Protective effect of hyaluronate on oxidative DNA damage in WI-38 and A549 cells

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Abstract. Progressive DNA damage in live cells by oxidants is the key factor contributing to cell aging and preconditioning to neoplastic transformation. The strategies to slow aging or prevent cancer rely on protection of DNA from the damage. Since cells reside within intercellular matrix it is of interest to know whether matrix constituents possess properties of modulating oxidative DNA damage. We explored, therefore, the effect of hyaluronate (HA), the ubiquitous component of the matrix, on extent of DNA damage induced by exogenous and endogenously generated oxidants. WI-38 and A549 cells were exposed to 200 μM H_2O_2 in the absence or presence of HA and induction of histone H2AX phosphorylation and activation of ATM, the reporters of DNA damage, was assessed by multiparameter cytometry. Also explored was effect of HA on constitutive H2AX phosphorylation that reflects DNA damage caused by endogenous oxidants generated during aerobic metabolism. HA of average MW 5.4 million (high MW) and 2 million (medium MW) at 0.1% (w/v) in culture medium totally prevented the H_2O_2-induced H2AX phosphorylation in both cell types whereas effect of 60,000 average MW (low MW) HA was somewhat less pronounced. Constitutive H2AX phosphorylation in WI-38 cells growing in the presence of 0.1% HA of low MW and medium MW was reduced by about 35 and 30%, respectively; no reduction was observed in A549 cells. The data indicate that HA protected DNA from damage caused by the exogenous oxidant H_2O_2. In WI-38 fibroblasts, the cells that express the HA-receptor CD44, HA also protected DNA from damage caused by endogenous oxidants. We postulate that expression of CD44 in some cell types such as stem cells may provide the means to internalize HA by endocytosis and one of the functions of the internalized HA may be protection of DNA from oxidants. The mechanism of protective effect of HA may either: i) involve entrapment of iron ions thereby inhibiting the Fenton's reaction that produces secondary oxidative species, and/or: ii) directly scavenging of primary and secondary ROIs, as an antioxidant, resulting in HA degradation. Since no significant degradation of HA upon its exposure in tissue culture medium to H_2O_2 was detected the scavenging may occur intracellularly.

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Introduction

DNA in live cells is being constantly damaged by reactive oxygen species (ROS) either generated during aerobic respiration in mitochondria (1-6), or originating from exogenous sources such as environmental pollutants (7), macrophage oxidative burst (8-10), and other factors (11). The by-products of oxidative phosphorylation reactions can diffuse from mitochondria and reach the nuclear DNA inducing its damage (12,13). Oxidation of all four constituent DNA bases with predominance of guanine [formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG)], base ring fragmentation, sugar modification, covalent crosslinking of DNA and protein, and induction of DNA strand breaks, all may occur as a result of oxidative DNA damage (14,15). Lipid peroxidation of cell membranes is still another injurious effect of endogenous and exogenous oxidants (16).

It has been estimated that during a single cell cycle of about 24-h duration approximately 5,000 DNA single-strand lesions (SSLs) per nucleus are generated by oxidants (6). Of them about 1% (~50 'endogenous DSBs') are converted, predominantly during DNA replication, to DNA double-strand breaks (DSBs). Recombinatorial repair and non-homologous DNA-end joining (NHEJ) are the two major pathways of DSB repair. Recombinatorial repair takes place in cells that have already replicated some portion of their DNA that can then serve as a template, i.e. in late S and G_2 phase cells. DNA repair in G_1 and early S phase cells that lack a template, however, relies on the NHEJ pathway, which is error-prone and may lead to deletion of some base pairs (16,17). Progressive alteration in molecular structure of DNA occurring by this mechanism in live cells is considered to be the main cause of aging and

senescence as well as predisposition to cancer (4,18-22). Therefore, the strategies designed to slow aging or prevent cancer rely on protection of DNA from oxidative damage, primarily by the use of anti-oxidants.

Since cells within tissues are embedded in the intercellular matrix it is of interest to know whether components of intercellular matrix possess properties of modulating extent of ROS and can affect oxidative DNA damage. Indeed, there is evidence in the literature that some glycosaminoglycans such as chondroitin-4-sulfate and hyaluronan (hyaluronic acid, HA) inhibit lipid peroxidation caused by oxidative stress and thereby decrease inflammatory reactions mediated by oxidants (23-26). The anti-inflammatory effects of various forms of HA seen after administration into arthritic joints was also observed and considered to be due to restoration of elastoviscosity of the synovial fluid of the inflamed joints (27). However, the protective effect of this biopolymer through neutralization of reactive oxidants generated during inflammation-driven oxidative burst in leukocytes and macrophages may also play a role (8-10).

In the early 1940s, it was discovered that ascorbic acid or H_2O_2 causes degradation of HA. It was also observed that Fe^{2+} and Cu^{2+} ions and molecular oxygen catalyzed this 'oxidative degradation' producing oligosaccharides (28,29). Later it was suggested that oxygen-derived free radicals initiated the reaction (30,31). The reaction can be inhibited by various scavengers of oxygen-derived free radicals such as superoxide dismutase, catalase, and mannitol, histidine or copper-penicillamine complex (32). The stimulatory effect of low concentration of H_2O_2 (1-5 μM) on HA synthesis by cultured synovial cells of osteoarthritic but not of rheumatoid arthritic patients (33) was reported.

