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The effects of hyaluronan and its fragments on lipid models exposed to UV irradiation

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Abstract

The effects of hyaluronan and its degradation products on irradiation-induced lipid peroxidation were investigated. Liposomal skin lipid models with increasing complexity were used. Hyaluronan and its fragments were able to reduce the amount of lipid peroxidation secondary products quantified by the thiobarbituric acid (TBA) assay. The qualitative changes were studied by mass spectrometry. To elucidate the nature of free radical involvement electron paramagnetic resonance (EPR) studies were carried out. The influence of hyaluronan and its fragments on the concentration of hydroxyl radicals generated by the Fenton system was examined using the spin trapping technique. Moreover, the mucopolysaccharide's ability to react with stable radicals was checked. The quantification assay of 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) showed no concentration changes of the stable radical caused by hyaluronan. Hyaluronan was found to exhibit prooxidative effects in the Fenton assay in a concentration dependent manner. A transition metal chelation was proposed as a mechanism of this behavior. Considering human skin and its constant exposure to UV light and oxygen and an increased pool of iron in irradiated skin the administration of hyaluronan or its fragments in cosmetic formulations or sunscreens could be helpful for the protection of the human skin.

Keywords: Hyaluronan; Thiobarbituric acid assay; Mass spectrometry; Electron paramagnetic resonance spectroscopy; Stratum corneum lipids; Ultraviolet irradiation

1. Introduction

Hyaluronic acid was first mentioned by Meyer and Palmer (1934). They discovered a glassy fluid in the bovine vitreous humor and named it because of its physicochemical behavior [hyalos—Greek for glassy] and the presence of an uronic acid. Later on hyaluronic acid was isolated from various tissues such as rooster comb, monkey foreskin, synovial fluid and umbilical cord (Balazs, 1970).

Balazs et al. suggested the use of the name hyaluronan when the polysaccharide is mentioned in general terms (Balazs et al., 1986). That way the difficulty

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of identifying the counter ion of hyaluronic acid salts is avoided and the occurrence of the glycosaminoglycan as a polyanion in vivo is considered (Hascall and Laurent, 1997). Therefore, in this paper the terms hyaluronic acid and hyaluronan are used interchangeably.

Hyaluronic acid is a linear polysaccharide containing alternating 1-β-4 linked units of 1-β-3 linked *N*-acetyl-D-glucosamine and D-glucuronic acid (Brimacombe and Webber, 1964). The glycosaminoglycan is an important chemical compound for living things (Lee and Spicer, 2000) and has got a lot of different functions which are not understood in detail yet (Laurent and Fraser, 1992). These functions range from simple ones like the function as a mechanical viscoelastic lubricant up to complex regulatory tasks controlling the cell activity (Fraser et al., 1997). Because of its high water binding properties hyaluronan along with elastin, urea and collagen is part of the natural moisturizing factor of the human skin. Therefore, some cosmetic formulations contain hyaluronan as a moisturizing agent (Raab and Kindl, 1997) but its functions in the human skin are much more complicated and also include wound healing (Goa and Benfield, 1994) and pathogenetic controlling of skin diseases (Tammi et al., 1994).

Recently, hyaluronan was found to occur also in the human stratum corneum (Sakai et al., 2000) raising the question for its function in the horny layer. Is hyaluronan acting as a radical scavenger in the skin under physiological conditions (Myint et al., 1987; Laurent et al., 1996) or is the mucopolysaccharide just another biomolecule which can be degraded by reactive oxygen species (ROS) and, therefore, a kind of a radical scavenger analogue (Kreisl, 1982)? Often high molecular weight hyaluronan is used as a tool to test the antioxidative activity of drugs (Orvisky et al., 1997). The ability of substances to protect hyaluronan from depolymerization is regarded as a measure for the free radical scavenging properties of the compounds tested (Facino et al., 1996). However, at present even the exact function of hyaluronan in the area of its main occurrence, the synovial fluid, is still under debate. The speculations include the role as a molecular sieve (Evered and Whelan, 1989) and the function as an anti-inflammatic substance (Saari, 1992).

In this paper the properties of hyaluronan and its fragments are studied using stratum corneum lipid model systems to test the effects of the polysaccharides upon exposure to UV light. The lipid models were used to avoid effect overlapping which could be the case when using native stratum corneum or skin. With liposomes closely resembling multilayer membrane structures it renders them an excellent model for in vitro investigations (Trommer et al., 2002).

As the biggest organ of the human body, the skin is constantly exposed to both ultraviolet radiation and oxygen. Hereby, transition metal ions play a key role because they are able to catalyze the biomolecule damaging effects of UV light. Iron is the most ubiquitous transition metal and its involvement in UV induced free radical generation has been shown (Buettner and Jurkiewicz, 1996). Therefore, iron ions have been added to the lipid samples.

The degradation of hyaluronan caused by UV-light was studied using electrospray ionization mass spectrometry (ESI-MS). The advantages of the soft electrospray ionization (ESI) avoiding early fragmentation and the ion trap possibilities of full scan mass spectrometry (MS), MS/MS and multiple stage MS experiments (Papac and Shahrokh, 2001) allowed the detection of the hyaluronan decomposition products.

