IN VITRO ASSESSMENT OF TWO TYPES OF HYALURONIC ACID’S ANTITUMOR POTENTIAL IN OSTEOSARCOMA CELLS

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Abstract

Even after the remarkable progress of the last decade, osteosarcoma remains a serious problem on a global scale, mainly affecting teenagers and elderly people. Hyaluronic acid is a ubiquitous polysaccharide in vertebrates and plays important physiological roles in the body, including the regulation of cell proliferation and inflammation. There are a variety of medical applications for hyaluronic acid; recent research has highlighted its role in the mineralization and growth of bones. Following these theoretical considerations, the present study sought to evaluate the antitumor potential of two types of hyaluronic acid in osteosarcoma cells. In order to achieve this goal, the viability and morphology of the cells, the effect on the structure of the nuclei, and the gene expression of some pro- and anti-apoptotic genes were evaluated. The results of the study indicated that hyaluronic acid causes a dose-dependent decrease in cell viability and induces changes in morphology and nuclei characteristic of cell apoptosis. Additionally, the highest concentrations tested resulted in an increase in pro-apoptotic genes (Bax and Bad) and a decrease in anti-apoptotic genes (Bcl-2). In conclusion, hyaluronic acid may represent a possible effective candidate for the treatment of osteosarcoma, but further studies are required in order to clarify the molecular mechanisms involved.

Rezumat

În ciuda progreselor remarcabile din ultima perioadă, osteosarcoma reprezintă un problema serioasă la nivel global, afectând în principal adolescenții și persoanele de peste 60 de ani. Acidul hialuronic este o polisaccharidă omniprezentă în vertebrate și joacă un rol important în organism, precum reglarea proliferării celulare și inflamării. În această perspectivă, această studiu a avut drept scop evaluarea potențialului antitumoral al dvs. tipuri de acid hialuronic, la nivelul celulelor de osteosarcomă. În acest scop au fost studiate viabilitatea și morfologia celulelor, impactul la nivelul structurii celulare și expresia genelor pro- și anti-apoptotice. Rezultatele studiului au indicat faptul că acidul hialuronic determina o scădere doze- și dosarăre, a viabilității celulare și inducând modificări la nivelul morfologia și structură nucleilor caracteristice apoptozei celulare. În plus, concentrația de acid hialuronic poate reprezenta un posibil candidat eficient în terapia antitumorala a osteosarcomului, însă sunt necesare studii suplimentare pentru elucidarea mecanismelor biologice.

Keywords: hyaluronic acid, osteosarcoma, cell morphology, nuclei structure, RT-PCR

Introduction

Osteosarcoma (OS) is a malignant tumour located in the bones that is more common in teenagers and elderly individuals over 60 years of age. A major location for osteosarcoma is the knee and the proximal humerus [2]. In terms of clinical manifestations, OS
is characterized by pain and inflammation at the local level. Despite the ambiguity of the manifestations, the malignancy is very high, as evidenced by the fact that almost 20% of OS cases developed metastases before onset of symptoms and, implicitly, diagnosis. The most common site of metastases is the lungs [26]. In addition, this type of cancer is associated with the early development of micro-metastases, which are difficult to diagnose using current diagnostic methods, posing a threat to the patient’s survival [39]. OS is classified according to several factors, such as location, type of cells involved and severity. According to the location of the disease, OS usually affects the centre of the marrow cavity of the long bones, as well as the soft tissues, periosteum and cortex of the bone [13]. Based on its cellular composition, OS is comprised of osteoblasts, chondroblasts, or fibroblasts, and the prevalence of these cells determines the type of tumour. The severity of OS is classified histologically into three categories, namely, grade 1, 2 and 3 [16].

Even though there have been studies in the field, the pathogenicity of OS has not yet been fully explained. Two hypotheses have been developed regarding the cellular origin of osteosarcoma based on studies conducted in vitro and in vivo. These hypotheses regard mesenchymal stem cells and osteoblasts, respectively [28].