Data in the literature indicated that the concentration of H_2O_2 used both *in vitro* and in cell culture experiments is an important factor on the HA degradation process. Therefore, we studied the effect of 200 μM and 200 mM H_2O_2 on the three different average MW hyaluronan preparations dissolved in the various culture media used. We found that the 200 mM H_2O_2 caused considerable degradation especially of the high and medium average MW HA preparations during 4-h incubation at 37˚C (data not shown). Our goal was to use a H_2O_2 concentration that does not cause significant HA degradation during the 4-h period and therefore the cells were exposed during the entire culturing time to an unchanged HA molecular environment, we selected the lower concentration of H_2O_2 (200 μM) for our study. Recently, the effect of anti-inflammatory drugs (naproxen, acetylsalicylic acid) was tested *in vitro* on the degradation of HA in the ascorbate, H_2O_2 and Cu^{2+}-induced oxidative systems. The presence of these drugs retarded or inhibited the degradation of HA in a dose-dependant manner. In these experiments 50-100 μM H_2O_2 was used (34).

Activation of ataxia telangiectasia mutated (ATM) protein kinase through its phosphorylation on *Ser*-1981 (ATM-S1981^P), and phosphorylation of one of the variants of histone H2A, histone H2AX on *Ser*-139 are early reporters of a cell's response to DNA damage, particularly the damage that involves formation of DNA double-strand breaks (DSBs) (35-40). Phosphorylated H2AX has been named γH2AX (35,36). These modifications of ATM and H2AX trigger signal transduction pathways that recruit DNA repair machinery to the DSB site and activate cell cycle checkpoints halting progression through the cycle. We have recently reported that immunocytochemical detection of ATM activation and H2AX phosphorylation when assessed by flow- or laser-scanning cytometry provides a very sensitive marker of DNA damage, including the damage-induced endogenous oxidants (8,41-44). In fact, the cytometric detection of DNA damage through the analysis of γH2AX expression has been reported to be by two orders of magnitude more sensitive than the alternative approach the comet methodology that is based on single cell DNA gel electrophoresis (45). In the present study, therefore, we have applied the H2AX phosphorylation assay to explore whether HA may exert protective effect on oxidative DNA damage induced either by exogenous or endogenous oxidants. Towards this end we tested the effect of HA preparations of three different average MW and polydipersity on the level of: a) H2AX phosphorylation induced by exogenously applied oxidative agent H_2O_2, and b) constitutive H2AX phosphorylation (CHP) which reports DNA damage caused by endogenous oxidants generated during metabolic mitochondrial activity (41-43,46).

Materials and methods

Cells, cell treatment. A549 and WI-38 cells, obtained from American Type Culture Collection (ATCC, Manassas, VA), were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Gibco/BRL) in 25 ml Falcon flasks (Becton-Dickinson Co., Franklin Lakes, NJ) at 37.5˚C in atmosphere of 95% air and 5% CO_2. The cells were maintained in exponential and asynchronous phase of growth by repeated trypsinization and reseeding before reaching subconfluency. The cells were then trypsinized and seeded at low density (about $5x10^4$ cells per chamber) in 2-chambered Falcon Culture Slides (Becton-Dickinson Labware). Twenty-four hours after seeding cells on slides the sterile solution of HA (0.5% w/v in PBS; Matrix Biology Institute, Edgewater, NJ) was added to cultures to obtain the final 0.1% w/v HA concentration; the respective control cultures were supplemented with the equivalent volumes of PBS. HA of thee different average MW: 5.4 million (defined thereafter as high MW), 2 million (defined as medium MW) and 60,000 (low MW) were used throughout the experiments, as listed in Results and in figure legends. The cells were treated with 200 μM H_2O_2 (Sigma Chemical Co., St. Louis, MO) for various periods of time as shown in figure legends. In some experiments the cells were exposed to HA for 23 h then rinsed and treated with H_2O_2 in the medium containing the same concentration of HA as used in the prior 23-h incubation.

Detection of ATM activation and H2AX phosphorylation. Following incubations with HA and/or H_2O_2 the cells were rinsed with PBS and then fixed by transferring the slides into Coplin jars containing 1% methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) in PBS for 15 min on ice followed by suspension in 70% ethanol where they were