Electron paramagnetic resonance (EPR) spectroscopy is more and more used in pharmaceutical research (Katzhendler et al., 2000; Kroll et al., 2001) because of the unique information which can be obtained by EPR spectra of both in vivo and in vitro experiments (Maeder, 1998). We used the spin trapping method by 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) to detect the highly reactive hydroxyl radicals (Gunther et al., 1998). The influence of hyaluronan and its fragments on the concentration of these free radicals generated by the Fenton system, as well as the mucopolysaccharide's properties regarding the stable 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical, were studied.

The aims of the present paper can be summarized as the following: (A) quantification of the effects of hyaluronan and hyaluronan fragments on skin lipid models after UV exposure in the presence of iron ions by the thiobarbituric acid (TBA) assay, (B) measurement of the qualitative changes of hyaluronan after UV irradiation by MS, (C) use of EPR spectroscopy to test the effects of hyaluronan on ROS and on stable free radicals.

2. Materials and methods

2.1. Reagents

α-Linolenic acid (LLA), L-α-dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), ceramide IV (C IV; *N*-2-hydroxyacyl-sphingosine from bovine brain), ferrous sulfate, ferrous chloride, malondialdehyde-bis-(dimethylacetal), 2-thiobarbituric acid and trichloroacetic acid for the TBA assay (all of analytical grade) were obtained from Sigma (Deisenhofen, Germany) as well as DPPH, DMPO, adenosine 5′-diphosphate sodium salt (ADP) as a chelating agent and 2,2,6,6-tetramethyl-1-piperidyl-*N*-oxyl (TEMPO) for EPR investigations. Analytical grade ceramide III (C III; *N*-stearyl-phytosphingosine) was provided by Cosmoferm (Delft, The Netherlands). Methanol of gradient grade and chloroform (LiChrosolv®) were purchased from Merck (Darmstadt, Germany).

2.2. Hyaluronan and hyaluronan fragments

Hyaluronic acid and the fragments from enzymatic degradation were a gift from the Institute of Experimental Microbiology, Jena. The source of the polysaccharides was the highly productive microorganism *Streptococcus zooepidemicus*. The biotechnological production of the hyaluronan was carried out via fermentation by a cyclic batch high performance procedure. A detailed description of this method as well as the parameters influencing the fermentation process are published (Tan et al., 1990). For the enzymatic degradation 10.0 g of hyaluronan were preincubated in 5.0 l pH 6 acetate buffer (0.05 mM) at 37 °C. Afterwards 0.5 l acetate buffer (37 °C) containing 10,000 units hyaluronate lyase of *Streptococcus agalactiae* were added. The termination of the enzymatic reaction was attained by fast heating (90 °C) for 10 min after the desired incubation times. The non-enzymatically exposed macromolecule was heated as well. After cooling the fragments were lyophilized. Molecular weight determinations (Table 1) were carried out by laser light scattering. Further characterization of the hyaluronan and its fragments was carried out by our group recently (Alkrad et al., 2002; Kühn et al., 2002).

For mass spectrometric investigations a fragment of a molecular weight of 22 kDa from the same source as described above has been used. The smaller fragments resulting from the enzymatic degradation process were not separated to allow detection in the mass range of the mass spectrometer used between *m*/*z* 50 and 2000.

Table 1
Characterization of the hyaluronan and the fragments used (additionally a fragment with the molecular weight of 22 kDa was used for the mass spectrometric investigations)

Abbreviation	Incubation with hyaluronate lyase (min)	Molecular weight (g/mol)
HYA 0	0	1×10^6
HYA 15	15	6×10^5
HYA 30	30	3.3×10^5
HYA 60	60	3.2×10^5
HYA 120	120	2.2×10^5

The high viscosity of the native hyaluronic acid and of the fragments with high molecular weights was narrowing the choice of the concentrations for the experiments. For the EPR investigations a concentration range consisting of 0.02, 0.04, 0.06, 0.08 and 0.1 μM solutions was used. Malondialdehyde (MDA) determinations via the TBA assay were carried out using 0.1 μM hyaluronan or hyaluronan fragment solutions.

2.3. Sample preparation

Lipid model systems were chosen for our experiments to allow investigations on a molecular level. There were two standards the systems should meet. They should be simple to allow mechanistic investigations and avoid overlapping of effects which would make data interpretation difficult or impossible. Despite this simplicity the main properties of the stratum corneum intercellular lipid matrix should be present. The lipids for the experiments were chosen according to the lipid composition of the horny layer lipid matrix. Chol, C III and C IV as sphingolipid models, LLA and DPPC as a liposome generator were used.

The simple system was an oil in water dispersion of LLA obtained by shaking the system for 120 min using an laboratory flask shaker (GFL 3006, Gesellschaft für Labortechnik, Burgwedel, Germany).

