ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 9, pp. 974-982. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 9, pp. 1196-1206.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-118, July 29, 2007.

Differential Effect of Growth Factors on Hyaluronan Synthase Gene Expression in Fibroblasts Exposed to Oxidative Stress

G. M. Campo[1]*, A. Avenoso[1], S. Campo[1], A. D'Ascola[1], A. M. Ferlazzo[2], and A. Calatroni[1]

[1]Department of Biochemical, Physiological, and Nutritional Sciences, School of Medicine, University of Messina, Policlinico Universitario, Torre Biologica, 5° piano, Via C. Valeria, 98125 Messina, Italy; fax: +39-90-221-3330; E-mail: gcampo@unime.it
[2]Department of Morphology, Biochemistry, Physiology and Animal Production, School of Veterinary Medicine, University of Messina, Contrada Annunziata, 98168 Messina, Italy

Received April 26, 2007

Abstract—The aim of this study was to evaluate how growth factors (PDGF-BB, EGF, and TGF-1β) modulate hyaluronan synthase (HAS) activities in normal or stressed cultured human skin fibroblasts. The effects of concomitant treatment with cytokines and $FeSO_4$ plus ascorbate on HAS mRNA expression, protein synthesis, and hyaluronic acid (HA) concentrations were also studied. Treatment of fibroblasts with growth factors up-regulated HAS gene expression and increased HAS enzymes and HA production. PDGF-BB induced HAS mRNA expression, protein synthesis, and HA production more efficiently than EGF and TGF-1β. EGF was less effective than TGF-1β. In addition, TGF-1β reduced the expression and synthesis of HAS3, while PDGF-BB and EGF had the opposite effect. Concomitant treatment with growth factors and the oxidant was able to further increase HAS mRNA expression, once again with the exception of HAS3 with TGF-1β. HAS protein synthesis was reduced, while HA levels were unaffected in comparison to those obtained from exposure to $FeSO_4$ plus ascorbate alone. In conclusion, although growth factors plus the oxidant synergistically induced HAS mRNA expression in part, enzyme production was not correlated with this increase. Moreover, the increase in HAS mRNA levels was not translated into a consequent rise in HA concentration.

DOI: 10.1134/S0006297907090088

Key words: free radicals, glycosaminoglycans, growth factors, cell cultures, fibroblasts, hyaluronan synthases, hyaluronan, PDGF-BB, EGF, TGF-1β

It is widely known that free radicals play multiple roles in physiological and pathological states in biological organisms [1]. These reactive molecules are thought to be involved in a variety of common human pathologies, and their production increases markedly in these states. The reactive oxygen species (ROS) are formed mainly during normal aerobic metabolism in cells, and following phagocyte activation during infection/inflammation. One of the consequences of the uncontrolled production of free radicals is damage to biomolecules, which leads to impaired function and disease [1].

Glycosaminoglycans (GAGs) are a family of complex acidic polysaccharides that are involved in a variety of physiological and pathological conditions [2]. These linear carbohydrate polymers (except for keratan sulfate) are composed of alternating hexosamine and hexuronic acid residues. Hyaluronan (HA) is a high molecular weight, non-sulfated GAG component of the extracellular matrix (ECM) present in many tissues, such as skin, synovial joints, and other connective tissues. HA has many structural, rheological, and physiological functions in tissues, including ECM and cellular interaction, growth factor interaction, and the regulation of osmotic pressure and tissue lubrication [3]. HA is also a key component of chronic wounds during each stage of the wound healing process, including the inflammatory, granulation, and reepithelialization stages [4].

HA is synthesized on the inner surface of the plasma membrane by three related isoenzymes, the hyaluronan synthases (HAS1, HAS2, and HAS3) [5]. Each isoform is

Abbreviations: DMEM) Dulbecco's modified Eagle's medium; ECM) extracellular matrix; EGF) epidermal growth factor; FBS) fetal bovine serum; GAGs) glycosaminoglycans; HA) hyaluronic acid; HASs) hyaluronan synthases; HRP) horseradish peroxidase; IFN-γ) interferon-γ; IL-1β) interleukin-1β; NADH) reduced nicotinamide adenine dinucleotide; PBS) phosphate-buffered saline; PCR) polymerase chain reaction; PDGF-BB) platelet derived growth factor BB; PGs) proteoglycans; ROS) reactive oxygen species; SDS) sodium dodecyl sulfate; TBS) tris-buffered solution; TGF-1β) transforming growth factor 1β; TNF-α) tumor necrosis factor α.

* To whom correspondence should be addressed.

able to synthesize HA molecules of a given size, exhibits different kinetic properties, and specific cell-type characteristics [6]. It has recently been reported that certain growth factors, such as platelet derived growth factor BB (PDGF-BB), epidermal growth factor (EGF), transforming growth factor 1β (TGF-1β), etc. can modulate HAS transcription in cell culture. HAS modulation is dependent upon the type of cell line [7-10]. Mononuclear cells are important regulators of fibroblast proliferation and collagen biosynthesis. Mononuclear cells are often predominant in inflammatory cell infiltrate following many injuries and in many pathological disorders. This fact led to speculation that mononuclear cells may also be important regulators of GAG biosynthesis via cytokine and ROS release. The release of these mediators then stimulates the production of growth factors, which are responsible for tissue regeneration and ECM deposition. This hypothesis was postulated some years ago from studies showing that supernatants from activated mononuclear cells stimulate fibroblast GAG biosynthesis [11, 12].

We have recently reported that tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β) can modulate HAS expression differently [13]. The treatment of fibroblasts with cytokines up-regulated HAS gene expression and increased HA production. IL-1β induced HAS mRNA expression and HA production more efficiently than TNF-α and IFN-γ. The exposure of fibroblasts to the oxidant system increased HAS activities markedly while affecting HA production only slightly. The concomitant treatment of cells with cytokines and the oxidant acted in synergy and was able to further enhance HAS mRNA expression. In contrast, HA levels were found to be unaffected by concomitant treatment and resembled those obtained with the exposure to $FeSO_4$ plus ascorbate alone. In light of these previous findings, we used the same protocol in order to investigate how PDGF-BB, EGF, and TGF-1β treatments modulate the activity of HASs and the changes in HA production in normal and stressed fibroblasts. The effects of the increase in HAS mRNA expression and the endogenous HA levels obtained from fibroblasts concomitantly exposed to cytokines and oxidative stress were also studied.