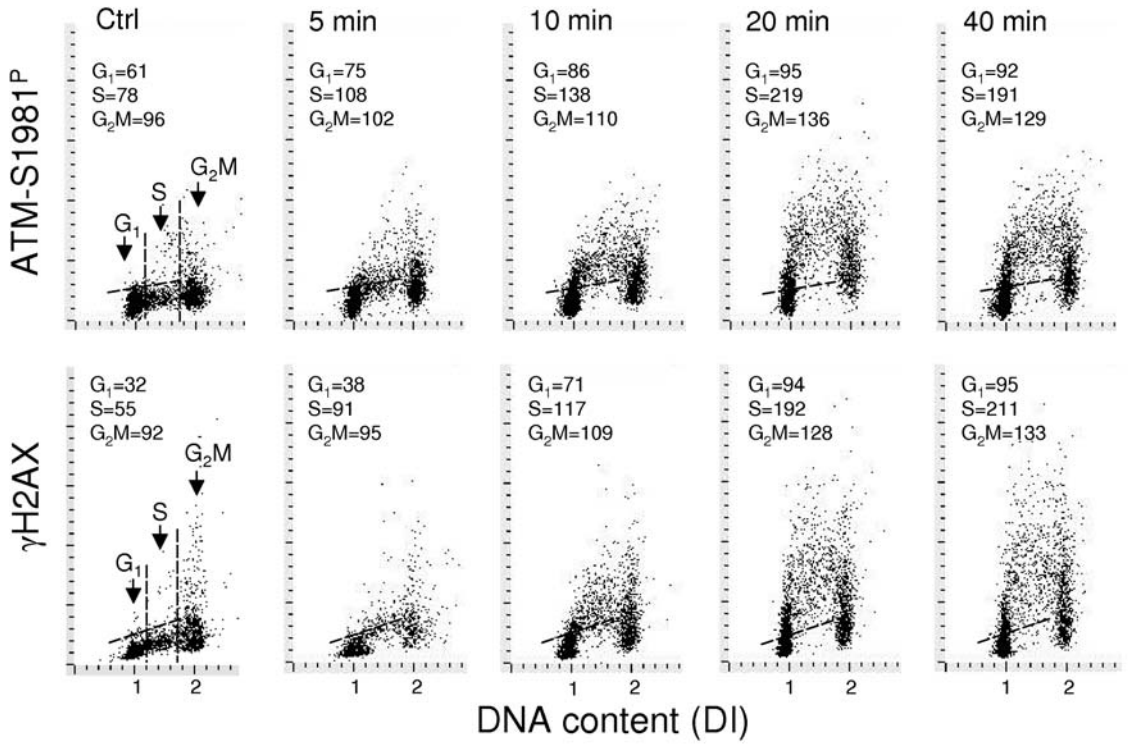

Figure 1. Induction of ATM activation and H2AX phosphorylation in A549 cells exposed to H_2O_2. A549 cells were untreated (Ctrl) or treated with 200 μM H_2O_2 for 5-40 min and expression of ATM-S1981^P and γH2AX, each detected immunocytochemically, was measured by laser scanning cytometry, concurrently with cellular DNA content. The latter was used to identify cells in G_1, S and G_2M phases of the cycle as shown in the left panels of these bivariate distributions (scatterplots). The dashed skewed lines show the upper threshold level of ATM-S1981^P and γH2AX expression for 97% of interphase cells in the untreated (Ctrl) cultures. By gating analysis based on differences in DNA content the mean values of γH2AX or ATM-S1981^P IF were calculated for cell populations in S phase (x) and are shown in each panel.

stored at -20˚C for 2-24 h. The fixed cells were then washed twice in PBS and treated on slides with 0.1% Triton X-100 (Sigma) in a 1% (w/v) solution of bovine serum albumin (BSA; Sigma) in PBS for 30 min to suppress non-specific antibody (Ab) binding. The cells were then incubated in 100 μl volume of 1% BSA containing 1:200 dilution of phospho-specific (*Ser*-139) histone H2AX (γH2AX) mouse monoclonal antibody (mAb) (Millipore/Upstate, Temecula, CA) or a 1:100 dilution of phospho-specific ATM (*Ser*-1981) mAb (Millipore/Upstate). After overnight incubation at 4˚C, the slides were washed twice with PBS and then incubated in 100 μl of 1:100 dilution of Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen/Molecular Probes, Eugene, OR) for 45 min at room temperature in the dark. The cells were then counterstained with either 2.8 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma) in PBS for 15 min, or 5 μg/ml PI (Invitrogen/Molecular Probes) in the presence of 100 μg/ml of RNase A (Sigma). Each experiment was performed with an IgG control in which cells were labeled only with secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (H+L) or Alexa Fluor 488 goat anti-rabbit IgG (H+L) without primary antibody incubation to estimate the extent of non-specific binding of the secondary antibody to the cells.

Measurement of cell fluorescence by Laser Scanning Cytometry. Cellular green fluorescence representing expression of γH2AX, or of activated (*Ser*-1981 phosphorylated) ATM and blue emission of DAPI was measured using a Laser Scanning Cytometer (LSC; iCys; CompuCyte, Cambridge, MA), utilizing standard filter settings; fluorescence was excited with 488-nm argon ion and violet diode lasers, respectively (47). The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3,000 cells were measured per sample. To express the treatment-induced change in mean values of the measured cells, the means values of the untreated as well as the treated cells were compensated for the level of non-specific fluorescence measured as described above. Each experiment was run in duplicate or triplicate and the experiments were repeated. Other details are given in figure legends.