The complex systems were prepared as liposomes using the thin layer method. The lipids were dissolved in 0.5 ml chloroform. The solvent was removed using a rotation vaporizer (Labo-Rota C-311, Resona Technics, Switzerland) and a vacuum pump (PIZ 100 Mini-Tower-MPC 050-Z, Saskia Hochvakuum- und Labortechnik, Ilmenau, Germany). To obtain a thin

Table 2
The systems studied (additionally 10 μM $FeSO_4$ were added to each sample)

System	Lipids	Concentration (μM)	Manufacturing process
Simple system (system A)	α-Linolenic acid	100	Shaking for 120 min
Complex system (system B)	α-Linolenic acid	100	Liposomes prepared by the thin film method
	DPPC	200	
	Cholesterol	100	
Complex system with ceramides			
Ceramide III (system C)	α-Linolenic acid	100	Liposomes prepared by the thin film method
	DPPC	200	
	Cholesterol	100	
	Ceramide III	100	
Ceramide IV (system D)	α-Linolenic acid	100	Liposomes prepared by the thin film method
	DPPC	200	
	Cholesterol	100	
	Ceramide IV	100	

and homogeneous lipid film a fast rotation was chosen (150 rpm). The remaining lipid film was dispersed in 20 ml of double distilled water by shaking intensively for 360 min. The remaining double distilled water was added to achieve the final concentration. The concentrations of the ingredients in the liposome stock solutions were: DPPC 1.0 mM; Chol, LLA, C III and C IV 0.5 mM.

To obtain vesicles of a uniform size the stock solution was passed through a 400 nm polycarbonate filter (Costar, Cambridge, UK) at 20 °C under nitrogen pressure using an extruder device (Lipex Biomembranes, Vancouver, BC, Canada). This step was repeated five times.

The exact composition of the samples as well as an overview of the manufacturing processes are shown in Table 2.

Ten micromole ferrous sulfate was added to the samples as an electron donator and catalyst of the Haber–Weiss reaction to initiate ROS generation via a Fenton type reaction. Previous investigations have shown that there is no effect when irradiating lipid model systems without transition metal catalysts (Trommer et al., 2001).

All the liposome suspensions and fatty acid dispersions were freshly prepared just before use.

2.4. Particle size determination

To control the vesicle size and the success of extrusion, the particle diameters were determined via photone correlation spectroscopy using the Malvern Instruments Autosizer 2c equipped with a series 7032 Multi-8 Correlator (Malvern, Worcester, UK). A detailed examination and interpretation of the influences of UV irradiation on the particle size distribution has been published recently (Trommer et al., 2001).

2.5. Ultraviolet irradiation

UV-B irradiation experiments were carried out using a UV irradiation chamber (Dr. Gröbel UV-Elektronik, Ettlingen, Germany) enabling a selective exposure to UV-B because of the special lamp F15/T8 15 W with a main emission range of 290–320 nm (Sankyo Co., Tokyo, Japan). Prior to irradiation, 5 ml sample (concentrations as given in Table 2) were transferred to 55 mm open glass dishes. Thereby, the optical pathlength was 2.1 mm and a homogeneous exposure was assumed in spite of light scattering. The samples were treated with an UV-B dose of 0.25 J/cm^2 which corresponds approximately with the two- to three-fold of the minimal erythemal dose (MED) of humans. This high dosage was required to test under high stress conditions.

2.6. Thiobarbituric acid assay

The TBA test is a quantitative assay for the detection of MDA, and is the most widely used technique to determine lipid peroxidation products.

In this study, the Buege–Aust method was used (Buege and Aust, 1978). Briefly, 2 ml of a stock TBA reagent containing 15% (w/v) trichloroacetic acid in 0.25 M HCl and 0.37% (w/v) thiobarbituric acid in 0.25 M HCl was added to 1 ml of the UV-B treated sample. After heating at 90 °C for 15 min and cooling down, the red TBA:MDA complex (2:1) appears allowing fluorescence measurement. A HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with an auto sampler AS-4000A, interface D-6000A, pump L-6200A, UV-Vis-Detector L-4250, fluorescence detector F-1080 was used to quantify the pigment. A reversed phase column (LiChrospher® 100, RP 8, particle size 5 μm) was used with a mobile phase methanol/water 30:70 for HPLC procedure. The wavelength for excitation was 515 nm and the emission measurement was performed at 555 nm.

A calibration curve was generated by preparing an aqueous solution of malondialdehyde-bis-(dimethylacetal). Under acidic conditions the acetal groups were hydrolyzed and MDA was formed.

2.7. Mass spectrometry

The mass spectrometric operations were carried out using a LCQ ion trap mass spectrometer with ESI interface and integrated syringe pump (Finnigan MAT, San Jose, CA, USA). ESI-MS was performed in the negative ion mode with an ESI voltage of −4.5 kV and a heated capillary temperature of 200 °C. The aqueous samples were mixed with methanol to obtain a stable ESI spray. Then the solution was injected via syringe pump (10 μl/min).

