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Research Article

Glycosaminoglycans Modify Elastase Action *In Vitro* and Enhance Elastase-Induced Cell Death in Cultured Fibroblasts

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Human neutrophil elastase (HNE) has been shown to be involved on death of different cell types, including epithelial lung cells, which is related to several pulmonary diseases. Since HNE activity may be influenced by extracellular matrix (ECM) molecules such as glycosaminoglycans (GAGs), and fibroblasts are the most common ECM-producing cells of lung connective tissue, the aim of this work was to verify if HNE can induce fibroblast death and to study the enzyme modulation by GAGs. HNE-like activity was mimicked by using human neutrophils conditioned medium (NCM). Heparan sulfate and chondroitin 6-sulfate reduce the enzyme activity and modify its secondary structure. NCM reduced cell viability, and this effect was higher in the presence of those GAGs. NCM also increased DNA fragmentation, suggesting the occurrence of apoptosis, but without influence of GAGs. These results can contribute to the understanding of HNE modulation in physio- and pathological processes where this enzyme is involved.

1. Introduction

Human neutrophil elastase (HNE) is a serine protease able to cleave fibrous elastin and other extracellular matrix (ECM) molecules, which plays essential structural function in lungs, arteries, skin, and ligaments [1, 2]. This enzyme is primarily located in the acidophilic granules of polymorphonuclear leucocytes, and its release is involved in tissue destruction and inflammation, despite the presence of potent endogenous inhibitors [3]. It has been implicated in several pulmonary diseases including emphysema, cystic fibrosis, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and chronic obstructive disease [3–6]. Corroborating these findings, HNE levels are increased in serum and bronchoalveolar lavage (BAL) fluid in clinical studies and animal models of ALI/ARDS [7].

HNE release has been associated to death of different cell types [8] such as lung epithelial and endothelial cells [2, 9]. The cell death promoted by HNE fits in the concept of *anoïkis* (from the Greek, homeless), which was defined as apoptosis resulting from the disruption of cell-matrix interactions, mainly induced by proteases, and whose integrity is essential for survival of adherent cells [10].

Besides the inhibitory mechanisms involved in the controlling of protease activity [11], some anionic polysaccharides named glycosaminoglycans (GAGs) can selectively affect that proteolytic activity [12–14]. Although GAGs have been shown to be able to modulate the activity and also

modify the structure of different proteases [15, 16], there is relatively little information on the biological role and mechanism of interaction between proteases and GAGs. However, this seems particularly interesting for HNE, which is able to cleave components of ECM where there are great amounts of GAGs.

Since fibroblasts represent a cell type that synthesizes ECM molecules and collagen and are the most common cells of connective tissue and structural framework of animal organs, including lung, and HNE has been shown to induce death of lung cells; it became interesting to verify if this enzyme is able to trigger fibroblast death, which could also contribute for genesis of some lung diseases. In addition to ECM components secreted by fibroblasts, we aimed to study the effect of exogenous GAGs on HNE activity using a simplified cell culture system. Results presented here may open perspectives for a better understanding of HNE action and its modulation by GAGs, which have a crucial role in many diseases.

2. Material and Methods

This project was approved and conducted under the supervision of the Ethics Committee of Universidade Federal de São Paulo. Human neutrophils were collected from peripheral venous blood of healthy individuals. Human fibroblasts were isolated of muscle biopsies (biceps) from a healthy adult donor and kindly provided by Dr. Mariz Vainzof from Human Genome Research Center, Instituto de Biociências of Universidade de São Paulo. These cells were used at passages 2–5.

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypan blue solution, and dimethylsulfoxide (DMSO) were obtained from Invitrogen Life Technologies (San Diego, Calif, EUA). Hanks balanced salt solution (HBSS), trypsin-EDTA solution, penicillin-streptomycin, solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 ± 0.001 g/mL (Histopaque-1077), andpropidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Human neutrophil elastase, N-(methoxysuccinyl)-Ala-Ala-Pro-Val-p-nitroanilide (MeOSuc-Ala-Ala-Pro-Val-pNA), N-(methoxysuccinyl)-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (MeOSuc-Ala-Ala-Pro-Val-AMC) and phenylmethylsulfonyl fluoride (PMSF) were purchased from EMD Biosciences (San Diego, Calif, EUA). Bovine lung heparin (Hep, 9.5–12 kDa) was obtained from Opocrin Research Laboratory (Corlo, Mo, Italy), shark chondroitin 6-sulfate (C6S, 40–60 kDa) and bovine intestinal dermatan sulfate (DS, 21.5 kDa) from Seikagaku Kogyo Co. (Tokyo, Japan), and bovine chondroitin 4-sulfate (C4S, 27 kDa) from Sigma-Aldrich (St. Louis, Mo, USA). Bovine cartilage keratan sulfate (KS) [17], bovine lung heparan sulfate (HS, 30–40 kDa) [18], and elastase inhibitor (CeEI) from *Caesalpinia echinata* seeds (unpublished data) were purified in our laboratory.