Determination of average MW and polydispersity of HA samples. Average MW and the polydispersity of the HA molecules were determined by agarose gel electrophoresis (34,48,49). The test samples contained H_2O_2 in two different concentrations (200 mM or 200 μM) in 0.1% (w/v) HA solution in the same culture media (including antibiotics and fetal bovine serum) as used for culturing the studied cells. The freshly prepared samples were placed either immediately (time 0) or were incubated for 2 or 4 h at 37˚C before being placed into the wells of the agarose gel. Depending on the average MW of HA either 0.5 or 1% agarose gel was used (34,48,49). Monodisperse internal standards (Hyalose, L.L.C., Oklahoma City, OH) were applied for each run.

Results

Exposure of A549 cells to 200 μM H_2O_2 resulted in rapid activation of ATM and induction of H2AX phosphorylation (Fig. 1). Expression of ATM-S1981^P was already apparent

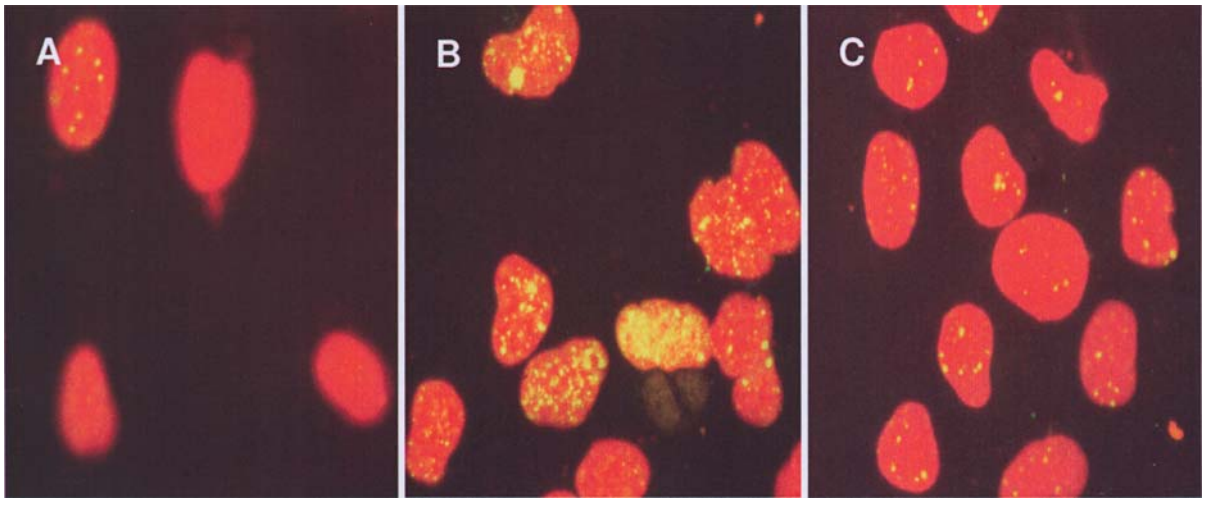

Figure 2. Effect of treatment of A549 cells with H_2O_2 in the absence and presence of high MW HA on induction of γH2AX. Photomicrographs of A549 cells, untreated (A), treated with 200 μM H_2O_2 for 20 min in the absence of HA (B), and treated with 200 μM H_2O_2 for 20 min in the presence of 0.1% (w/v) of HA of high MW (C); HA was administered into the culture 1 h prior to H_2O_2. Expression of γH2AX is shown using Alexa 488 Fluor-tagged secondary Ab, DNA is counterstained with PI.

