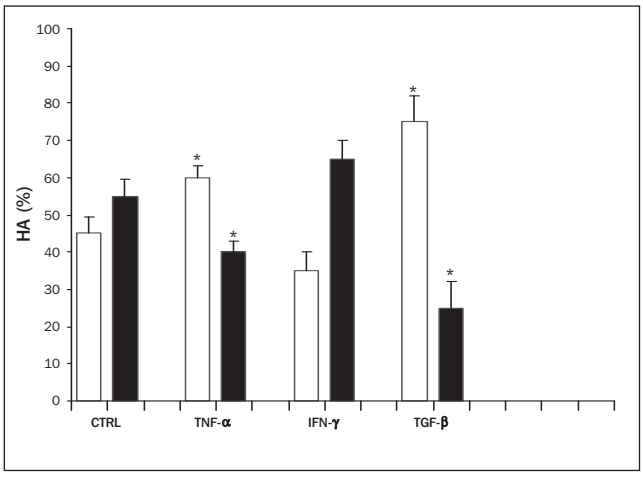

Fig. 10. Effect of cytokine treatment on fibroblast HA size production. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL). Then fibroblasts were incubated for 48 h and recovered for HA size determination. White bars indicate HA at high molecular weight (>500 kDa), black bars indicate HA at low molecular weight (100–500 kDa). Values are mean±SD of seven experiments ($^{*}P<0.001$ *vs*. IFN-γ).

Results

HAS1, HAS2 and HAS3 mRNA expression and protein evaluation

The amount of HAS1, HAS2 and HAS3 mRNA in fibroblasts (both normal and those exposed to $FeSO_4$ plus ascorbate) was measured in order to assess the effects of TNFα, IFNγ, and TGF1β treatment on gene expression and protein synthesis (Figs. 1, 2 and 3).

A significant positive effect was observed with TNFα and TGF1β, while the treatment with IFNγ produced only a slight increase in HAS2 at the highest dose. The lowest dose of TNFα failed to induce HAS1 expression (Fig. 1). The effect on HAS3 expression and protein production was positive, except with TGF1β treatment (Fig. 2).

HAS mRNA evaluation in fibroblasts exposed to oxidative showed a marked increase in expression and protein formation with HAS when compared to the non-stressed cells (Fig. 3).

Hyaluronan levels

Hyaluronan concentration was measured in control and stressed cells to evaluate the effects of cytokine treatment on HA synthesis (Figs. 4 and 5) and to determine the protective effect associated with enhanced HA accumulation.

Accumulation of HA after 48-h treatment with TNFα and TGF1β was higher than in the non-treated cells, although it differed depending on the cytokine and dosage. Hyaluronan concentration in fibroblasts treated with IFNγ was unchanged except in cells treated with the highest dose (Fig. 4).

Hyaluronan production measured in fibroblasts 24 h after the induction of oxidative stress was significantly higher than in the control cells (Fig. 5). The HA values measured in fibroblasts treated with cytokines and then exposed to oxidative stress were higher than for fibroblasts exposed to oxidative stress alone. In this case, the increase in HA was induced by all three cytokines at all doses used. This effect was found in unstressed cells and stressed cells by TNFα and TGF1β, while the HA increase induced by IFNγ was more significant in stressed cells (Fig. 5) than in unstressed cells (Fig. 4).

Cell viability

Exposure of cells to $FeSO_4$ plus ascorbate produced fibroblast death and growth inhibition as shown in Figure 6, with cell viability approximately 57%. No change in cell viability in comparison to the normal cells was seen in fibroblasts treated with cytokines but not exposed to $FeSO_4$ plus ascorbate. In contrast, when the three cytokines were administered to the fibroblasts exposed to oxidative stress, only treatment with TGF1β protected fibroblasts (in a dose-dependent manner) (Fig. 6). Tumour necrosis factor-α afforded moderate protection (exception at the lowest dose), while IFNγ exerted no effect at any of the doses tested.

Lactate dehydrogenase activity

Lactate dehydrogenase activity was determined as an indicator of cytotoxicity in fibroblast cultures (Fig. 7). Release of LDH in unexposed wells was approximately 10% and this was considered to be physiological.[39] In contrast, a marked increase in this enzyme was observed in the fibroblasts exposed to oxidative stress. No changes in LDH release were seen in fibroblasts treated with cytokines but not exposed to $FeSO_4$ plus ascorbate. However, treatment with TGF1β reduced LDH activity in a dose-dependent manner (Fig. 7). Tumour necrosis factor-α administration reduced LDH release at the highest dose, while IFNγ failed to protect fibroblasts from free radical injury.