2.1. Effect of GAGs in the Activity and Structure of HNE. HNE was previously titrated with CeEI, assuming a "slow-tight-binding" mechanism of enzyme and inhibitor forming a stoichiometric complex [19]. Briefly, HNE was preincubated in 200 mM Tris buffer pH 8.0, for 10 min at 37°C, with increasing amounts of CeEI, previously titrated using a standard trypsin solution. The residual activity of the enzyme was assayed with 0.20 mM MeOSuc-Ala-Ala-Pro-Val-pNA as substrate that hydrolysis was followed by the absorbance at 405 nm in a microplate reader Synergy HT (Biotek, Winooski, Vt, USA). Kinetic experiments were performed according to Gozzo et al. [12]. The enzyme (6.0 nM) was prewarmed in 200 mM Tris buffer pH 8.0 for 5 min at 37°C, in the absence or presence of Hep, HS, KS, DS, C4S, and C6S (300 nM). After that, MeOSuc-Ala-Ala-Pro-Val-pNA (0.10 to 2.5 mM) was added. The hydrolysis was followed by the increasing in the absorbance at 405 nm. The enzyme concentration for initial rate determinations was chosen at levels which would allow hydrolysis of less than 5.0% of the substrate [20]. Corresponding slopes were converted into μmol of hydrolyzed substrate/min, assuming 8,900 $M^{-1}\,cm^{-1}$ to be the p-nitroaniline molar extinction coefficient at 405 nm. The values of K_m, k_{cat}, V_{max}, and catalytic efficiency (k_{cat}/K_m) were determined according to Wilkinson [21].

Circular dichroism spectra were recorded in a JASCO-810 spectropolarimeter (Easton, Md, USA) equipped with a stopped flow chamber and thermostated cell holder. Far ultraviolet measurements (260–200 nm) were performed at 36°C scanning at rate of 50 nm/min on HNE solution (3.9 μM) in 0.05 cm-cells. Circular dichroism spectra of HNE-GAGs interaction were done in 200 mM Tris buffer pH 8.0. The observed ellipticity was normalized to units of degrees × cm^2/dmol. Base-line recordings in the absence or presence of GAGs (134 μM) were routinely made and used to correct HNE spectra, and they were analyzed for the percentage of secondary structural elements as previously described [22].

2.2. Cell Cultures, Neutrophils Isolation, and NCM Preparation. Fibroblasts were cultured in DMEM containing 4.0 mM glucose, 2.0 mM L-glutamine, 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and grown at 37°C in 5.0% CO_2. Fresh medium was replaced every two days. NCM was prepared according to Oltmanns et al. [8]. Neutrophils were obtained from heparinized peripheral venous blood (10 mL). Blood was layered onto a Histopaque-1077 and centrifuged at 500 ×g for 30 min. Neutrophils were sedimented on 3% dextran sulfate 500 and harvested as the cellular band above the red cell pellet. Cells were washed twice with HBSS and resuspended in DMEM without serum. This methodology allowed the preparation of neutrophils in high yield and at purity level greater than 96%. The viability of cells was higher than 99%, as judged by the ability of the cells to exclude trypan blue. NCM was obtained by culturing neutrophils (1×10^6 cell/mL) in serum-free DMEM for 24 h at 37°C. Supernatants were collected by centrifugation at 400 ×g for 10 min and cellular debris was discarded. The NCM was immediately used for the experiments. The activity of HNE in NCM was determined as described below and was adjusted to 8.0 and 15 nM.

Table 1: Effect of GAGs on the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA by HNE.

GAG	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (10^3 M^{-1} s^{-1})
No GAG	4.73 ± 0.38	0.854 ± 0.001	5.54
Hep	5.55 ± 0.58	1.11 ± 0.08	5.00
KS	3.75 ± 0.18	0.614 ± 0.001	6.11
C4S	3.20 ± 0.03	0.681 ± 0.004	4.70
HS	1.95 ± 0.12	0.468 ± 0.021	3.31
DS	1.83 ± 0.69	0.574 ± 0.088	3.19
C6S	1.49 ± 0.33	0.653 ± 0.001	2.28

HNE (6.0 nM) in 200 mM Tris buffer pH 8.0 was preincubated for 5 min at 37°C with or without GAGs (300 nM). After that, MeOSuc-Ala-Ala-Pro-Val-pNA (0.10 to 2.5 mM) was added, and the hydrolysis was followed by the absorbance in 405 nm. Hep: heparin; HS: heparan sulfate; KS: keratan sulfate; C4S: chondroitin 4-sulfate; C6S: chondroitin 6-sulfate; DS: dermatan sulfate.

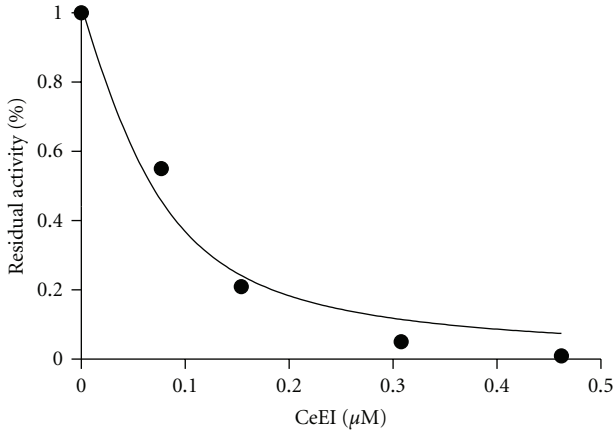

Figure 1: HNE titration by CeEI. HNE was preincubated in 200 mM Tris buffer pH 8.0, for 10 min at 37°C, with increasing amounts of CeEI, previously titrated using a standard trypsin solution. The residual activity of the enzyme was assayed with 0.20 mM MeOSuc-Ala-Ala-Pro-Val-pNA as substrate and followed by the absorbance at 405 nm.