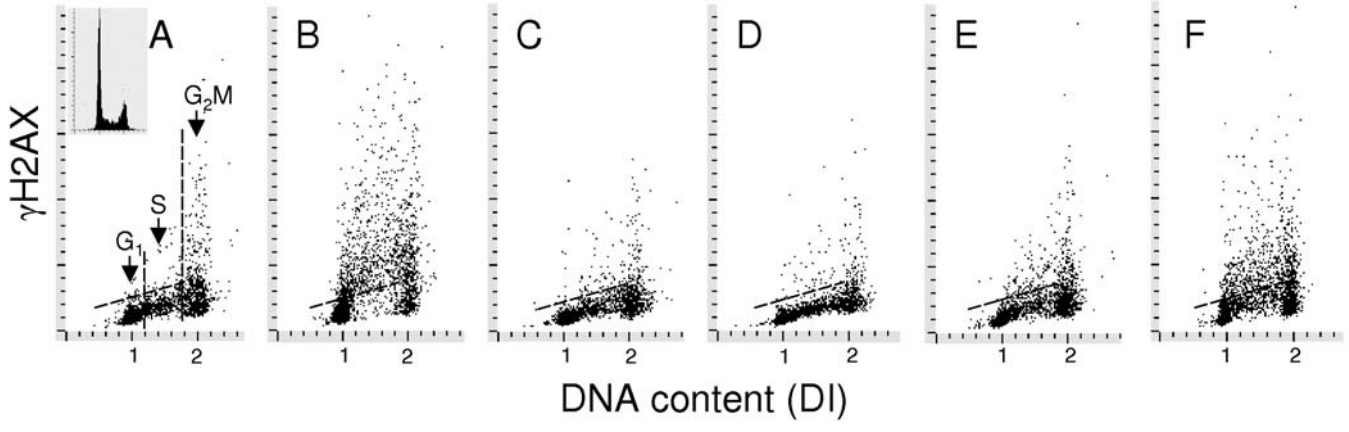

Figure 3. Effect of HA on the H_2O_2-induced H2AX phosphorylation in A549 cells. The bivariate DNA content vs. γH2AX distributions illustrate expression of γH2AX with respect to the cell cycle phase of the untreated cells (A), cells treated with H_2O_2 in the absence of HA (B) or treated with H_2O_2 in the presence of 0.1% HA of high MW (different batches, C and D), medium MW (E) and low MW (F), as described in Materials and methods. The dashed skewed lines show the upper threshold level of γH2AX expression for 97% of interphase cells in the untreated (Ctrl) culture (A). Based on differences in cellular DNA content it was possible to discriminate cells in G_1, S and G_2M phases of the cell cycle as shown in A. The inset in A shows cellular DNA content histogram of the studied cultures; cell treatment with HA for 24 h had no detectable effect on DNA histogram (cell cycle distribution), regardless of MW of HA.

after 5 min of treatment and the level of γH2AX expression was elevated as well. Prolongation of the treatment up to 20-40 min led to further rise in the level of expression of both ATM-S1981^P and γH2AX. Because cellular DNA content, the marker that identifies the cell cycle position, was measured concurrently with expression of ATM-S1981^P and γH2AX, it was possible on the bivariate ATM-S1981^P or γH2AX versus DNA content distributions to correlate activation of ATM and phosphorylation with cell cycle phase (Fig. 1, left panels). The data distinctly show that the H_2O_2-induced increase in expression of ATM-S1981^P or γH2AX was maximal for the S phase cells. This is evident by the fact that following H_2O_2 treatment the mean values of ATM-S1981^P or γH2AX IF were more elevated in S-phase than in G_1 or G_2M cells. Also, the proportion of cells with ATM-S1981^P or γH2AX expression above the maximal level representing untreated (Ctrl) cells (threshold defined by the dashed lines) was the highest for cells in S-phase. Similar set of data was obtained for WI-38 cells treated with H_2O_2 (not shown).

The induction of γH2AX in cells exposed to H_2O_2 manifested in form of IF foci (Fig. 2). The microphotographs shown in this figure demonstrate formation of a large number of γH2AX IF foci in the cells that were exposed to H_2O_2 in the absence of HA (Fig. 2B). However, there was almost total suppression of γH2AX IF foci formation in the cells that were exposed to H_2O_2 in the presence of high MW HA.

Figs. 3 and 4 illustrate effect of HA on the H_2O_2-induced phosphorylation of histone H2AX in A549 cells. It is quite evident from the raw data presented as cellular DNA content

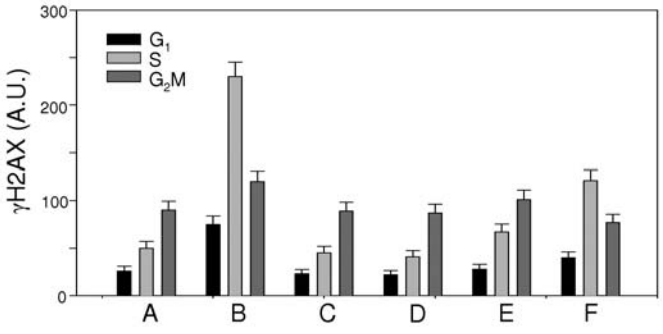

Figure 4. Mean values of γH2AX expression in A549 cells [in arbitrary units (AU); fluorescence channels] untreated or treated with H_2O_2 in the absence and presence of HA of different average MW in relation to the cell cycle phase. The data represent quantitative analysis of the experiment shown in Fig. 3 as the raw data represented by bivariate DNA content vs. γH2AX expression distributions. Based on differences in DNA content populations of cells in G_1, S and G_2M phases were gated, their mean value of γH2AX expression was calculated and is presented (± SE) as bar graphs.

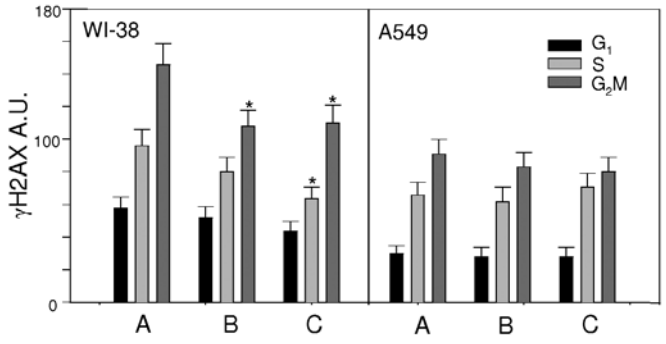

Figure 6. Mean values of γH2AX expression in WI-38 and A549 cells [in arbitrary units (AU); fluorescence channels] untreated (A) or treated with medium (B) or low (C) MW HA for 24 h, in relation to the cell cycle phase. The data represent quantitative analysis of the experiment shown in Fig. 5. Populations of cells in G_1, S and G_2M phases were gated, their mean value of γH2AX expression was calculated and is presented (± SE) as bar graphs.