MATERIALS AND METHODS

Reagents. Recombinant human PDGF-BB, EGF, and TGF-1β obtained from *Escherichia coli* source were purchased from Peprotech Inc. (USA). HAS1, HAS2, and HAS3 monoclonal antibodies were obtained from Cell Signaling (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypsin-EDTA solution, and phosphate-buffered saline (PBS) were obtained from GibcoBRL (USA). All cell culture plastics were obtained from Falcon (USA). Ascorbic acid, iron(II) sulfate, sucrose, EDTA, sodium dodecyl sulfate (SDS), glycerol, dithiothreitol (DTT), bromophenol blue, methanol, Tween 20, potassium phosphate, RNase, and all other general laboratory chemicals were obtained from Sigma-Aldrich S.r.l. (Italy).

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

Oxidative stress induction and cytokine treatment. Fibroblasts were cultured in six-well culture plates at a density of $1.3 \cdot 10^5$ cells/well. Twelve hours after plating (time 0), when cells were firmly attached to the substratum (about $1 \cdot 10^5$ cells/well), the culture medium was replaced with 2.0 ml of fresh medium containing PDGF-BB, EGF, or TGF-1β at three different concentrations: 5.0, 10.0, and 20.0 ng/ml for each. After 3 h of incubation, fibroblasts were recovered for HAS mRNA evaluation. Another set of plates, treated with the three growth factors, each one again at the three concentration levels, were incubated for 24 h, and then fibroblasts were recovered for HA level quantization. A further set of plates was first treated with the three growth factors and, at the same time, oxidative stress was induced in the cells as follows: 10 μl of 100 μM $FeSO_4$ was added to wells (final concentration 0.5 μM), and 15 min later 10 μl of 50 mM ascorbic acid was added for free radical production [14]. Three hours later cells were subjected to HAS mRNA evaluation. Once again, a separate set of plates were treated with the three growth factors and exposed to $FeSO_4$ plus ascorbate and incubated for 24 h in order to evaluate HA production. Finally, two additional set of plates were exposed to oxidative stress only and incubated for 3 and 24 h in order to evaluate HAS mRNA expression and HA levels, respectively.

RNA isolation, cDNA synthesis, and real-time quantitative PCR amplification. For reverse-PCR real time analysis (RealTime PCR system, Model 7500; Applied Biosystems, USA) of HAS1, HAS2, and HAS3, total RNA was isolated from $(4\text{-}5) \cdot 10^6$ cells using the Omnizol Reagent kit (Euroclone, UK). The first strand of cDNA was synthesized from 1.0 μg total RNA using a high capacity cDNA Archive kit (Applied Biosystems). β-Actin mRNA was used as an endogenous control to allow the relative quantification of HAS mRNAs [15]. PCR RealTime was performed with ready-to-use assays (Assays on demand; Applied Biosystems) for both targets and endogenous controls. The amplified PCR products were quantified by measuring the HASs 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 analyzed. The amount of

specific mRNA in samples was calculated from the standard curve and normalized with the β-actin mRNA. After normalization, the mean of normal fibroblasts target levels became the calibrator (one per sample) and the results were expressed as an *n*-fold difference relative to normal controls (relative expression levels).

Western blot assay of HAS proteins. For SDS-PAGE and Western blotting, the cells were washed twice in ice-cold PBS and subsequently dissolved in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Aliquots of whole cell protein extract (10-25 µl/well) were separated on a mini gel (10%). The proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences) using a semi-dry apparatus (Bio-Rad, USA). The blots were flushed with double distilled H_2O, dipped into methanol, and dried for 20 min before proceeding with the next steps. Subsequently, the blots were transferred to a blocking buffer solution (1× PBS, 0.1% Tween 20, 5% w/v nonfat 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 antibody goat anti-rabbit conjugated with peroxidase, in TBS/Tween-20 buffer containing 5% nonfat dried milk. After 45 min of gentle shaking, the blots were washed five times in wash buffer, and the proteins were made visible using LumiGLO (New England Biolabs, USA) and Kodak BioMax MR films.

Hyaluronic acid assay. HA analysis was performed in the lysate of (4-5)·10^5 cell samples obtained 24 h after cytokine treatment. HA levels were analyzed as a product of HAS activity. The assay was carried out using a specific enzyme-linked binding protein assay test kit (Cat. No. 029-001; Corgenix UK, UK). In brief, cell lysate samples were added to a microtiter well together with a primary antibody and incubated at room temperature for 1 h. After washing, a secondary antibody conjugated with peroxidase (HRP-conjugated) was added to the well and incubated at room temperature for 30 min. Subsequently,