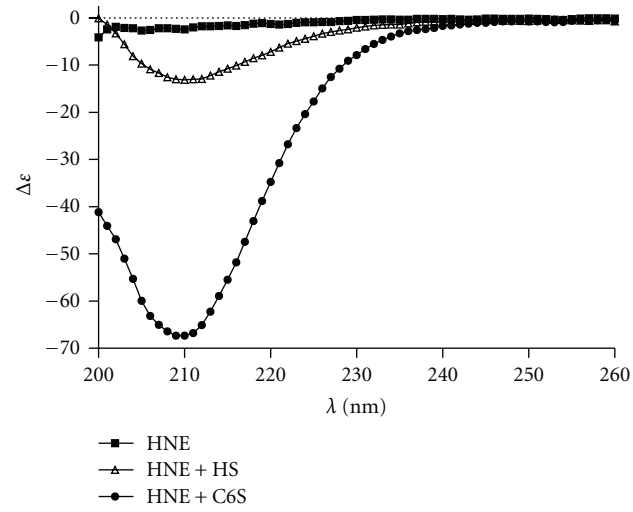

Figure 2: Effect of GAGs on HNE circular dichroism spectra. Spectra of HNE (3.9 μM) in absence or presence of GAGs (134 μM) were determined in 200 mM Tris buffer pH 8.0, at 260–200 nm and 36°C, as described in Section 2. Percentage values of secondary structures are shown in Table 2. HS and specially C6S changed the HNE secondary structure. HS: heparan sulfate; C6S: chondroitin 6-sulfate.

2.3. Detection of HNE Activity in NCM. The activity of HNE in NCM in different concentrations was detected using a fluorogenic peptide substrate- (MeOSuc-Ala-Ala-Pro-Val-AMC-) based assay and compared to a standard curve of the substrate hydrolysis by commercial HNE. Briefly, NCM was incubated with 100 mM HEPES buffer pH 7.5, 500 mM NaCl, 10% DMSO, and the substrate (4.0 μM) at 37°C. The substrate hydrolysis was followed fluorometrically (λ_{exc} = 360 nm and λ_{em} = 460 nm) in a Hitachi Spectrofluorimeter (Tokyo, Japan).

2.4. Cell Viability of NCM-Exposed Human Fibroblasts. Cells were growth until confluence and arrested by FBS deprivation for 24 h. Different amounts of NCM (in final volumes that correspond to HNE activity of 1.6, 2.0, 4.0, 8.0, and 15 nM) were used for screening tests to determine cell viability based on trypan blue exclusion assay. After 16 h of treatment, cells were washed twice with cold phosphate-buffered saline (PBS), harvested with 0.25% trypsin solution containing 2.0 mM EDTA, centrifuged at 400 ×g for 5 min and stained with 0.2% trypan blue solution. The unstained (live) and stained (dead) cells were counted and cell viability was expressed as percentage of total number of cells.

2.5. Effect of GAGs on Cell Viability and DNA Fragmentation. For flow cytometry experiments, cells were incubated with NCM (in final volumes that correspond to HNE activity of 8.0 and 15 nM) for 16 h, in absence or presence of C6S or HS (10 μM). After this period, cells were collected by trypsinization (0.25% trypsin, 2.0 mM EDTA), centrifuged at 400 ×g for 5 min at 4°C, and the pellet was resuspended in 500 μL PBS containing 4.0 μg/mL PI. Samples were kept in darkness for 30 min until the analysis by flow cytometry. Fluorescence was measured using the FL2 channel (orange-red fluorescence 585/42 nm).

For DNA fragmentation studies, fibroblasts were washed with PBS and suspended in the same buffer containing 0.01% Nonidet P-40 and 20 μg/mL PI. Cell suspensions were then kept in the dark for 30 min at 4°C. The extension of DNA

Table 2: Effect of GAGs on secondary structure of elastase.

Sample	α helix (%)	Antiparallel β sheet (%)	Parallel β sheet (%)	β turn (%)	Randon (%)	Total (%)
HNE	4.50	40.0	5.30	20.2	35.0	105
HNE + HS	11.3	29.2	5.50	20.0	35.5	101
HNE + C6S	86.5	0.200	2.90	2.9	2.30	94.7

HS: heparan sulfate; C6S: chondroitin 6-sulfate.

fragmentation was assessed by the hypodiploid peaks (sub-G1 peaks) on DNA histograms. A total of 10.000 events were analyzed for each sample in a FACScan flow cytometer (Becton-Dickinson, NJ, USA). Graphics were obtained by the program Cell Quest and analyzed using the free software WinMDI, version 2.8.