versus γH2AX bivariate distributions (Fig. 3) that expression of γH2AX in H_2O_2-treated cells was markedly reduced in the cells that were growing in the presence of HA. The most pronounced were changes in γH2AX expression in S-phase cells. Maximal reduction was seen in cells treated with HA of high MW (Figs. 2C and 3C and D). Distinctly lower reduction was observed in the culture containing HA of low MW (Fig. 3F). The mean values of γH2AX expression calculated for cells in particular phases of cell cycle are presented in Fig. 4. The nearly 5-fold rise in γH2AX expression was apparent for the S-phase cells exposed to H_2O_2 in the absence of HA (Fig. 4B). This increase was totally abolished when the cells were treated with H_2O_2 in the presence of high MW HA (Fig. 4C and D) and was significantly reduced in the presence of medium (Fig. 4E) and low MW HA (Fig. 4F).

Marked proportion of constitutive histone H2AX phosphorylation seen in normal or tumor cells not treated by any exogenous genotoxic agent was shown to reflect oxidative DNA damage caused by ROS generated in mitochondria as by-products of aerobic metabolism (41-44,46). We have tested whether HA may affect the level of constitutive H2AX phosphorylation by growing WI-38 and A549 cells in its presence and comparing expression of γH2AX in so treated cells with that of the untreated control cells (Figs. 5 and 6). It is evident from the raw data shown in Fig. 5 that expression of γH2AX was diminished in WI-38 cells grown in the presence of HA for 24 h. The decrease was observed in cells treated with both medium and low MW HA, but the extent of decrease was somewhat more pronounced in the case of HA of the low MW. When expressed as mean values of γH2AX for particular phase of the cell cycle the decrease was statistically significant ($p<0.05$) for G_2M-phase cells in the cultures treated with HA of both medium and low MW and

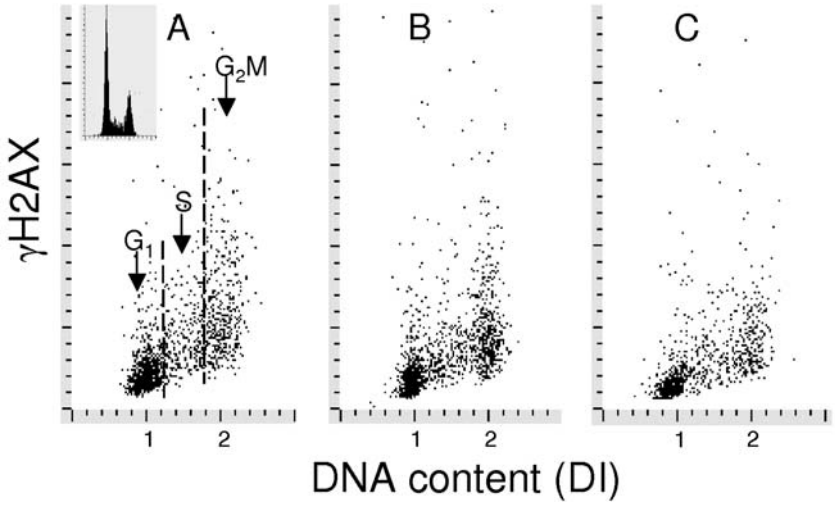

Figure 5. Effect of HA on the level of constitutive expression of γH2AX in WI-38 cells. Medium (B) and low (C) MW HA was present at 0.1% (w/v) concentration for 24 h in cultures of exponentially growing WI-38 cells; control (A) cells were left untreated. The cells were then harvested and expression of γH2AX as well as DNA content was measured by laser scanning cytometry. The inset in A shows DNA content histogram of the studied cultures; cell treatment with HA for 24 h had no detectable effect on DNA histogram regardless of its MW. Note a decrease in overall γH2AX level of the HA treated cells, more pronounced in cells treated with low MW HA.