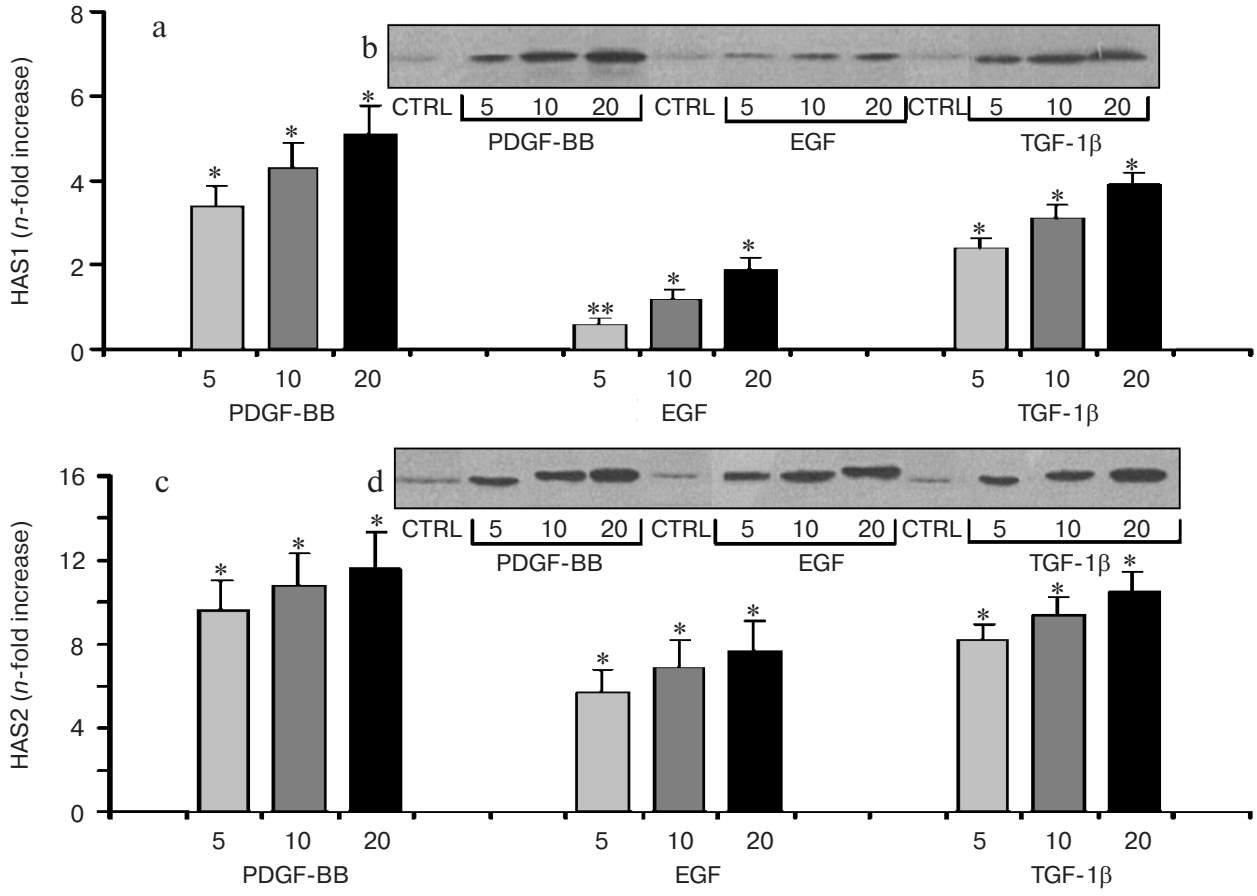

Fig. 1. Effect of growth factors treatment on fibroblast HAS1 and HAS2 mRNA expression and protein activity. a, c) mRNA levels of HAS1 and HAS2, respectively. Values are reported as *n*-fold increase with respect to control cells. b, d) Protein levels of HAS1 and HAS2, respectively. Here and in Figs. 2, 4, 5, 6, and 7, PDGF-BB, EGF, and TGF-1β were each used at doses of 5.0, 10.0, and 20.0 ng/ml. Values are the mean ± S.D. of seven experiments. * $p < 0.001$ and ** $p < 0.005$ versus control (CTRL).

after adding a one-component substrate solution (3,3′,5,5′-tetramethylbenzidine plus hydrogen peroxide) and a further incubation period (at room temperature for 30 min) the reaction was stopped with 0.36 N H_2SO_4 and the absorbance was read at 450 nm using a microplate reader (DAS S.r.l., Italy). The concentration of HA in each sample was determined by interpolation from a standard curve ranging from 0 to 800 ng/ml.

Protein analysis. The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad) and bovine serum albumin as a standard in accordance with the published method [16].

Statistical analysis. Data are expressed as means ± S.D. 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. The statistical significance of differences was set at $p < 0.05$.

RESULTS

HAS-1, HAS-2, and HAS-3 mRNA expression and protein evaluation. The treatment of normal fibroblasts with PDGF-BB and EGF significantly increased the expression and protein production of all of the synthases. HAS2 was stimulated more effectively than HAS1, and PDGF-BB was more effective than EGF. TGF-1β was also able to increase HAS1 and HAS2 but this growth factor significantly inhibited HAS3 expression and reduced protein synthesis compared to PDGF-BB and EGF (Figs. 1 and 2).

HAS mRNA evaluation in fibroblasts exposed to oxidative stress alone also showed a marked increase in the expression of all mRNAs tested. Of these, HAS2 and HAS3 mRNA were induced more efficiently than HAS1 mRNA (Fig. 3a). Indeed, HAS protein synthesis was not correlated with mRNA expression, although the protein content was higher than controls (Fig. 3b).

The concomitant treatment of fibroblasts with $FeSO_4$ plus ascorbate and the three growth factors was able to increase HAS mRNA expression synergistically, with the exception of HAS3 as this was inhibited by TGF-1β (Figs. 4a, 4c, and 5a). These data were significantly different from normal basal fibroblast values as well as to the values for fibroblasts exposed to oxidative stress. Nevertheless, HAS protein levels were much lower than those obtained for the fibroblast unexposed to oxidative stress (Figs. 4b, 4d, and 5b).

Hyaluronic acid levels. HA levels measured 24 h after PDGF-BB, EGF, and TGF-1β stimulation were higher