2.6. Statistical Analysis. Data of cell viability are presented as means ± S.D. of duplicates of at least three separate experiments. Differences between means were analyzed one-way analysis of variance followed by Tukey's multiple comparison tests. The level of significance was set at $\rho < 0.05$.

3. Results

3.1. Effect of GAGs in the Activity and Structure of Elastase. HNE was efficiently titrated by CeEI as showed in Figure 1. Michaelis-Menten plots were obtained for MeOSuc-Ala-Ala-Pro-Val-pNA hydrolysis by HNE without or with GAGs. The K_m value for the hydrolysis of the substrate by HNE in the absence of any GAGS was determined to be 0.854 mM, and the k_{cat} value obtained was 4.73 s^{-1}. Our results demonstrated that HS, DS, and C6S reduced the catalytic efficiency of HNE on the substrate hydrolysis more than 1.5-folds (1.7-, 1.7-, and 2.4-folds, resp.). Hep, C4S, and KS did have no effect on HNE activity (Table 1).

The interaction of GAGs with HNE was monitored by circular dichroism spectroscopy. The structural composition of the protein was estimated by the method of Bohm et al. [22] using a reference data set of 37 proteins. Figure 2 and Table 2 show that HS and C6S modify the secondary structure of HNE.

3.2. Effect of GAGs on Cell Viability and DNA Fragmentation of NCM-Exposed Human Fibroblasts. HNE-like peptidase activity in NCM in two different concentrations was determined in the MeOSuc-Ala-Ala-Pro-Val-AMC hydrolysis (Figure 3).

Different amounts of NCM (in final volumes that correspond to HNE activity of 1.6, 2.0, 4.0, 8.0, and 15 nM) were used for cell viability screening tests. Cell membrane integrity was reduced in the incubation of fibroblasts with NCM in a concentration-dependent manner, as verified by trypan blue exclusion assay. Unstained (live) and stained (dead) cells were counted, and cell viability was expressed as percentage of total number of cells (Figure 4).

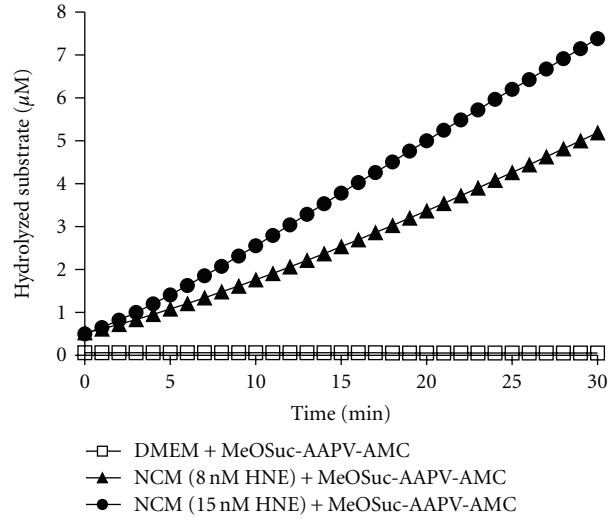

Figure 3: HNE-like peptidase activity in NCM. Supernatants from incubation of neutrophils with DMEM were kept in 100 mM HEPES buffer pH 7.5, containing 500 mM NaCl and 10% DMSO for 10 min at 37°C. After that, the substrate MeOSuc-Ala-Ala-Pro-Val-AMC (4.0 μM) was added. Substrate hydrolysis was followed by the increasing in the fluorescence (λ_{exc} = 360 nm and λ_{em} = 460 nm) in the spectrofluorimeter. HNE concentration in NCM was determined using a standard curve of MeOSuc-Ala-Ala-Pro-Val-AMC hydrolysis by commercial HNE.

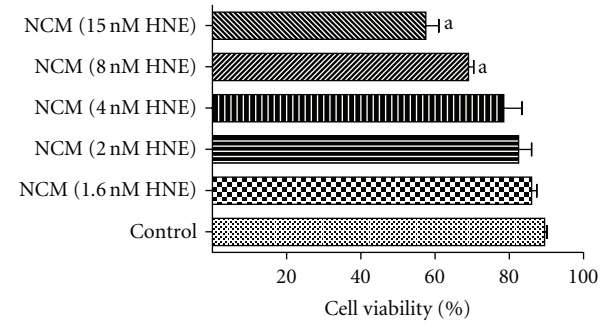

Figure 4: Cell viability of NCM-exposed fibroblasts. Different amounts of NCM were incubated with fibroblasts for 16 h in the cell culture conditions. Loss of cell membrane integrity was evaluated by trypan blue exclusion assay. Unstained (live) and stained (dead) cells were counted and cell viability was expressed as percentage of total number of cells. (a) statistically differs from control, $\rho < 0.05$. Cell viability was reduced in the incubation of fibroblasts with NCM, in a concentration-dependent manner, in comparison to untreated cells (Control).