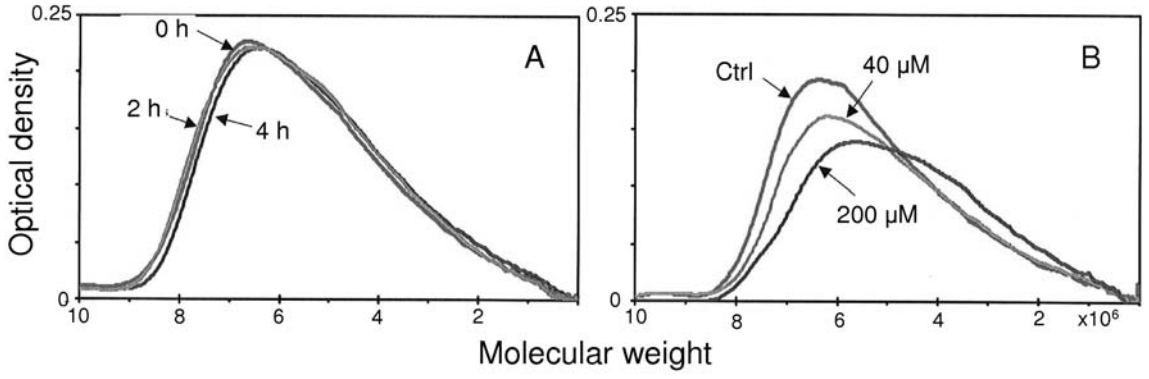

Figure 7. Different effects of 200 μM H_2O_2 on degradation of HA in the absence and presence of $FeSO_4$. A, HA of average MW 5.4 million was dissolved in culture medium (RPMI-1640 with penicillin, streptomycin and FBS) at 0.1% (w/v) concentration containing H_2O_2 and was incubated for 0, 2 and 4 h at 37˚C. Note lack of the effect. B, The effect of 40 and 200 μM $FeSO_4$ on the same HA preparation dissolved in the same culture media as in A. The average MW after 2 and 4 h of incubation at 37˚C decreased by 5 and 14% respectively. The effect was most noticeable in the presence of 200 μM of $FeSO_4$.

for S-phase cells in the cultures treated with low MW HA (Fig. 6). However, no such reduction was observed in the case of A549 (Fig. 6).

We also assessed as to whether HA was degraded when exposed to high (200 mM) or low (200 μM) H_2O_2 concentration. At 2 mM H_2O_2 concentration, the effect was significant, as after 4-h exposure the average MW of the high MW HA decreased 19% and that of the medium MW HA decreased 23%. However, the low MW HA did not show any change (data not shown). On the other hand, we could not observe any effect of the low concentration H_2O_2 (200 μM) on HA degradation regardless of the MW (Fig. 7A). However, when $FeSO_4$ was added to the reaction mixture, as expected, a small but significant degradation was observed (Fig. 7B).

Discussion

The present data indicate that exposure of A549 or WI-38 cells to H_2O_2 led to rapid induction of ATM activation and H2AX phosphorylation. The maximal response was observed for S-phase cells (Fig. 1). This observation is compatible with the known mechanism of induction of DNA damage by oxidants that initially leads to formation of DNA single strand lesions, predominantly consisting of 8-oxo-dG, followed by conversion of some of these lesions into DSBs during DNA replication (1-6,50). Because DSBs are the most potent inducers of ATM activation and H2AX phosphorylation (35-40) it would be expected that cells in S-phase responded maximally to short pulse of H_2O_2 (Fig. 1).

A good correlation was seen between the rise in expression of both γH2AX and ATM-S1981^P induced by H_2O_2 which suggests that H2AX phosphorylation was mediated by ATM. The data also imply that H2AX phosphorylation was not caused by replication stress because in the latter case phosphorylation is mediated by ATR and/or DNA PKcs and not by ATM (51). Activation of ATM and phosphorylation of H2AX when occurring concurrently are considered to be a more reliable marker of DNA damage that involves formation of DSBs than phosphorylation of H2AX alone (52,53). Consistent with the induction of DSBs by H_2O_2 was also observation of the presence of γH2AX in form of distinct foci (Fig. 2) instead of its dispersed localization. The number of individual γH2AX foci per nucleus is being considered to report the number of DSBs (35-38). All the above evidence points out, therefore, that in the cells treated with H_2O_2 the induction of γH2AX reports oxidative DNA damage, some of each leading to formation of DSBs.

Oxidative DNA damage induced by H_2O_2 as reflected by induction of γH2AX was distinctly reduced in cells that were exposed to H_2O_2 in the presence of HA. The most pronounced reduction was seen in the presence of high MW HA; in fact, exposure of cells to H_2O_2 under these conditions essentially totally prevented the induction of γH2AX as the level of expression of γH2AX in these cells was similar to that on the H_2O_2-untreated cells. The protective effect was still apparent but was of lesser degree when HA of medium and low MW were used. Similar degree of protection, also related to MW of HA, was observed in WI-38 cells treated with H_2O_2 (data not shown).

The data (Figs. 3 and 4) report effect of HA on γH2AX induction by H_2O_2 under conditions when the cells were grown in the presence of HA for 24 h and were treated with H_2O_2 during their final hour of growth. These conditions mimic situation *in vivo* when cells are growing within intercellular matrix containing HA and are exposed to an exogenous oxidizing agent. In parallel experiments we have also tested the effect of HA added concurrently with H_2O_2 for 1 h on the cells that were prior growing either in absence or presence of HA. In either case a marked protective effect, in terms of reduction of γH2AX induced by H_2O_2, of all HA preparations, but the most pronounced of the high MW, was observed (data not shown).

It has been reported by Campo *et al* (54), that HA and chondroitin-4-sulphate could reduce DNA fragmentation caused by iron plus ascorbate-induced oxidative stress in human fibroblasts. Their observations, however, were based on measurement of DNA damage in bulk, upon its extraction from large populations of cells. Unlike in the present study, therefore, the authors were unable to relate DNA damage to individual cells and to the cell cycle phase. Furthermore,

the oxidative stress lasted for 24 h. It is possible that in the course of the stress lasting for so long some cells were undergoing apoptosis. Because during apoptosis DNA is extensively fragmented (55) the DNA fragmentation observed by these authors could be caused not only directly by the oxidants but also could be the result of apoptotic cell death. Nevertheless, their studies provide evidence for the protective effect of HA on cells stressed by oxidants.