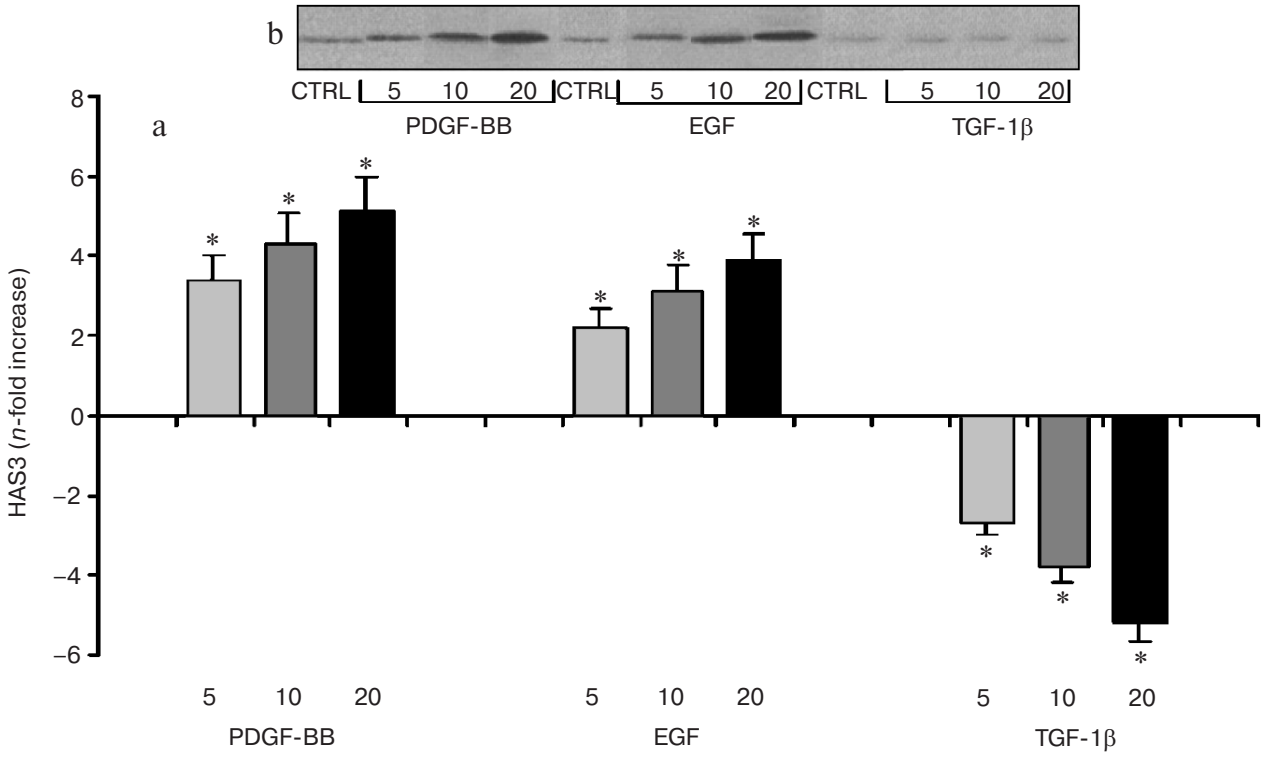

Fig. 2. Effect of growth factor treatment on fibroblast HAS3 mRNA expression and protein activity. a) mRNA levels of HAS3. Values are reported as *n*-fold increase with respect to control cells. b) Protein levels of HAS3. Values are the mean ± S.D. of seven experiments. * $p < 0.001$ versus CTRL.

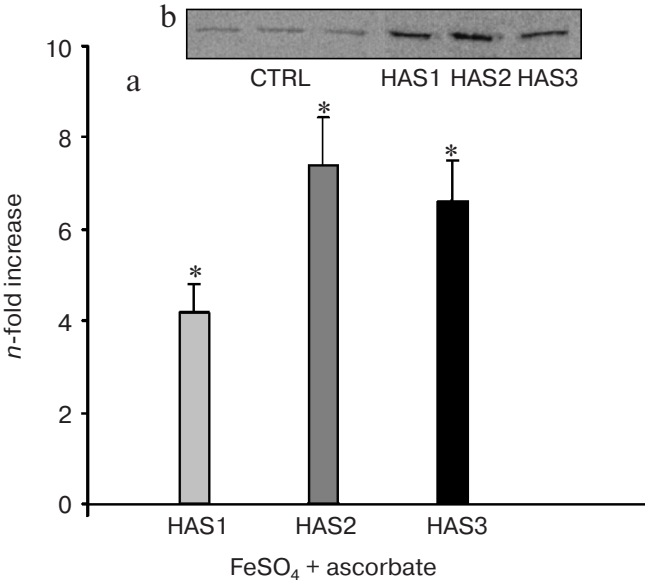

Fig. 3. Effect of $FeSO_4$ plus ascorbate on fibroblast HAS1, HAS2, and HAS3 mRNA expression and protein activity. a) mRNA levels of HAS1, HAS2, and HAS3, respectively. Values are reported as *n*-fold increase with respect to control cells. b) Protein levels of HAS1, HAS2, and HAS3, respectively. Values are the mean ± S.D. of seven experiments. * $p < 0.001$ versus CTRL.

than those of non-stimulated cells. This increment was produced by all growth factors. However, the effect produced by EGF at doses of 5.0 and 10.0 ng/ml was less significant than PDGF-BB and TGF-1β (Fig. 6). In addition, since TGF-1β reduced HAS3, in this case HA production arose only from HAS2 and HAS1 stimulation. The difference in HA production measured in fibroblasts 24 h after the induction of oxidative stress was significant in comparison to the control cells (Fig. 7). The HA levels measured in fibroblasts treated with the growth factors and concomitantly exposed to oxidative stress were significantly higher than those not exposed. HA levels of fibroblasts treated with the three growth factors at all doses used were similar to those obtained after the treatment with $FeSO_4$ plus ascorbate alone (Fig. 7).

DISCUSSION

Hyaluronic acid is a linear GAG consisting of repeating units in which the sugars glucuronic acid and N-acetyl glucosamine alternate. It exists as the tertiary structure of a random coil with a high molecular weight [17]. Concentrations of HA are high in musculoskeletal tissues, skin embryological tissues, and synovial fluid. Many biological functions, such as space filling and lubrication have been attributed to it, as well as other more specific effects relating to cell functions. Its simple chemical structure belies the range of unique viscoelastic and physiological properties that this polysaccharide possesses [2]. HA is involved in numerous biological functions, including the lubrication of joints, the regulation of molecular permeation into various tissues, proliferation, adhesion, and motility of cells, and the development of embryos [18]. It has also been demonstrated to play a role in cancer metastasis, wound healing, and inflammation [19]. It is synthesized by most cells of the human body and it can be found in all tissues in a wide range of concentrations [20]. HA is of vital importance as there are no known viable individuals that lack the ability to synthesize this molecule. Evidence of this was provided by the early embryonic lethality of HAS2 knockout mice [21]. It is also an excellent hydrated matrix, through which cells can migrate [22]. This and other actions are mediated by its interaction with other ECM components, as well as receptor and intracellular proteins [23].

Previous reports have shown that HA plays several roles in the activation and modulation of the inflammatory response, including the antioxidant scavenging of ROS derived from polymorphonuclear leukocytes and other sources [24-28]. HA and other GAG structures, such as chondroitin sulfates and heparan sulfate, are commonly found in human plasma [29]. Nevertheless, a marked increase in plasma HA and other GAG levels have been observed in a large number of diseases, especially those involving free radical damage [30-35]. This increase in native plasma GAGs over the course of diseases could be a biological cellular response in an attempt to reduce the damage produced by oxidative stress. Nevertheless, the increase in antioxidant activity produced by GAGs is probably insufficient to neutralize the massive amount of ROS released and the consequent cell injury. However, the exact meaning of the increase in GAG is at the moment unclear.