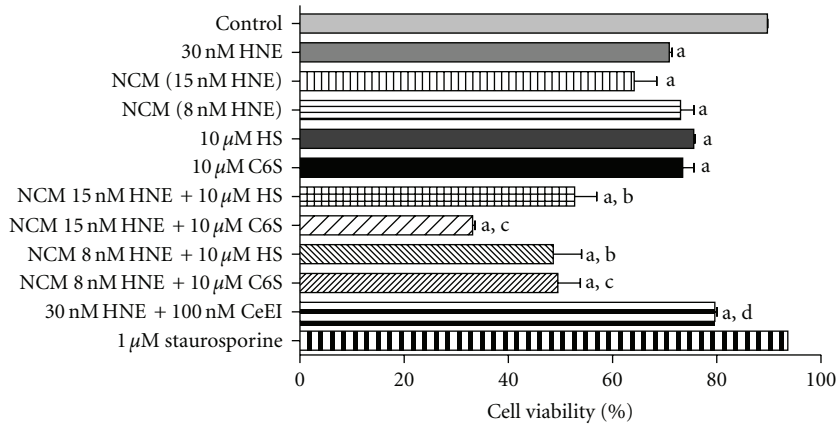

Figure 5: Effect of GAGs on membrane integrity of NCM-exposed fibroblasts. Cells were incubated with NCM (HNE activity of 8.0 or 15 nM), in the absence or presence of C6S or HS (10 μM), for 16 h in the cell culture conditions. After this period, cells were collected by trypsinization, stained by PI, and analyzed by flow cytometry. A total of 10,000 events were registered. (a) statistically differs from Control (untreated cells); (b) differs from NCM and HS groups; (c) differs from NCM and C6S groups; (d) differs from HNE group ($\rho < 0.05$).

NCM samples corresponding to HNE-like activity of 8.0 and 15 nM, without or with GAGs (10 μM), were used for flow cytometry experiments. HS and C6S statistically reduced cell viability by 24.5 and 26.6% in comparison to control as shown in Figure 5. NCM and commercial HNE also reduced fibroblasts viability, however, the mixture of NCM plus GAGs caused an important reduction of cell membrane integrity, which was significantly different from control and from groups with only GAGs or NCM. CeEI was able to inhibit the effect of commercial HNE. Staurosporine, a typical apoptosis inducer, did not cause changes in cell membrane integrity in the assay conditions.

Both cell viability and DNA fragmentation were statistically changed in the incubation with NCM (8.0 or 15 nM HNE). The mixture of NCM plus C6S or HS caused a more important reduction in the cell membrane integrity (51.4 and 50.5%, resp.), which was significantly different from control and from groups with only GAGs or NCM. DNA fragmentation was higher in NCM-treated cells (approximately 20.0%) compared to controls without NCM or GAGs alone (Figure 6). However, the percentage of apoptotic nuclei was almost not modified in the group incubated with NCM in the presence of HS or C6S. Staurosporine (1.0 μM) promoted DNA fragmentation in 33.5% of stimulated cells.

4. Discussion

Several evidences have suggested that some of lung diseases are caused by destruction of elastic fibers by elastase. Laurell and Eriksson [23] have firstly described five patients with deficiency of α1-antiprotease, an inhibitor of HNE; three of them presented emphysema. Subsequently, other investigators have instilled a variety of proteases into animal lungs [24–26] demonstrating that elastolytic proteases, including pancreatic elastase and HNE, were able to cause emphysema. These experiments provided the basis for the elastase-antielastase hypothesis, which states that the relative balance between elastases and their inhibitors determines the susceptibility of the lung to develop emphysema. This hypothesis remains a prevailing; however, other several important factors such as matrix metalloproteinases [27] and apoptosis [28] are considered to contribute to the development of the pulmonary destruction.

Although proteases such as HNE and their involvement in physio- and pathological processes are much studied, there is relatively little information on the biological role of the proteases interaction with carbohydrates and the mechanism of interaction between them. Nonetheless, it has been shown that GAGs are able to modulate the activity and also to change the structure of different proteases [15, 16]. Our group has already reported that GAGs influence the action of human plasma kallikrein on the hydrolysis of its natural substrate—kininogen—and in inflammation [12], affect the interaction of kallikrein with various substrates and inhibitors [13, 14] and interfere with the binding of the kininogen and prekallikrein on the surface of the endothelial cells [29].

Because of its strong cationic nature (it is consisted of 218 amino acids, being 19 arginine residues) it will be not surprise that HNE could interact with GAGs. In fact, this enzyme may bind to a variety of polyanions [30]. Once bound to HNE or elastase from hamster or rat, GAGs are able to inhibit the proteolytic activity of the enzyme [31, 32], as well as prevent HNE release from leukocytes [33].

Our results have showed that GAGs were able to modify the catalytic efficiency of HNE on small peptide substrate hydrolysis. This efficiency was not affected in the presence of Hep, KS, or C4S, but was reduced by 1.7-, 1.7-, and 2.4-folds in the presence of HS, DS, and C6S, respectively. These results are in agreement with the obtained by Walsh et al. [32] and Baici and Bradamante [34], showing that reactions involving HNE are slower in the presence of GAGs and this fact could be responsible for the protection of the tissue from the deleterious effect of this enzyme in the disease.