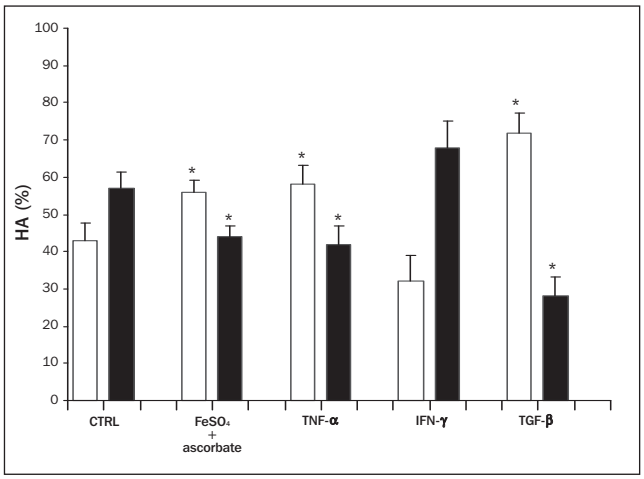

Fig. 11. Effect of cytokine pretreatment on fibroblast HA size production during oxidative stress. At time 0 the fibroblast culture medium was replaced with 2 mL fresh medium containing TNF-α or IFN-γ or TGF-1β (each at 1, 2 and 4 ng/mL). After 24 h, oxidative stress was induced, and 48-h later cells were recovered for HA size determination. White bars indicate HA at high molecular weight (>500 kDa), black bars indicate HA at low molecular weight (100–500 kDa). Values are mean±SD of seven experiments (*$P<0.001$ *vs*. IFN-γ).

Malondialdehyde levels

Evaluation of MDA levels was performed to estimate the degree of membrane lipid peroxidation produced in culture cells by oxidative injury (Fig. 8). A significant increase in MDA production was found in cells exposed to $FeSO_4$ plus ascorbate, while low levels of MDA were found in the untreated fibroblasts. No variation in MDA concentration was found in fibroblasts treated with cytokines and not exposed to oxidative stress. Treatment with TGF1β reduce lipid peroxidation at all doses used (Fig. 8). The TNFα treatment produced a moderate reduction, while IFNγ had no significant positive effect.

Endogenous antioxidants

Concentrations of SOD and GSH were assayed in order to evaluate the antioxidant balance after free-radical production (Figs. 9a and 9b). In the control wells, SOD and GSH were 26–38 units/mg protein and 8–14 nmol/mg protein, respectively. These levels were considered physiological.[40] In contrast, a significant reduction in the activity of both antioxidants was observed in stressed fibroblasts. No variation in SOD and GSH concentrations was found in fibroblasts treated with cytokines and not exposed to oxidative stress. Only the addition of TGF1β to fibroblasts prevented endogenous antioxidant depletion in a dose-dependent manner (Figs. 9a and 9b). Tumour necrosis factor-α blunted antioxidant depletion at the highest dose, while IFNγ was unable to improve antioxidant defences.

Hyaluronan molecular weight

Figures 10 and 11 show the detection of HA fragment size produced throughout the experiment. Normal fibroblasts produced comparable amounts of HA of both low and high molecular weight. Tumour necrosis factor-α induced an increase in HA generation at high molecular weight. However, IFNγ mainly induced low-molecular-weight HA production, while TGF1β induced production of HA at high molecular weight. (Fig. 10). Treatment of fibroblasts with $FeSO_4$ plus ascorbate induced a significant inversion in HA molecular weight with respect to the control cells (Fig. 11). Tumour necrosis factor-α plus oxidative stress also produced the same inversion. Finally, the size distribution of HA molecules showed little changed in HA produced by fibroblasts pretreated with cytokines and then exposed to oxidative stress (Fig. 11).

Discussion

The present study examined first the effect of TNFα, IFNγ and TGF1β treatment on HAS mRNA expression and protein levels in fibroblast cultures. Then HA concentration and size after HAS induction were measured and the protective effect of the increased HA levels after exposure of fibroblasts to the pro-oxidant $FeSO_4$ plus ascorbate was investigated.