Osteosarcoma has a wide geographical distribution, but the incidence rate differs according to the region. It has been observed that OS cases have increased in Southern Europe, particularly among the adult population younger than 24 years of age. Alternatively, in Northern Europe, more cases were recorded among the elderly because of malignant transformation of pre-existing diseases [23]. Risk factors include age, gender, socio-economic status, genetics and environmental factors [31]. As far as current therapeutic strategies are concerned, they involve the use of chemotherapy with various therapeutic agents, such as methotrexate or cisplatin, followed by surgical resection and adjuvant chemotherapy. There are a number of problems inherent in chemotherapy treatment, most notably those arising from adverse reactions, such as kidney or liver failure, leukoencephalopathy and damage to the white matter of the brain [12, 27].

Hyaluronic acid (HA) is a non-branched polysaccharide composed of D-glucuronic and N-acetyl-D-glucosamine disaccharides, with a high molecular weight (2 x 10^7 Da). HA was initially isolated from the vitreous body of the bovine eye in 1934 but is found in a wide variety of bacterial strains and is ubiquitous in vertebrates, particularly in embryonic tissues and extracellular matrix [22]. Despite being considered an extracellular molecule, HA can also be found intracellularly, within smooth muscle cells in the aorta. Hyaluronic acid has not yet been fully elucidated as to its physiological functions, but it is known that it plays an important role in the regulation of cell proliferation and inflammation [1]. As a result of its polymer structure and chemical structure, HA has undergone numerous derivatizations that have been of significant value to medical practice over the years. Additionally, different therapeutic molecules can be attached to the HA structure to facilitate their delivery to the target [32]. Hyaluronic acid has found its usefulness in a variety of therapeutic applications, including tissue regeneration, cosmetics and orthopaedics. It is important to note that HA’s role is crucial when it comes to bone regeneration [36]. HA is commonly used in the treatment of knee pathologies as well as temporomandibular osteoarthritis in medicine. Furthermore, HA has been shown to be effective as an antioxidant, an anti-inflammatory and an antibacterial in the dental field. It has been demonstrated recently that composites made of bone grafts and HA improve the mineralization and growth of bone [4].

Since the blood supply to bones is weaker than that to other tissues, delivering chemotherapeutic agents to the target is an important challenge in osteosarcoma therapy. This has led to the development of drug delivery systems that target bone tissue, with HA being one of the most promising candidates [21]. Starting from these premises, the aim of the present study was the evaluation of two types of hyaluronic acid (Fluicondrial 80 and Juvederm) at the level of osteosarcoma cells (SAOS-2) in terms of viability, cell morphology, structure of the nuclei and the impact at the level of markers with implications in cell apoptosis (Bax, Bad and Bcl-2).

Materials and Methods

Reagents

Fluicondrial (HA-Fu) was purchased from TheWave and Juvederm Ultra 3 (HA-Ju) was acquired from Allergan. Reagents used in the present study: trypsin-EDTA solution, dimethyl sulfoxide (DMSO), foetal calf serum (FCS), penicillin/streptomycin mixture, phosphate saline buffer (PBS), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent (MTT), 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma Aldrich, Merck KgaA (Darmstadt, Germany).

The osteosarcoma cell line - SAOS-2 was cultivated in a specific McCoy’s 5A Medium culture medium purchased from PAN Biotech (Aidenbach, Germany). To determine the markers involved in the apoptosis process, the following primers were used in the RT-PCR technique: 18s, Bcl-2, Bax purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and Bad bought from Eurogentec (Seraing, Belgium). All reagents used were pure and suitable for use in cell culture.

Cell Culture

The potential impact HA-Fu and HA-Ju on cell proliferation was evaluated using the osteosarcoma
cell line - SAOS-2 (catalog number: HTB-85™) purchased as a frozen vial from ATCC (American Type Cell Collection, Lomianki, Poland). The cells were cultured in McCoy’s 5A culture medium supplemented with 15% FCS and penicillin/streptomycin mixture. The cells were incubated in standard conditions (37°C and 5% CO₂).