2.8. Electron paramagnetic resonance spectroscopy

EPR studies were carried out in the X-Band using a Bruker ESP 380 E FT-EPR spectrometer (Bruker, Rheinstetten, Germany). The quantitative investigations were performed using the double resonator technique in the rectangular cavity ER 4105 DR with corresponding aqueous sample cells ER 160 FC (Rototec Spintec, Spektroskopie Vertriebs-GmbH, Remshalden). The double resonator settings were: microwave frequency 9.78 GHz; microwave power 13.6 mW; modulation frequency 100 kHz; modulation amplitude 0.3 mT; sweep width 15 mT; conversion time 40.96 ms; time constant 10.24 ms; sweep time 41.94 s. For evaluating the hydroxyl radical scavenging activity, the spin trapping method was used. Therefore, the reactive hydroxyl radicals were produced via the Fenton system consisting of hydrogen peroxide and iron(II) ions and trapped by DMPO. Before transferring the specimen into the cavity 1 ml of each stock solution was added in the order: (I) ADP (2 mM), (II) ferrous sulfate (0.5 mM), (III) test substance or double distilled water as a blank control, (IV) the spin trap DMPO (40 mM), (V) H_2O_2 (0.3%).

Exactly 120 s after the addition of hydrogen peroxide the recording of the spectra was started measuring the specimen in the resonator 1 of the cavity and TEMPO (50 μM in methanol) as the reference in the resonator 2 of the double resonator.

The double resonator technique is a method to study aqueous samples in a reproducibly quantitative manner. Every measured signal was set in proportion to a reference signal of TEMPO to avoid analytical artifacts due to changes in the Q-factor of the resonator.

DPPH (0.1 mM in methanol) was chosen as a stable free radical. We mixed different concentrations of hyaluronan or fragments (a range from 0.02 to 0.1 μM; 2.0 ml of each specimen) with 3.0 ml of the DPPH stock solution and started the spectrum recording again after 120 s against TEMPO as a reference sample in the double resonator. All signals are the results of five time accumulations.

The quantitative analysis of the EPR spectra were carried out by double integration of the EPR signals using the WinEPR software (Bruker, Rheinstetten, Germany).

3. Results and discussion

3.1. Thiobarbituric acid assay

Fig. 1 shows the results of the thiobarbituric acid assay of the stratum corneum lipid systems. The effects of 0.1 μM hyaluronan or hyaluronan fragments on a LLA dispersion are shown in Fig. 1A. Secondary lipid peroxidation products (measured as MDA units) are significantly decreased. The TBA reaction product concentration level is decreased by hyaluronan from 675 ng/ml for the irradiated sample to 260 ng/ml for the sample containing 0.1 μM of HYA 0.