HAS1, HAS2, and HAS3 are the enzymes responsible for HA production [5]. These synthases are similar in terms of amino acid sequence and molecular structural characteristics. The discovery of these distinct mammalian HASs raised additional questions regarding the potential differences in their activities and the exact biological roles they play.

Activated mononuclear cells produce interleukins [36]. Among them growth factors play an important role during fibroblast growth and collagen biosynthesis [7]. The best characterized growth factors that act on connective tissue cells are PDGF, EGF, FGF, and TGF-1β [7, 37]. The results relating to cell proliferation have been clarified by a number of authors, since these growth factors can modulate HA production by inducing HA synthases [38-40]. Although, the three HASs were induced differently by each growth factor and the effect of the stimulation differed in relation to the type of cell used, HA concentrations were well correlated with HAS mRNA levels for all these molecules [8, 41, 42].

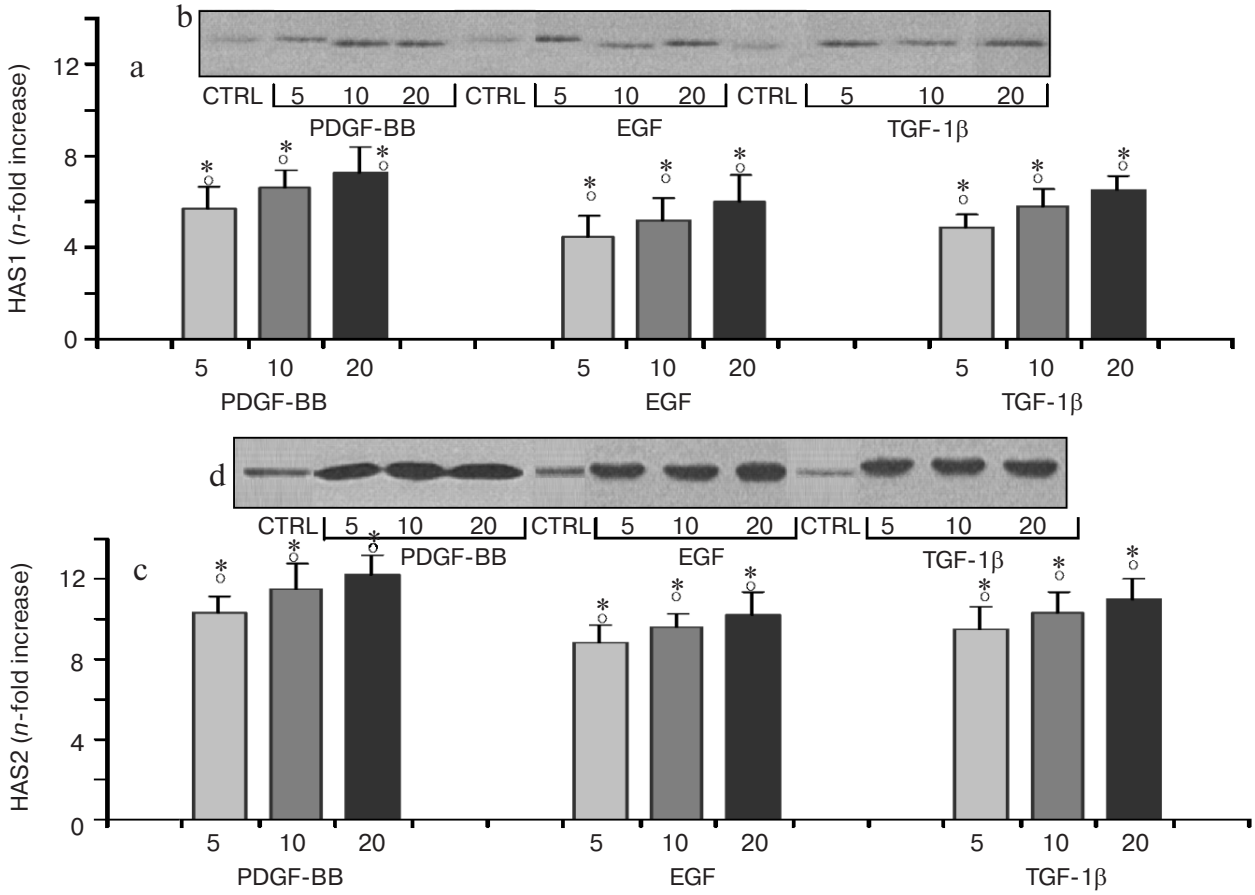

Fig. 4. Effect of growth factors and $FeSO_4$ plus ascorbate treatment on fibroblast HAS1 and HAS2 mRNA expression and protein activity. a, c) mRNA levels of HAS1 and HAS2, respectively. Values are reported as *n*-fold increase with respect to control cells. b, d) Protein levels of HAS1 and HAS2, respectively. Values are the mean ± S.D. of seven experiments. ° $p < 0.001$ versus CTRL; * $p < 0.001$ versus $FeSO_4$ + ascorbate treatment and cytokine treatment alone.

In our study, HA levels obtained in fibroblasts treated with growth factors and concomitantly exposed to oxidative stress were significantly higher than those not exposed. In contrast, HA levels were unchanged with respect to cells exposed to oxidative stress. Indeed, HA levels of fibroblasts treated with the three growth factors at all doses used were similar to those obtained after the treatment with $FeSO_4$ plus ascorbate alone (Fig. 7).

Since oxidative stress by itself was also able to increase mRNA HAS levels, this mechanism can be explained by the fact that oxidative stress can directly induce endogenous cytokine production [43, 44].