**Cellular Viability Assessment**

The impact on cellular viability was determined by applying the MTT method [25]. For this purpose, the cells were cultured in 96-well plates and stimulated with five concentrations (50, 100, 250, 500 and 1000 μg/mL) of the two types of hyaluronic acid for a period of 24 hours. After the incubation time, the culture medium was replaced with a fresh one and 10 μL/well of MTT reagent, and the cells were incubated for 3 hours. Finally, the solubilizing solution was added in a volume of 100 μL/well and the absorbance was read at 570 nm for the calculation of cell viability using the Cytation 5 device (BioTek Instruments Inc., Winooski, VT, USA).

**Cellular Morphology**

The effect of the two types of HA on cell morphology was analysed by photographing the cells after 24 hours of stimulation using an Olympus IX73 inverted microscope (Olympus, Tokyo, Japan). The images were processed and analysed using The cellSens Dimensions v.1.8 software package (Olympus, Tokyo, Japan).

**Nuclear Staining**

To determine the impact at the nuclear level, immunofluorescence methods were applied. To achieve this objective, the cells were cultured in 12-well plates and stimulated with two concentrations of HA (250 and 1000 μg/mL) for a time interval of 24 hours. The cells were then washed with ice-cold PBS, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X. Nuclei images were taken with an inverted microscope (Olympus, Tokyo, Japan) through fluorescence methods were applied. To achieve this effect, the nuclei images were photographed after 24 hours.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

For qRT-PCR analysis, the PeqGold RNAPure™ Package purchased from Peqlab Biotechnology GmbH, Erlangen, Germany was used for RNA extraction, and the quantitative determination of the extracted RNA was measured by means of a DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA). Reverse transcription was performed using the kit purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA (Maxima® First Strand cDNA Synthesis Kit) and samples were then incubated in the Advanced Biometra Product line (Analytik Jena AG, Göttingen, Germany) for 10 min at 25°C, 15 min at 50°C and 5 min at 85°C. qRT-PCR analysis was performed with Power SYBR-Green PCR Master Mix, samples’ cDNA, sense and antisense primers and pure water, using a Quant Studio 5 real-time PCR system. For these experiments, the following primers were used: 18S (as housekeeping genes), Bax, Bad and Bcl-2.

**Statistical Analysis**

The statistical analysis was performed using GraphPad Prism version 9.3.1 software (GraphPad Soft-ware, San Diego, CA, USA, www.graphpad.com). The results were then expressed as ± standard deviation as the result of three individual experiments. The differences between the groups were analyzed by applying the ANOVA method followed by Dunnett’s multiple post-test and were expressed in the form of * (* p < 0.1; ** p < 0.01; *** p < 0.001; **** p < 0.0001).

**Results and Discussion**

According to the results, both samples (HA-Fu and HA-Ju) have a concentration-dependent effect on cellular viability, but with a different intensity. Consequently, HA-Fu concentrations of 50 and 100 μg/mL did not cause a significant decrease in viability, comparable to the control, unstimulated cells. Alternatively, the concentrations of 500 and 1000 μg/mL caused a more marked decline in the percentage of viable cells, which was 81% and 80%, respectively (Figure 1).

![Figure 1](image-url)

Evaluation of the cytotoxic effect on SAOS-2 cells after 24 hours of stimulation with HA-Fu and HA-Ju (50, 100, 250, 500 and 1000 μg/mL)

The results are expressed as a percentage, ± standard deviation of three individual experiments. Statistical analysis was conducted using the one-way ANOVA method and Dunnett’s multiple-comparisons post-test (* p < 0.1; *** p < 0.001; **** p < 0.0001).