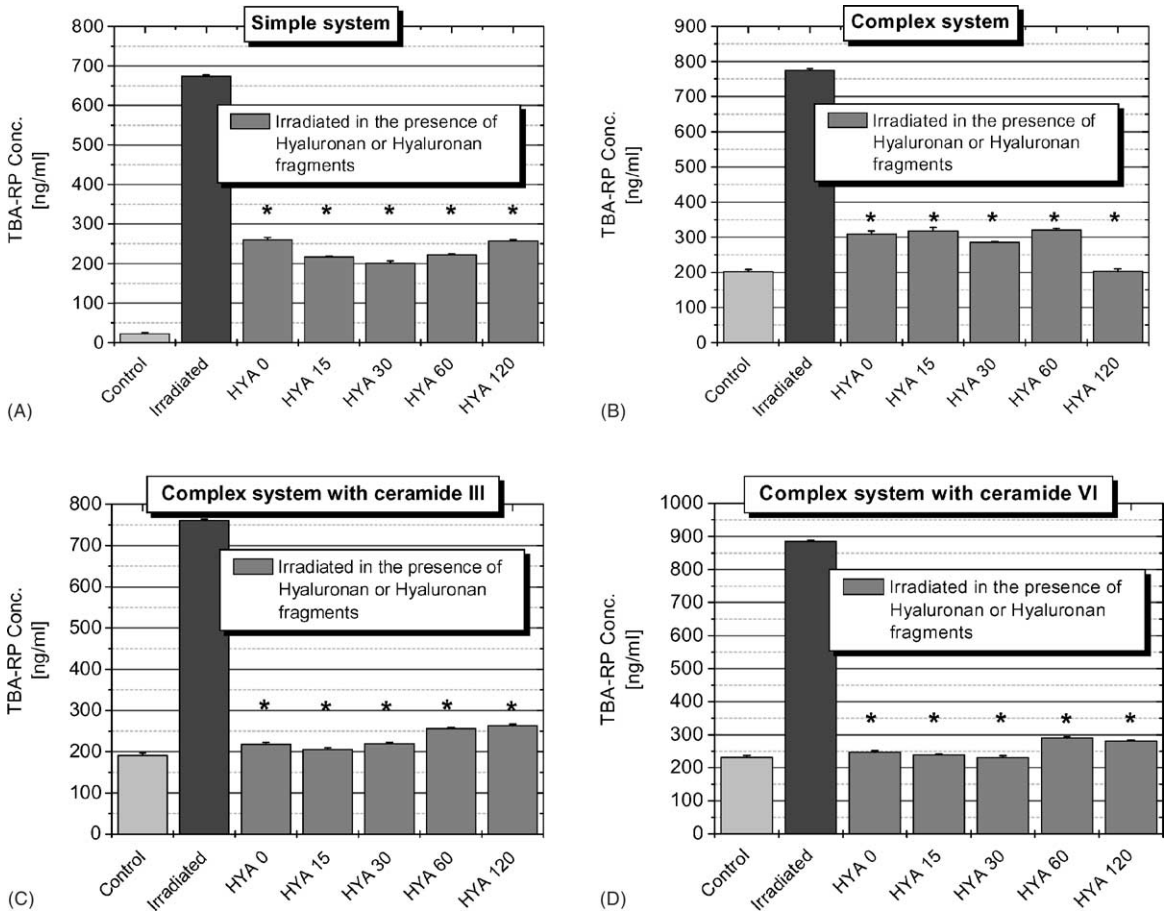

Fig. 1. Concentration of the thiobarbituric acid reaction products (TBA-RP Conc.) and influence of ultraviolet irradiation and hyaluronan and its fragments from enzymatic degradation in the systems used. (A) Simple system, (B) complex system, (C) complex system with ceramide III, (D) complex system with ceramide IV. $^{*}P < 0.05$, UV exposed samples in the presence of hyaluronan or fragments (light grey columns) vs. samples irradiated without hyaluronan (dark grey columns), two-tailed test. All data represent the mean values ± S.D. of sextuple measurements ($n = 6$).

Fig. 1B shows the effects of the polysaccharide measured using the complex system including the phospholipid DPPC, Chol and LLA. Hyaluronan and its fragments are acting antioxidatively as well, but the level of peroxidative damage after irradiation is increased. Only LLA is sensitive to UV light, transition metals and ROS because it is the only compound containing allylic double bonds. The enhanced sensitivity of unsaturated compounds when encapsulated into liposomes has been reported (Trommer et al., 2001).

The addition of C III and C IV to the lipid systems made the models more complex, making them more similar to the lipid matrix of the human stratum corneum (Fig. 1C and D). The addition of the sphingolipids caused nearly no additional effect on the TBA level of the complex system. This can be explained by the lack of conjugated or allylic double bonds, and the relative stability against oxidative attack arising from the sphingolipid structure.

Hyaluronan and its fragments from enzymatic degradation decrease the amount of lipid peroxidation products measured as MDA content in all skin lipid model systems studied. Interestingly, the different fragments of hyaluronan show nearly similar effects on the level of the generated lipid peroxidation products. A dependence between polysaccharide