It should be noted that HA is promoting wound healing (56,57) and modulating inflammatory processes (24,25,58-60). Because reactive oxidants play key role in inflammation, particularly during oxidative burst of phagocytes/macrophages (8,61), it is likely that the palliative effect of HA in inflammation is mediated to a large extent by its ability to protect cells from reactive oxidants.

As mentioned, the constitutive phosphorylation of histone H2AX observed in untreated cells reflects to a large extent the ongoing DNA damage induced by endogenous oxidants produced primarily in mitochondria during aerobic metabolism (41-44,46). We observed that HA was able to reduce the level of γH2AX expression in the untreated WI-38 but not in A549 cells (Figs. 5 and 6). In contrast to the protection from the exogenous H_2O_2, that was distinctly lower when the HA of low MW was used, compared to high MW HA (Figs. 3 and 4), no such pattern was apparent in the case of constitutive DNA damage in WI-38 cells. In fact, HA of low MW appeared to have more pronounced effect compared to medium (Fig. 5) or high (not shown) HA. The protective effect of HA from the endogenous oxidants in WI-38 cells would suggest that this biopolymer was more effectively internalized. The hyaluronate transmembrane receptor CD44 is present on plasma membrane of fibroblasts, including WI-38 cells (62), and it has been shown that this receptor mediates internalization of HA via endocytotic pathway (63-65). It is likely, therefore, that in the present study HA was internalized by WI-38 cells by this mechanism and it exerted its protective effects after the internalization. If this is indeed a mechanism of the observed protection, it appears then that the internalization of HA of low MW was more effective than that of the medium MW.

It is unclear why we observed the protective effect of HA on constitutive DNA damage in WI-38 fibroblasts but not of adenocarcinoma A549 cells. One of the mechanisms could be that HA the internalization in the latter cells was less effective. However, most normal epithelial cells express CD44 (66) and its presence on A549 cells have also been reported (67). It was shown, however, that chemical modification such as palmitoylation of CD44 is needed to associate this receptor with lipid rafts and effectively internalize HA via endocytosis (65). It is possible therefore, that A549 cells lack this mechanism and therefore were unable to internalize HA.

Two possible mechanisms may account for the observed protective effect of HA against oxidative DNA damage. One mechanism may be associated with the ability of the polyanion HA to chelate ions such ad Fe^{++} and Cu^{++} (68,69). These ions are critical in Fenton's reaction (70,71) in which the superoxide and hydrogen peroxide, which themselves are not strongly reactive towards DNA, are converted into the highly reactive hydroxyl radical (OH^*) known to produce a multiplicity of chemical modifications in DNA. Another mechanism may involve direct scavenging by HA of reactive oxidant molecules, particularly the reactive products of Fenton's reaction such as OH^*. The observed *in vitro* fragmentation of HA by H_2O_2 in the presence of $FeSO_4$ in tissue culture media (Fig. 7B) is consistent with the second mechanism. Namely, the radical scavenging activity by HA results in its breakdown and depletes the pool of oxidants that otherwise will damage DNA. This supports the proposition that one of the HA biological functions is to provide protection against cellular damage caused by radicals produced by oxidative reductive systems or ionizing radiation (72). Consistent with this interpretation are findings of Hutadilok *et al* (33), who observed that exposure of synovial fibroblasts to low concentrations of H_2O_2 stimulated synthesis of HA. Apparently the initiation of HA degradation by H_2O_2 triggered compensatory production of high MW HA thereby enhancing the antioxidant defense.

The presence of the hyaluronate-receptor CD44 on the surface of stem cells is a well recognized fact (73) although its function in these cells is not entirely understood (74). However, it is known that stem cells are equipped in variety of defense mechanisms, particularly protecting their DNA from oxidative damage (75). In light of the evidence presented by us that hyaluronate prevents DNA damage by endogenous oxidants in WI-38 cells, the cells that express CD44, it is tempting to speculate that one of the functions of CD44 in stem cells may be to facilitate endocytosis of hyaluronate which then may act as protector of their DNA from oxidants. Interestingly, the lipid raft modification (clustering) on stem cells seems to be the key event triggering their entrance to the cell cycle (76). The internalization of CD44-bound hyaluronan requires CD44 association (upon its palmitoylation) with lipid rafts (65). Modifications of lipid rafts on stem cells, thereby, provide signals controlling both the cell cycle status (quiescence vs. proliferation) and internalization of HA. The stem cell thus may have several diverse mechanisms to protect integrity of its DNA, such as low metabolic activity that ensures minimal level of oxidative DNA damage, highly effective efflux pump that rapidly removes genotoxic agents from the cell (77), and possibly internalization of HA which protects DNA from oxidants. These mechanisms may be coordinated with each other and with the cell cycle status.

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