The mechanism by which GAGs reduced HNE activity is not known. However, several mechanisms can be postulated

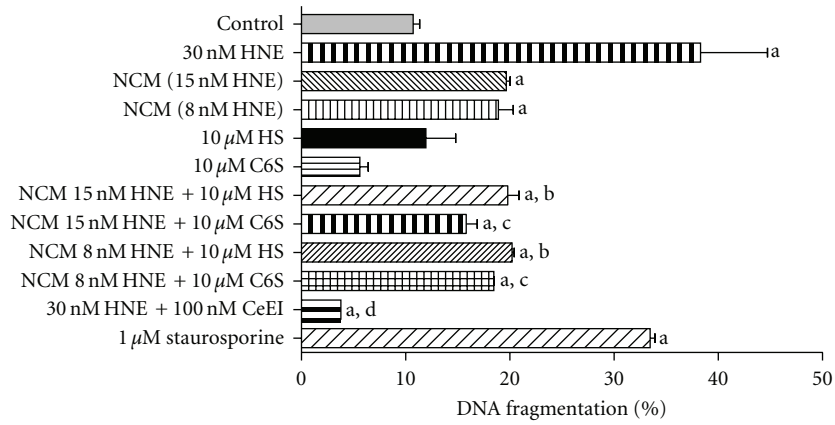

Figure 6: Effect of GAGs on DNA fragmentation of NCM-exposed fibroblasts. Cells were incubated with NCM (HNE activity of 8.0 or 15 nM), in the absence or presence of C6S or HS (10 μM), for 16 h in the cell culture conditions. After this period, cells were collected by trypsinization, stained by PI, in the presence of Nonidet P-40, and analyzed by flow cytometry. A total of 10,000 events were registered. (a) statistically differs from Control (untreated cells); (b) differs from HS group; (c) differs from C6S group; (d) differs from HNE group ($\rho < 0.05$).

and one of them is that GAGs may induce conformational changes in the enzyme, rendering it less active. To confirm it, we tested the effect of GAGs on elastase secondary structure by circular dichroism. We observed that HS, and specially C6S, increased α-helix and reduced β-sheet content in HNE. Regarding to that, some authors have related that heparin can increase ordered structure in proteins, usually inducing significant α-helix conformation [15, 35, 36].

Since HNE activity was *in vitro* modulated by GAGs and soluble fragments of GAGs are generated by proteoglycans cleavage in HNE-mediated inflammatory processes [37], we tested the ability of these compounds to affect the enzyme activity on the cell death induction, using a simplified cell culture system. Fibroblasts were the chosen cell type because they are able to produce ECM molecules. Also, HS and C6S were the chosen GAGs because they presented higher effect of HNE action *in vitro*.

HNE was obtained from neutrophils in NCM as this methodology appeared to be an easy procedure to obtain the necessary high amounts of the enzyme. In fact, cathepsin-like activity was also detected in that medium; however, the use of a specific elastase inhibitor (CeEI) in cell culture experiments confirmed that the observed events were mainly mediated by HNE.

NCM has similar effect of commercial HNE, which was inhibited by CeEI. Staurosporine, a typical apoptosis inducer, has no effect in the cell viability in the assay conditions.

To better characterize fibroblasts death after incubation with NCM, cell membrane integrity and DNA fragmentation were evaluated by flow cytometry, since those methods have been used with good results as indicators of the occurrence of necrosis and apoptosis, respectively, in different cell types. Based on flow cytometry results, we provided evidence that HNE is able to induce cell death of human fibroblasts. Addition of NCM, containing 8.0 or 15 nM HNE, caused a significant reduction (73.0 and 64.2%, resp.) in the fibroblasts membrane integrity. However, the mixture of NCM plus HS or C6S caused a more important reduction in the cell membrane integrity (51.4 and 50.5%, resp.), which was significantly different from control and from groups with only GAGs or NCM. On the other hand, NCM alone (HNE activity of 8.0 or 15 nM) promoted an increase in DNA fragmentation, an effect that was not modified by GAGs. When commercial HNE was used (about 32 nM), it was observed an increase in the percentage of cells with fragmented DNA (2.8 folds), which was prevented by CeEI. Staurosporine, which is an agent that typically induces apoptosis in several cell types, was used as control and for the characterization of the events involved in cell death. Cells incubated with staurosporine did not display important alterations in membrane integrity; nevertheless, DNA fragmentation was a remarkable event in these cells.

It has been shown that HNE cleaves important molecules such as fibronectin, thrombospondin, and *von Willebrand* factor, participating actively in the loss of cell-matrix interactions [1] and contributing for *anoïkis*, which is a reasonable hypothesis to explain the effect of HNE in the induction of cell death. Other enzymes such as matrix metalloproteases (MMPs) also have been shown to be involved in the apoptotic cell death [10]. In this context, Isnard et al. [38] verified that incubation of some GAGs, including HS, CS, and DS, to the corneal explants cultures promoted an increase in the expression and activation of MMPs. This effect, which is mediated through the binding of the GAGs to CD44 receptor—the major receptor for hyaluronan, was not correlated to cell death by those authors. However, the upregulation of MMP induced by GAGs may contribute for the results found in our work; even MMP expression and activation were not evaluated.