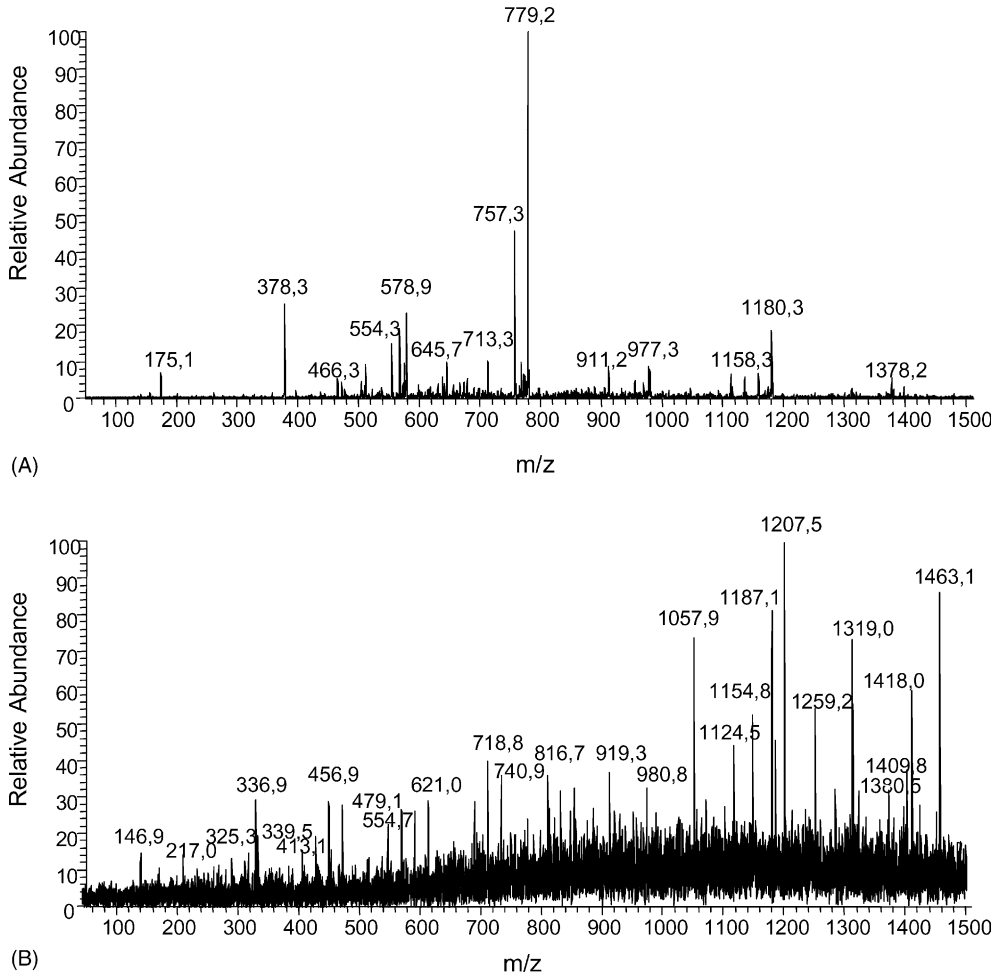

Fig. 2. (A) Negative ion mode ESI mass spectrum of the 22 kDa hyaluronan fragment with non-separated smaller degradation products. (B) Negative ion mode ESI mass spectrum of the 22 kDa hyaluronan fragment irradiated with 0.25 J/cm^2 UV-B.

molecular weight and the degree of the antioxidative action was not detectable. This is of importance when interpreting the mechanism of the protection behavior.

3.2. Mass spectrometry

For the qualitative characterization of the UV irradiation effects on hyaluronan mass spectrometric investigations were performed using an ESI-MS device. Fig. 2A shows the negative ion mode spectrum of the 22 kDa hyluronan fragment with non-separated smaller fragments from enzymatic degradation in the m/z range 50–1500.

The single charged tetramer at $m/z = 757$, the sodium adduct of the double charged tetramer at $m/z = 779$, the sodium adduct of the triple charged sixmer with $m/z = 579$, the single charged dimer with $m/z = 378$ (the hyalobiuronic acid), the single charged trimer with $m/z = 554$ as well as the single charged glucuronic acid anion at $m/z = 175$ were detected as the six main signals in the range of $m/z = 0$–1000 (Viseux et al., 1997; Takagaki et al., 1998). One has to consider that the peaks in the mass spectrum can be assigned to more than one carbohydrate structure (Price et al., 1997; Mahoney et al., 2001). So the peak at $m/z = 378$ can not only be interpreted as the single charged

dimer. It can also be assigned to the double charged tetramer which can be proved by the distance between the main peak and its C13 satellite of 0.5 mass units instead of 1 mass unit. Another structural explanation for the 378 peak is the triple charged hexamer.

Fig. 2B shows the negative ion mode mass spectrum of the 22 kDa fragment after UV irradiation. The treatment was very effective leading to many additional ion signals which are due to a photo degradation of the macromolecule. As a result smaller fragments are generated which are visible in the m/z range of the mass spectrometer used ($m/z = 50$–2000).

3.3. Electron paramagnetic resonance spectroscopy

In 1996, Hawkins and Davies carried out EPR experiments and could detect hyaluronan radicals as intermediates of hydroxyl radical induced degradation of hyaluronic acid (Hawkins and Davies, 1996). Photo degradation products of hyaluronan has been visualized by EPR spin trapping (Lapcik et al., 1991).

Using EPR spectroscopy we were able to elucidate the radical processes involved in the systems studied. Experiments with hydroxyl radicals using the spin trapping technique with DMPO as a trap for the ROS were carried out. Hydroxyl radicals are the most reactive ROS, hence it is impossible to detect them directly even by EPR because of their high reaction constants. An indirect determination can be achieved by the reaction with spin traps such as DMPO. The decrease in the signal intensity is regarded as a measure of the antioxidative potential of compounds. Fig. 3A shows the EPR spectrum of the DMPO-OH adduct with its characteristic spin Hamiltonian hfs parameter $a^{H} = a^{N} = 1.49$ mT (Hadjur and Jeunet, 1995).

The DPPH radical was used as an example of stable organic free radicals. The X-Band EPR spectrum of a 0.1 mM solution is shown in Fig. 3B. Often, the antioxidative properties of compounds are referred to their ability to reduce the concentration of the DPPH free radical.

TEMPO is a stable free radical and was used as a reference specimen in the double resonator for both DMPO-OH determinations and DPPH studies. Fig. 3C illustrates the EPR spectrum of a 50 μM methanolic solution with a characteristic splitting of $a^{N} = 1.74$ mT.