The ECM and its components have been documented to control the growth, state of differentiation, development, and metabolic responses of a cell [17]. However, the effects of the ECM on the cell do not occur in a milieu that is devoid of other influences that affect the biology of a cell. At the same time as studies documented the role of ECM in controlling cell biology, similar investigations established the importance of cytokines, growth factors, hormones, vitamins, and cell-to-cell contact as regulators of cell phenotype. Two important concepts have emerged from previous studies: first, one of the major effect of cytokines, growth factors, vitamins and hormones, and cell-to-cell contact on the cell is the regulation of ECM component production; and, second, many of the biological changes attributed to the effects of cytokines and growth factors on a cell are similar, if not identical, to the effect of ECM on cell components. These findings can be interpreted, beyond basic genetic programming, as four main types of interactions (cytokines, growth factor hormones/vitamins, cell-to-cell contacts, and ECM) that regulate the growth, shape, state of differentiation, development, and biochemical responses of the cell [45]. Hence, our current knowledge indicates that control of the biological phenotype of a cell, both in normal development and function as well as in pathological responses, represents a complex interplay between soluble and insoluble mediators in which the ECM plays a pivotal role.

In our study, the significant amount of HA generated by HAS stimulation in fibroblasts exposed to oxidative

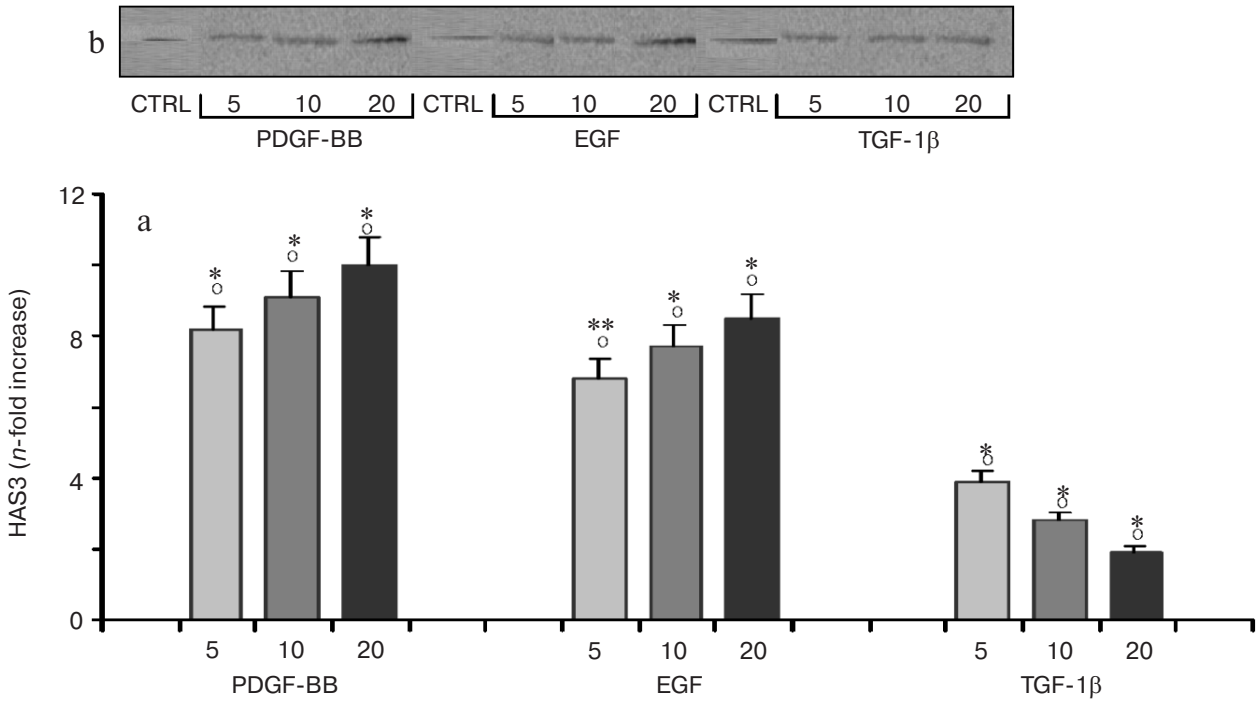

Fig. 5. Effect of growth factors and $FeSO_4$ plus ascorbate treatment on fibroblast HAS3 mRNA expression and protein activity. a) mRNA levels of HAS3. Values are reported as *n*-fold increase with respect to control cells. b) Protein levels of HAS3. Values are the mean ± S.D. of seven experiments. ° $p < 0.001$ versus CTRL; * $p < 0.001$ versus $FeSO_4$ + ascorbate treatment and cytokine treatment alone; ** $p < 0.001$ versus cytokine treatment.

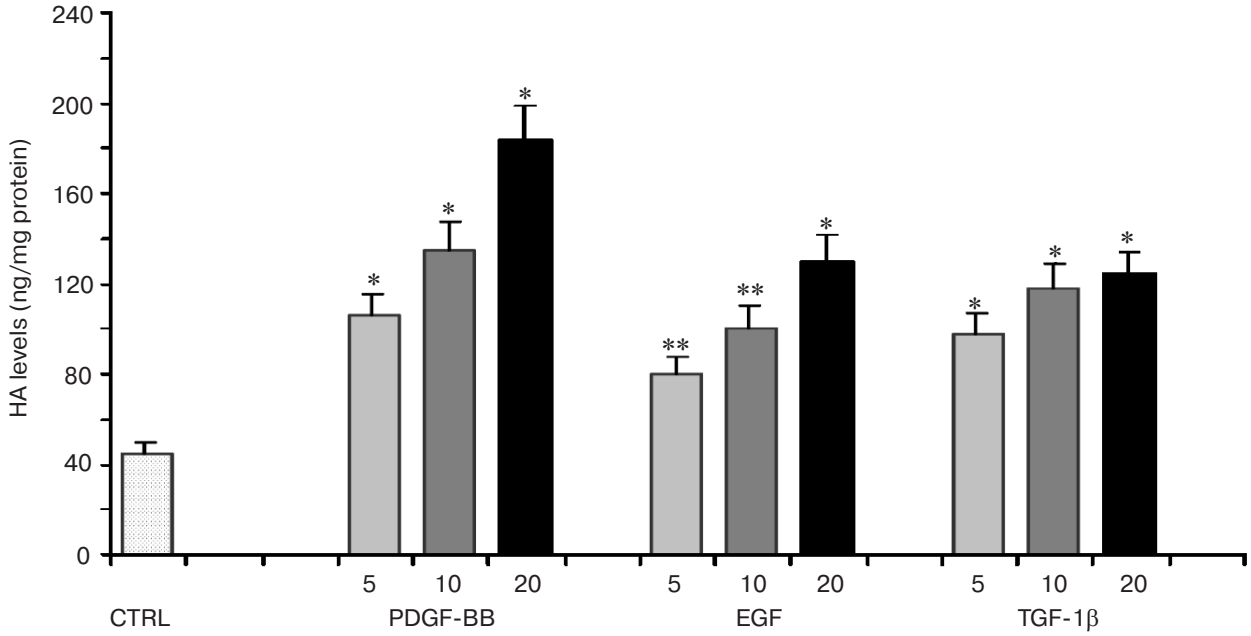

Fig. 6. Effect of growth factor treatment on fibroblast HA production. Values are the mean ± S.D. of seven experiments. * $p < 0001$ and ** $p < 0.005$ versus CTRL.