As compared to HA-Fu, HA-Ju exhibits a stronger cytotoxic effect. The viability of cells was significantly decreased even at the lowest concentration tested (50 μg/mL). Cell viability dropped dramatically to approximately 49% at a concentration of 1000 μg/mL, which produced the strongest cytotoxic effect (Figure 2). The use of HA as a delivery system for cancellous bone allografts is quite common. A variety of types of HA have been evaluated in vitro for their potential effects on osteoblasts. Huang and colleagues assessed...
**in vitro** the effect of HA on rat calvarial-derived cell cultures, with the results of the study indicating that concentrations between 0.5 and 2 mg/mL of HA increased cell proliferation [10]. In a similar manner, Bonifacio and colleagues evaluated HA at the level of human osteoblasts - NHOst, emphasizing its capacity to stimulate cell proliferation [3]. Also, Kyyak et al. evaluated the effect of HA on human osteoblasts - HOBs **in vitro**, highlighting the stimulation of cell viability, proliferation and migration [17]. In this study, concentrations of HA were chosen in accordance with these studies. In this regard, the concentrations were selected following a review of the literature and selection of concentrations that are considered safe for use at the level of healthy bone cells. So far, we are unaware of any studies examining the effect of HA alone on osteosarcoma cells. Studies have focused on the potential of HA in transporting active substances to their target sites. Researchers Xu and colleagues developed a transport system for zoledronic acid used in the treatment of osteosarcoma based on hyaluronic acid. As a result of the association of HA with a therapeutic agent, tumour recurrence is prevented, improving the response to treatment [35]. The potential antitumor effect of a transport system containing HA and doxorubicin was also evaluated in an **in vitro** study on osteosarcoma cells. Results of the study indicated that the association releases the drug at the site of action, thereby inhibiting bone metastasis [38]. Similarly, Zhang et al. made nano-particles based on calcium carbonate and hyaluronic acid used to release doxycycline at the tumour level. This formulation proved useful in the treatment of advanced osteosarcoma, tumour growth being strongly inhibited [37].

Following the observation of cytotoxic effects on cell viability, the next step in the study was to evaluate the impact on cell morphology. Cell morphology provides information regarding the type of cell death, which can be classified as necrosis or apoptosis. Apoptosis is characterized by the preservation of cellular organization and membranes for a longer period of time, while necrosis involves the degradation of cell membranes and organelles [7].

A dose-dependent change in cell morphology was observed in response to HA-Fu. Hence, at low concentrations (50 and 100 μg/mL), no significant changes were observed, with the cells' morphology being similar to that of the control cells. Alternatively, at a concentration of 1000 μg/mL, cells rounded off, detached from the plate, confluence decreased and connections with neighbouring cells were lost (Figure 2). HA-Ju also induced significant morphological changes; however, in this case, the first signs of altered cell morphology were observed at a concentration of 250 μg/mL. Moreover, the 1000 μg/mL concentration induced the most significant cytotoxic signs at the level of cell morphology (rounding of cells, detachment from the plaque and significant decrease in cell confluency) (Figure 2).

![Figure 2. Evaluation of the effects of 24 h treatments with HA-Fu and HA-Ju on the morphologies of SAOS-2 cells. Scale bars indicate 50 μm](image)

Apoptosis is characterized by cell contraction, rounding of cells and the loss of connections with neighbouring cells [30]. In SAOS-2 cells, all changes were observed following HA treatment, particularly at a concentration of 1000 μg/mL. Gallorini and his colleagues highlighted the potential pro-apoptotic effect of HA in tendon-derived cells [9]. The use of HA in a targeted transport system of doxorubicin led to similar morphological changes in breast cancer cells - MCF-7 [34]. The next step in evaluating the effects of HA-Fu and HA-Ju on osteosarcoma cells was to determine the impact on the nuclei's structure. The microscopic examination of nuclei with fluorescence offers a number of advantages, including low costs and simplicity of the method for detection and quantification of the cell apoptosis process [20]. In order to visualize nuclei, 4',6-diamidino-2-phenylindole (DAPI) is one of the most commonly used fluorescent dyes. DAPI is suitable for use on both live and fixed cells, and increases fluorescence by more than 20 times when bound to DNA. As a result of its affinity for DNA, this technique provides the advantage of being able
to directly correlate the staining intensity with DNA content, nuclear condensation degree and nuclear area measurement [7]. The concentrations of 250 μg/mL and 1000 μg/mL were selected based on the fact that the first significant signs of cell viability and morphology damage were observed at 250 μg/mL and at 1000 μg/mL, the strongest cytotoxic effects were observed. Therefore, the HA-Fu induced structural changes in the nuclei starting at a concentration of 250 μg/mL. Compared to the control cells, there was a decrease in nuclei number, and chromatin was slightly condensed.