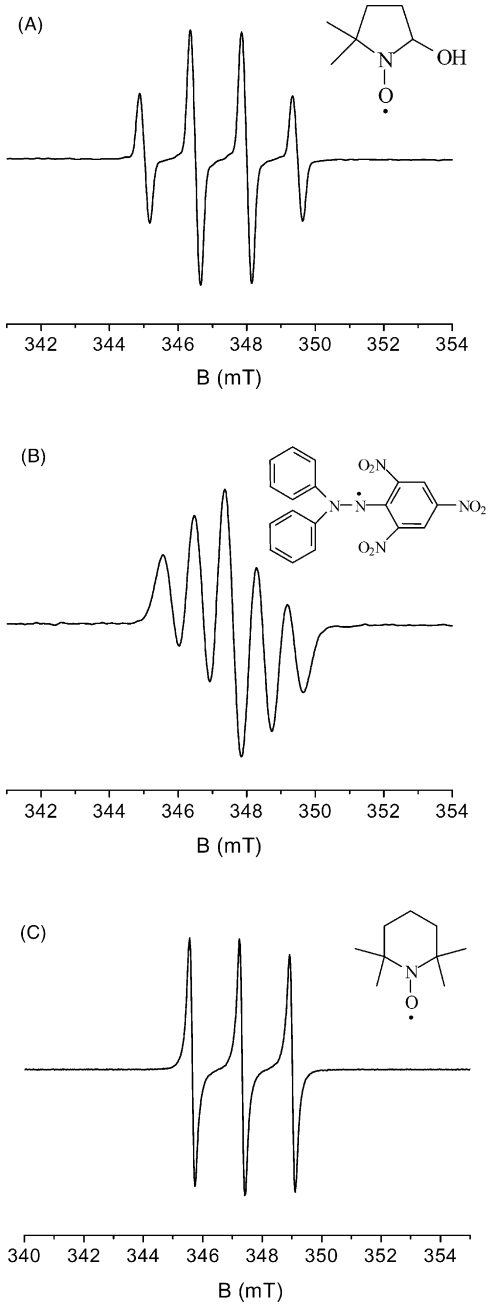

Fig. 3. X-Band EPR spectra (A) hydroxyl radicals trapped by DMPO, (B) the DPPH radical, (C) the spin probe TEMPO (instrument settings are described in Section 2).

By adding hyaluronan or its fragments to a DPPH solution in several concentrations, no changes of the EPR signal intensities of the stable radical in relation to the TEMPO reference could be observed. We conclude that neither hyaluronan nor its enzymatically degraded fragments are able to reduce stable free radicals.

Fig. 4 shows the results of the DMPO-OH spin adduct quantification using the double resonator technique. As examples for all fragments investigated the data for native hyaluronan HYA 0 (Fig. 4A), the fragment HYA 30 (Fig. 4B) and the fragment HYA 120 (Fig. 4C) are shown. Hyaluronan and all its fragments from enzymatic degradation showed prooxidative effects in the DMPO-OH assay quantifying Fenton generated hydroxyl radicals via spin trapping dependent on the polysaccharide concentration. These results are helpful to interprete the mechanism of the antioxidative effects observed in the thiobarbituric acid assay. The prooxidative behavior in the Fenton assay can be explained by the chelation of iron ions, a theoretical approach which has been proposed also for other glycosaminoglycanes and proteoglycanes (Albertini et al., 2000). There are prooxidative and antioxidative effects of iron chelators described in the literature (Elstner, 1990). Davies et al. could show that uric acid as another polyhydroxy compound was able to form stable co-ordination complexes with iron ions and inhibit lipid peroxidation in liposomes by this (Davies et al., 1986). Transition metal chelation has been classified along with direct radical scavenging, reduction by electron donation and enzymatic antioxidativity as an important part of antioxidative behavior of compounds (Voegeli et al., 1993). In the lipid model systems the iron complexation leads to a lipid protection and a decrease of the amount of lipid peroxidation products. In the Fenton system used for EPR investigations an iron ion chelation showed opposite results. The addition of a second transition metal chelator besides ADP had an additional prooxidative effect. Apparently, an iron chelation in the Fenton system increased the availability or rather the transfer of the transition metal electron to oxygen resulting in increased hydroxyl radical concentrations. This interpretation is supported by the results of Agren et al. The affinity between hyaluronan and iron ions has been proved by adding superoxide dismutase, catalase and Detapac to