stress could represent a biological response that is an attempt to reduce cell damage from free radicals. In contrast, HA produced by HAS stimulation after growth factor treatment represents a mechanism for tissue repair during inflammation [46, 47]. Since ROS are produced abundantly during inflammation [48], both these mechanisms seem to have the common aim of maintaining/preserving cell homeostasis. Nevertheless, these biological

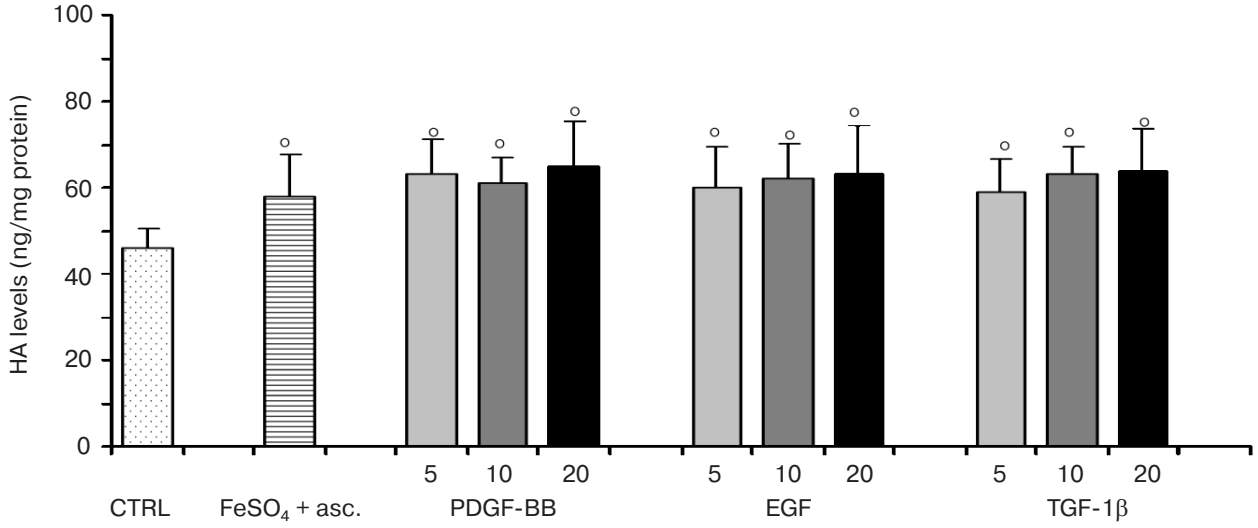

Fig. 7. Effect of growth factors and $FeSO_4$ plus ascorbate treatment on fibroblast HA production. Values are the mean ± S.D. of seven experiments. ° $p < 0.05$ versus CTRL.

responses are often useless by themselves. In fact, as overproduction of free radicals is closely correlated with the severity of the pathological state, cell damage is often inevitable. In our experiments, the concomitant treatment of fibroblasts with growth factors and $FeSO_4$ plus ascorbate showed a synergistic effect leading to a marked increase of HAS expression. Nevertheless, this increase was not able to synthesize corresponding levels of the HAS enzymes. In addition, the reduction in HAS levels produced a consequent decrease in HA concentrations. This reduction in protein could be explained by the fact that the HAS mRNA and the related proteins could be affected by the deleterious action of free radicals. In this way, HAS expression was activated, but at this stage or at protein level, the mechanism was no longer working because the mRNA or HAS enzymes were damaged.

Although further investigations are needed to confirm these findings, this study suggests that the increment in fibroblast HAS expression and HA synthesis after cytokine/growth factor treatment or oxidant exposure could be a biological mechanism to preserve tissue against injury during inflammation.

This study was supported in part by a grant ex 40% (COFIN 2004) from the MIUR, Italy, and in part by a grant PRA (Research Athenaeum Project 2003) from the University of Messina, Italy.

REFERENCES

1. Droge, W. (2002) *Physiol. Rev.*, **82**, 47-95.
2. Templeton, D. M. (1992) *Crit. Rev. Clin. Lab. Sci.*, **29**, 141-184.
3. Laurent, T. C. (1998) *The Chemistry, Biology and Medical Application of Hyaluronan and Its Derivatives*, Vol. 72, Wenner-Green International Series, Portland Press, London.
4. Chen, W. Y., and Abatangelo, G. (1999) *Wound Rep. Reg.*, **7**, 79-89.
5. Weigel, P. H., Hascall, V. C., and Tammi, M. (1997) *J. Biol. Chem.*, **272**, 13997-14000.
6. Itano, N., Sawait, T., Yoshida, M., Lenas, P., Yamada, Y., Imagawa, M., Shinomura, T., Hamaguchi, M., Yoshida, Y., Ohnuki, Y., Miyauchi, S., Spider, P. A., McDonald, A. J., and Kimata, K. (1999) *J. Biol. Chem.*, **274**, 25085-25092.
7. Heldin, P., Laurent, C. T., and Heldin, C. H. (1989) *Biochem. J.*, **258**, 919-922.
8. Jacobson, A., Brinck, J., Briskin, M. J., Spicer, A. P., and Heldin, P. (2000) *Biochem. J.*, **348**, 29-35.
9. Stuhlmeier, K. M., and Pollaschek, C. (2004) *J. Biol. Chem.*, **279**, 8753-8760.
10. Yamada, Y., Itano, N., Hata, K., Ueda, M., and Kimata, K. (2004). *J. Invest. Dermatol.*, **122**, 631-639.
11. Anastassiades, T. P., and Wood, A. (1981) *J. Clin. Invest.*, **68**, 792-802.
12. Whiteside, T. L., Worrall, J. G., Prince, R. K., Buckingham, R. B., and Rodman, G. P. (1985) *Arthritis Rheum.*, **28**, 188-197.
13. Campo, G. M., Avenoso, A., Campo, S., D'Ascola, A., Ferlazzo, A. M., and Calatroni, A. (2006) *Mol. Cell. Biochem.*, **292**, 169-178.
14. Campo, G. M., D'Ascola, A., Avenoso, A., Campo, S., Ferlazzo, A. M., Micali, C., Zanghi, L., and Calatroni, A. (2004) *Glycoconj. J.*, **20**, 133-141.
15. Bustin, S. A. (2000) *J. Mol. Endocrinol.*, **25**, 169-193.
16. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
17. Iozzo, R. V. (1998) *Annu. Rev. Biochem.*, **67**, 609-652.
18. Knudson, C. B., and Knudson, W. (1993) *FASEB J.*, **7**, 1233-1241.
19. Hascall, V. C., Majors, A. K., De La Motte, C. A., Evanko, S. P., Wang, A., Drazba, J. A., Strong, S. A., and Wight, T. N. (2004) *Biochim. Biophys. Acta*, **1763**, 3-12.