Regarding to the molecular mechanisms involved in HNE-induced cell death, it has been also showed that this enzyme can activate the apoptotic program via mitochondrial membrane permeability changes, which is accompanied by an early increase followed by a decrease in the phosphorylation of epithelial Akt, an important kinase involved in cell survival [39].

Although the molecular events involved in the cell death induced by HNE have not so far been clarified, our data suggest that HNE and GAGs are able to promote fibroblasts *anoikis* in culture. Nevertheless, to analyze fibroblast death in the current model we have to keep in mind the GAGs action on HNE activity, HNE effect on cell-matrix interactions that are crucial for cell survival, and also a possible MMP induction by GAGs. Combination of all these effects might contribute to the results presented here, which open perspectives for the best understanding of the HNE involvement in several patho- and physiological processes as well as for the development of agents capable to interfere in those processes.

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References

[1] A. Bonnefoy and C. Legrand, "Proteolysis of subendothelial adhesive glycoproteins (fibronectin, thrombospondin, and von Willebrand factor) by plasmin, leukocyte cathepsin G, and elastase," *Thrombosis Research*, vol. 98, no. 4, pp. 323–332, 2000.

[2] K. Kawabata, T. Hagio, and S. Matsuoka, "The role of neutrophil elastase in acute lung injury," *European Journal of Pharmacology*, vol. 451, no. 1, pp. 1–10, 2002.

[3] W. L. Lee and G. P. Downey, "Leukocyte elastase: physiological functions and role in acute lung injury," *American Journal of Respiratory and Critical Care Medicine*, vol. 164, no. 5, pp. 896–904, 2001.

[4] M. Horwitz, F. Q. Li, D. Albani et al., "Leukemia in severe congenital neutropenia: defective proteolysis suggests new pathways to malignancy and opportunities for therapy," *Cancer Investigation*, vol. 21, no. 4, pp. 579–587, 2003.

[5] T. J. Moraes, C. W. Chow, and G. P. Downey, "Proteases and lung injury," *Critical Care Medicine*, vol. 31, no. 4, pp. S189–S194, 2003.

[6] P. P. Scaglioni and P. P. Pandolfi, "Taking apart a cancer protein," *Nature*, vol. 426, no. 6966, pp. 512–513, 2003.

[7] P. M. Suter, S. Suter, E. Girardin, P. Roux-Lombard, G. E. Grau, and J. M. Dayer, "High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis," *American Review of Respiratory Disease*, vol. 145, no. 5, pp. 1016–1022, 1992.

[8] U. Oltmanns, M. B. Sukkar, S. Xie, M. John, and K. F. Chung, "Induction of human airway smooth muscle apoptosis by neutrophils and neutrophil elastase," *American Journal of Respiratory Cell and Molecular Biology*, vol. 32, no. 4, pp. 334–341, 2005.

[9] T. Suzuki, T. J. Moraes, E. Vachon et al., "Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells," *American Journal of Respiratory Cell and Molecular Biology*, vol. 33, no. 3, pp. 231–247, 2005.

[10] F. Mannello, F. Luchetti, E. Falcieri, and S. Papa, "Multiple roles of matrix metalloproteinases during apoptosis," *Apoptosis*, vol. 10, no. 1, pp. 19–24, 2005.

[11] I. Cruz-Silva, A. J. Gozzo, V. A. Nunes et al., "A proteinase inhibitor from *Caesalpinia echinata* (pau-brasil) seeds for plasma kallikrein, plasmin and factor XIIa," *Biological Chemistry*, vol. 385, no. 11, pp. 1083–1086, 2004.

[12] A. J. Gozzo, V. A. Nunes, A. K. Carmona et al., "Glycosaminoglycans affect the action of human plasma kallikrein on kininogen hydrolysis and inflammation," *International Immunopharmacology*, vol. 2, no. 13-14, pp. 1861–1865, 2002.

[13] A. J. Gozzo, V. A. Nunes, H. B. Nader et al., "Glycosaminoglycans affect the interaction of human plasma kallikrein with plasminogen, factor XII and inhibitors," *Brazilian Journal of Medical and Biological Research*, vol. 36, no. 8, pp. 1055–1059, 2003.

[14] A. J. Gozzo, V. A. Nunes, I. Cruz-Silva et al., "Heparin modulation of human plasma kallikrein on different substrates and inhibitors," *Biological Chemistry*, vol. 387, no. 8, pp. 1129–1138, 2006.

[15] P. C. Almeida, I. L. Nantes, J. R. Chagas et al., "Cathepsin B activity regulation. Heparin-like glycosaminoglycans protect human cathepsin B from alkaline pH-induced inactivation," *Journal of Biological Chemistry*, vol. 276, no. 2, pp. 944–951, 2001.

[16] I. L. S. Tersariol, D. C. Pimenta, J. R. Chagas, and P. C. Almeida, "Proteinase activity regulation by glycosaminoglycans," *Brazilian Journal of Medical and Biological Research*, vol. 35, no. 2, pp. 135–144, 2002.

[17] Y. M. Michelacci and D. S. P. Q. Horton, "Proteoglycans from the cartilage of young hammerhead shark *Sphyrna lewini*," *Comparative Biochemistry and Physiology. Part B*, vol. 92, no. 4, pp. 651–658, 1989.