In this research, low cytokine concentrations (1, 2 and 4 ng/mL) with respect to those used in a previous experiment were selected.[23] This choice was related to the fact that cytokines may regulate the expression of several compounds involved in the inflammatory response. In fact, among them, one is the increase in HAS and HA. For instance, other detrimental pathways that TNFα and IFNγ may activate are those of metalloproteases (MMPs), MAP/ERK or of the apoptotic caspases.[41]

Activation of these pathways depends on cytokine concentration and on exposure time to cytokines. In order to minimise the effect of these other pathways, reduced cytokine concentrations were used and cell exposure to cytokines was extended. At the doses and incubation times used, these pathways are not fully activated and higher doses (up to 25 ng/mL) are necessary to promote these events.[42,43] It has been found that the effects of cytokines are evident at 20 ng/mL and at 5 ng/mL, although at 20 ng/mL the effect is greater. Doses of 1, 2 and 4 ng/mL were selected, as, with these low doses, the effect on HAS and HA production is evident, but the activation of other pathways is minimised.

Data obtained for the treatment of fibroblasts with TNFα and TGF1β showed a significant increase in expression and protein synthesis of HAS1 and HAS2. Treatment with IFNγ did not cause any change, although higher concentrations of IFNγ have been shown to be effective.[23] In contrast, both TNFα and IFNγ significantly induced HAS3 in a dose-dependent manner, while TGF1β significantly inhibited HAS3 expression and protein formation. All three cytokines were able to boost HA levels, with TNFα and IFNγ showing comparable effects. Oxidative stress alone was also able to increase HAS mRNA level and HA concentration, as previously observed.[23] However, in the present study, pretreatment with TNFα, IFNγ and TGF1β yielded a further significant increase in HA level in stressed fibroblasts.

Even if HA levels were sufficiently high to reduce free-radical damage in all fibroblast groups, only HA production induced by TGF1β protected fibroblasts significantly from the oxidative burst – an effect that was dose-dependent. Tumour necrosis factor-α partially limited free-radical injury, while IFNγ, although able to increase HAS activity and HA concentration, produced no beneficial effect.

The protective effect of cytokine treatment was tested using cell viability and oxidative injury parameters (i.e., MDA concentration and endogenous antioxidants such as SOD activity and GSH concentration).

Only TGF1β showed a positive dose-dependent effect in the amelioration of morphological and biochemical fibroblast parameters after exposure to oxidative stress, despite the fact that the cytokines used globally increased HAS activities and HA levels. In order to explain this finding, we suggest that the answer lies in significant down-regulation of HAS3 by TGF1β and the differences in the distribution of HA size produced. HAS1 and HAS2 induction mainly favours production of high-molecular-weight HA, while HAS3 produces predominantly low-molecular-weight HA.[19] Moreover, it has been shown that high-molecular-weight HA has a better antioxidant effect than low-molecular-weight HA.[44]

The present results showed that TNFα and IFNγ mainly stimulated HAS3 mRNA production that in turn was responsible for accumulation of low-molecular-weight HA. In contrast, HAS1 and HAS2 were induced poorly (TNFα) or not at all (IFNγ) by these two cytokines. However, TGF1β mainly stimulated HAS1 and HAS2 mRNA expression and the related protein formation, which are expected to increase high-molecular-weight HA production. Therefore, as TGF1β was also able to inhibit HAS3 mRNA levels and protein synthesis in a dose-dependent manner, the final effect of TGF1β is thought to be very low production of low-molecular-weight HA and high production of high-molecular-weight HA, which is the better antioxidant.

It is hypothesised that high-molecular-weight HA protects stressed fibroblasts better than does low-molecular-weight HA because it is more efficient at chelating pro-oxidant metal ions. The use of metal chelating agents may have a therapeutic effect by reducing oxidative burst and consequent cell damage.[45] The presence of the carboxylic group in the HA structure is believed to confer antioxidant activity, as this charged group could interact with transition metal ions (e.g., Cu^{++} or Fe^{++}) that play a role in the lipid peroxidation mechanism.[46]

The ability of HA to chelate different ions and transition metals has been reported by several authors.[23,47,48] Solid complexes of iron(III) with HA are highly stable and show a binding constant log K of approximately 8.[48] All these data strongly suggest that HA is able to bind iron and copper cations in solution. This would decrease their availability for oxidation processes.

We observed an antioxidant effect with commercial HA,[4,6] although no conclusion can be drawn about the effect of HA molecular weight because commercial HA is a mixture of chains of different sizes.

The finding that fibroblasts with increased production of endogenous high-molecular-weight HA might be better protected than those in which low-molecular-weight HA production is increased is of particular interest. Although further investigations are needed to confirm these findings, this study suggests that the increment in HA synthesis may represent a significant biological mechanism to reduce oxidative processes. □

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