20. Laurent, T. C., and Fraser, J. R. E. (1991) *Catabolism of Hyaluronan*, in *Degradation of Bioactive Substances: Physiology and Pathology* (Henriksen, J. H., ed.) RC Press, Boca Raton, pp. 249-265.
21. Spicer, A. P., and Nguyen, T. K. (1999) *Biochem. Soc. Trans.*, **27**, 109-115.
22. Laurent, T. C., and Fraser, J. R. E. (1992) *FASEB J.*, **6**, 2397-2404.
23. Iozzo, R. V., and Murdoch, A. D. (1996) *FASEB J.*, **10**, 598-614.
24. Campo, G. M., Avenoso, A., Campo, S., Ferlazzo, A. M., Altavilla, D., and Calatroni, A. (2003) *Arthritis Res. Ther.*, **5**, R122-R131.
25. Campo, G. M., Avenoso, A., D'Ascola, A., Campo, S., Ferlazzo, A. M., Sama, D., and Calatroni, A. (2005) *Toxicol. In Vitro*, **19**, 561-572.
26. Fukuda, K., Takayama, M., Ueno, M., Oh, M., Asada, S., Kumano, F., and Tanaka, S. (1997) *Inflamm. Res.*, **46**, 114-117.
27. Kvam, B. J., Fragonas, E., Degrassi, A., Kvam, C., Matulova, M., Pollesello, P., Zanetti, F., and Vittur, F. (1995) *Exp. Cell Res.*, **218**, 79-86.
28. Suzuki, Y., and Yamaguchi, T. (1993) *Agents Actions*, **38**, 32-37.
29. Calatroni, A. (2002) *Extraction and Purification of Glycosaminoglycans (GAGs) from Biological Fluids*, in *Analytical Techniques to Evaluate the Structure and Function of Natural Polysaccharides*, *Glycosaminoglycans* (Volpi, N., ed.) Research Signpost Press, Trivandrum, pp. 15-22.
30. Calabro, L., Musolino, C., Spatari, G., Vinci, R., and Calatroni, A. (1998) *Clin. Chim. Acta*, **269**, 185-99.
31. Friman, C., Nordstrom, D., and Eronen, I. (1987) *J. Rheumatol.*, **14**, 1132-1134.
32. Laurent, T. C., Laurent, U. B. G., and Fraser, J. R. E. (1996) *Ann. Med.*, **28**, 241-253.
33. Plevris, J. N., Haydon, G. H., Simpson, K. J., Dawkes, R., Ludlum, C. A., Harrison, D. J., and Hayes, P. C. (2000) *Eur. J. Gastroenterol. Hepatol.*, **12**, 1121-1127.
34. Radhakrishnamurthy, B., Tracy, R. E., Dalferes, Jr., and Berenson, J. R. (1998) *Exp. Mol. Pathol.*, **65**, 1-8.
35. Roughley, P. J. (2001) *Arthritis Res.*, **3**, 342-347.
36. Palladino, Jr., and Shepard, H. M. (1985) *Science*, **230**, 943-945.
37. Schlessinger, J. (1986) *J. Cell. Biol.*, **103**, 2067-2072.
38. Recklies, A. D., White, C., Melching, L., and Roughley, P. J. (2001) *Biochem. J.*, **354**, 17-24.
39. Sugiyama, Y., Shimada, A., Sayo, T., Sakai, S., and Inoue, S. (1998) *J. Invest. Dermatol.*, **110**, 116-121.
40. Tirone, E., D'Alessandris, C., Hascall, V. C., Siracusa, G., and Salustri, A. (1997) *J. Biol. Chem.*, **272**, 4787-4794.
41. Ducale, A. E., Ward, S. I., Dechert, T., and Yager, T. L. (2005) *Am. J. Physiol. Gastrointest. Liver Physiol.*, **289**, G462-G470.
42. Oguchi, T., and Ishiguro, N. (2004) *Connect. Tissue Res.*, **45**, 197-205.
43. Mata-Greenwood, E., Grobe, A., Kumar, S., Noskina, J., and Black, S. M. (2005) *Am. J. Physiol. Lung Cell Mol. Physiol.*, **289**, L288-289.
44. Suzuki, Y. J., Nagase, H., Nie, K., and Park, A. M. (2005) *Antiox. Redox Signal.*, **7**, 829-834.
45. Haralson, M. A., and Hassell, J. R. (1995) *The Extracellular Matrix: an Overview*, in *Extracellular Matrix – a Practical Approach* (Haralson, M. A., and Hassell, J. R., eds.) IRL Press, New York, pp. 1-30.
46. Gao, B. (2005) *Cell. Mol. Immunol.*, **2**, 92-100.
47. Tarnawski, A. S. (2005) *Dig. Dis. Sci.*, **50**, Suppl. 1, S24-33.
48. Wang, J., and Slungaard, A. (2006) *Arch. Biochem. Biophys.*, **445**, 256-260.