[18] H. B. Nader, T. M. P. C. Ferreira, and J. F. Paiva, "Isolation and structural studies of heparan sulfates and chondroitin sulfates from three species of molluscs," *Journal of Biological Chemistry*, vol. 259, no. 3, pp. 1431–1435, 1984.

[19] J. F. Morrison, "The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions," *Trends in Biochemical Sciences*, vol. 7, no. 3, pp. 102–105, 1982.

[20] J. R. Chagas, L. Juliano, and E. S. Prado, "Intramolecularly quenched fluorogenic tetrapeptide substrates for tissue and plasma kallikreins," *Analytical Biochemistry*, vol. 192, no. 2, pp. 419–425, 1991.

[21] G. N. Wilkinson, "Statistical estimations in enzyme kinetics," *The Biochemical journal*, vol. 80, pp. 324–332, 1961.

[22] G. Bohm, R. Muhr, and R. Jaenicke, "Quantitative analysis of protein far UV circular dichroism spectra by neural networks," *Protein Engineering*, vol. 5, no. 3, pp. 191–195, 1992.

[23] C.B. Laurell and S. Eriksson, "The electrophoretic alpha-globulin pattern of serum in alpha-antitrypsin deficiency," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 15, pp. 132–140, 1963.

[24] C. Kuhn, S. Y. Yu, and M. Chraplyvy, "The induction of emphysema with elastase. II. Changes in connective tissue," *Laboratory Investigation*, vol. 34, no. 4, pp. 372–380, 1976.

[25] R. M. Senior, H. Tegner, and C. Kuhn, "The induction of pulmonary emphysema with human leukocyte elastase," *American Review of Respiratory Disease*, vol. 116, no. 3, pp. 469–475, 1977.

[26] G. L. Snider, E. C. Lucey, and T. G. Christensen, "Emphysema and bronchial secretory cell metaplasia induced in hamsters by human neutrophil products," *American Review of Respiratory Disease*, vol. 129, no. 1, pp. 155–160, 1984.

[27] S. D. Shapiro, D. K. Kobayashi, and T. J. Ley, "Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages," *Journal of Biological Chemistry*, vol. 268, no. 32, pp. 23824–23829, 1993.

[28] K. Aoshiba, N. Yokohori, and A. Nagai, "Alveolar wall apoptosis causes lung destruction and emphysematous changes," *American Journal of Respiratory Cell and Molecular Biology*, vol. 28, no. 5, pp. 555–562, 2003.

[29] A. J. Gozzo, G. Motta, I. Cruz-Silva et al., "Heparin affects the interaction of kininogen on endothelial cells," *Biochimie*, vol. 93, no. 10, pp. 1839–1845, 2011.

[30] N. Volpi, "Inhibition of human leukocyte elastase activity by heparins: influence of charge density," *Biochimica et Biophysica Acta*, vol. 1290, no. 3, pp. 299–307, 1996.

[31] F. Redini, J. M. Tixier, M. Petitou, J. Choay, L. Robert, and W. Hornebeck, "Inhibition of leucocyte elastase by heparin and its derivatives," *Biochemical Journal*, vol. 252, no. 2, pp. 515–519, 1988.

[32] R. L. Walsh, T. J. Dillon, R. Scicchitano, and G. McLennan, "Heparin and heparan sulphate are inhibitors of human leucocyte elastase," *Clinical Science*, vol. 81, no. 3, pp. 341–346, 1991.

[33] D. Mikulikova and K. Trnavsky, "Influence of a glycosaminoglycan polysulfate (Arteparon®) on lysosomal enzyme release from human polymorphonuclear leukocytes," *Zeitschrift fur Rheumatologie*, vol. 41, no. 2, pp. 50–53, 1982.

[34] A. Baici and P. Bradamante, "Interaction between human leukocyte elastase and chondroitin sulfate," *Chemico-Biological Interactions*, vol. 51, no. 1, pp. 1–11, 1984.

[35] A. D. Cardin, D. A. Demeter, H. J. R. Weintraub, and R. L. Jackson, "Molecular design and modeling of protein-heparin interactions," *Methods in Enzymology*, vol. 203, pp. 556–583, 1991.

[36] R. Tyler-Cross, M. Sobel, D. Marques, and R. B. Harris, "Heparin binding domain peptides of antithrombin III: analysis by isothermal titration calorimetry and circular dichroism spectroscopy," *Protein Science*, vol. 3, no. 4, pp. 620–627, 1994.

[37] M. Bernfield, M. Götte, P. W. Park et al., "Functions of cell surface heparan sulfate proteoglycans," *Annual Review of Biochemistry*, vol. 68, pp. 729–777, 1999.

[38] N. Isnard, L. Robert, and G. Renard, "Effect of sulfated GAGs on the expression and activation of MMP-2 and MMP-9 in corneal and dermal explant cultures," *Cell Biology International*, vol. 27, no. 9, pp. 779–784, 2003.

[39] H. H. Ginzberg, P. T. Shannon, T. Suzuki et al., "Leukocyte elastase induces epithelial apoptosis: role of mitochondial permeability changes and Akt," *American Journal of Physiology*, vol. 287, no. 1, pp. G286–G298, 2004.