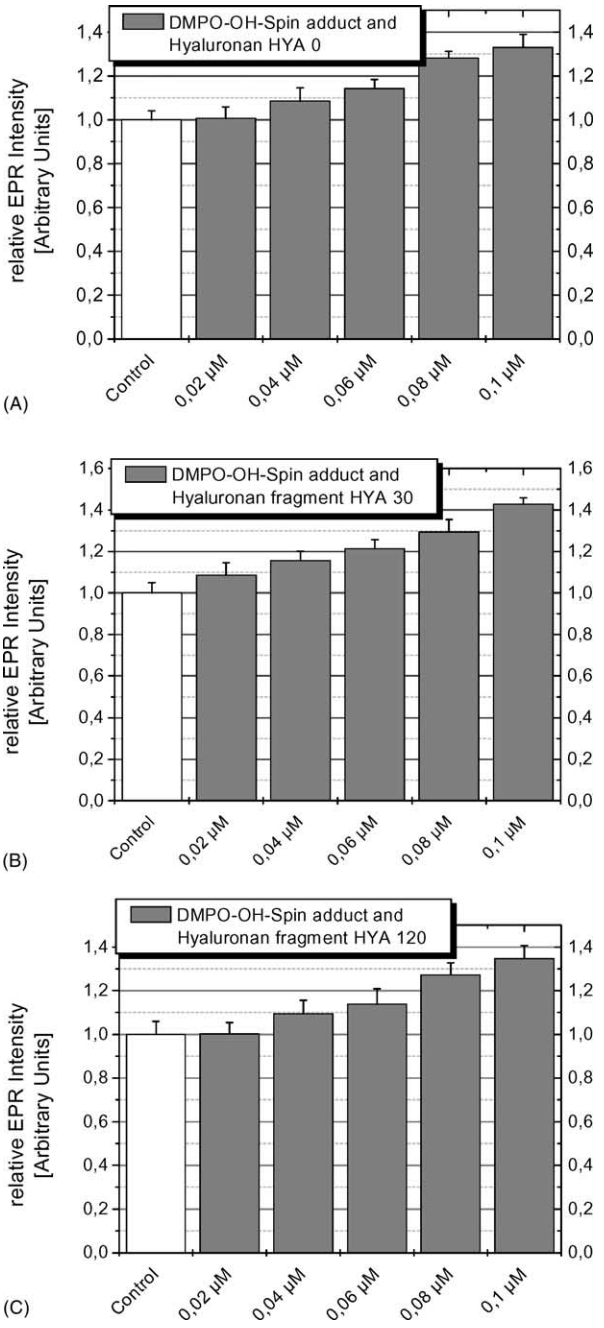

Fig. 4. Effect of hyaluronan and fragments on the concentration of the DMPO-OH spin adduct measured using the EPR double resonator technique in relation to the TEMPO reference. The error bars represent the standard deviation (±S.D.) obtained by quadruple measurements ($n = 4$) which was always below 5% (S.D. < 5%). (A) Fragment HYA 0, (B) fragment HYA 30, (C) fragment HYA 120.

skin explants (Agren et al., 1997). The iron chelator Detapac was the most effective compound in preventing ^{3}H-labeled hyaluronan from degradation which shows the interaction between the polysaccharide and iron before hyaluronan degradation. This transition metal chelation theory also explains the similarity of the protective effects of hyaluronan and its fragments observed in the TBA assay. Assuming iron chelation as the main reason for the antioxidative behavior of hyaluronan and transition metal catalysis as the key step in lipid peroxidation the similarity is easily explained. All samples contain the same concentrations of iron ions and the chelation of them leads to a similar protection from UV-induced lipid peroxidation.

The iron concentration used in this study when finding the harmful effects (10 μM) was relatively high towards the lipids. However, Green et al. (1968) demonstrated that human skin is a significant side of iron excretion. In a collaborative study a total iron content in the epidermal skin of 22.5 ± 17.8 mg was calculated from material obtained from eccrine areas. The involvement of iron ions in UV irradiation-induced free radical formation in the skin was confirmed in 1996 (Buettner and Jurkiewicz, 1996). The treatment of skin samples with the iron chelator Desferal® resulted in a significant reduction of a spin adduct after irradiation suggesting a key role for iron in UV-mediated free radical formation.

UV radiation itself is able to increase the skin pool of non-heme iron (Fe(II)) in dermis and epidermis significantly. Authors have measured 18 ppm per dry weight of unexposed body parts (buttocks, thigh) versus 53 ppm per dry weight in epidermis biopsies of exposed parts such as cheek, forehead and neck (Guy et al., 1999). Additional to direct damage of biomolecules by UV, this is an indirect pathway leading to dangerous oxygen radicals.

We have shown in this article that hyaluronan and hyaluronan fragments from enzymatic degradation exhibit antioxidative effects on lipid model systems of differing complexity. The polysaccharide's degradation after UV irradiation was demonstrated by mass spectrometry.

The results of EPR investigations were useful for detailed studies regarding the mechanism of hyaluronan protecting stratum corneum lipids from UV irradiation. Iron chelation is suggested to be an important part of the mechanism of the antioxidative properties of hyaluronan and fragments.

4. Conclusion

Several techniques were applied to investigate the complex matter of hyaluronan redox behavior using in vitro systems. The TBA assay was applied to quantify lipid peroxidation products. It was shown that hyaluronan and also its degradation products with reduced molecular weights exert antioxidative effects on the models.

To elucidate the mechanism of these effects, mass spectrometric studies were carried out, and have shown that the mucopolysaccharide is degraded after acting antioxidatively.

To learn more about the radical involvement during these processes EPR experiments were applied. Hyaluronan and hyaluronic acid fragments increased the concentration of spin trapped hydroxyl radicals and had no influences on the content of the stable organic radical DPPH. The first observation results from hyaluronan and its fragments acting as iron chelators which explains the results of both TBA assay and EPR experiments.

Considering human skin and its constant exposure to UV light and oxygen, combined with the increased iron content of the exposed skin, exclusive topical administration of hyaluronan in cosmetic and pharmaceutical semisolid formulations could be protective for the lipids within the largest human organ. The hyaluronan fragments from enzymatic degradation showed a lipid protective potential as well. This could be of advantage. Because of their reduced molecular weight the incorporation of them into semisolid formulations should be facilitated.

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