In addition to chromatin condensation and the decrease in the number of nuclei, the concentration of 1000 μg/mL caused massive changes which included the appearance of apoptotic bodies and nuclear fragmentation. Comparatively, HA-Ju caused more substantial alterations than HA-Fu. A massive condensation of chromatin and the appearance of apoptotic bodies could be observed even at the concentration of 250 μg/mL, whereas at the concentration of 1000 μg/mL, the signs of nuclear damage were even more evident, suggesting apoptotic effects (Figure 3).

Apoptosis is characterized by chromatin condensation, one of the hallmarks of the process. When apoptosis occurs, genomic DNA condenses together with nuclear proteins, forming the so-called “poptotic bodies” [33]. In the present study, all of these changes were also observed, especially when HA was tested at a concentration of 1000 μg/mL.

In a recent study, the impact of hyaluronic acid used in two concentrations (100 μM and 500 μM) was evaluated at the level of chondrocyte cell nuclei, by using DAPI staining, and no changes were observed at this level [24]. In addition, Della Sala et al. investigated the potential role of hyaluronic acid in the regeneration of alveoli in association with mesenchymal stem cells. According to the researchers, DAPI staining did not reveal any significant changes at the level of the nucleus [29]. It is important to note that the studies previously mentioned were conducted on healthy cells. When hyaluronic acid was examined in lung cancer cells – A549, DAPI staining demonstrated condensation of the chromatin in the nuclei, similar to the findings of this study [8]. On the basis of the calculation of the apoptotic index, it was found that Ha-Ju has a more intense apoptotic-

Figure 3.
SAOS-2 nuclei stained with DAPI dye after 24 h treatment with HA-Fu and HA-Ju (250 and 1000 μg/mL)
The orange arrows indicate signs of apoptosis. The scale bars represent 100 μm.
like effect than Ha-Fu, especially at the concentration of 1000 μg/mL (Figure 4).

Figure 4.
Evaluation of the apoptotic index (AI) of osteosarcoma cells, SAOS-2, after stimulation with HA-Fu and HA-Ju at 250 and 1000 μg/mL for a period of 24 h.

The results are expressed as percentages and standard deviation as a result of three independent experiments.

The statistical analysis involved one-way ANOVA analysis and a Dunnett’s multiple-comparisons post-test (* p < 0.1; *** p < 0.001; **** p < 0.0001)

Due to previous studies suggesting that both types of hyaluronic acid promote apoptosis, the next step was to examine their impact on the expression of pro-apoptotic (Bax and Bad) and anti-apoptotic genes (Bcl-2). Bcl-2 proteins play a major role in mediating cell death, most notably by regulating the permeability of the outer mitochondrial membrane, which facilitates the release of proteins associated with cell death from the intermembrane space. There is a link between the activity of the members of the Bcl-2 family and the changes occurring in the nuclei previously described. Therefore, members of the Bcl-2 family are associated with the nuclear apoptotic machinery and can either promote or inhibit cell apoptosis [11]. Pro-apoptotic proteins include Bax, Bak, Bad, Bid, etc., whereas anti-apoptotic proteins include Bcl-2, Bcl-XL, etc. In the end, the fate of a cell is determined by the ratio between these two categories of Bcl-2 members. External apoptotic stimuli play a significant role in determining the interaction between pro- and anti-apoptotic cells [14]. Both types of tested samples revealed an increase in the expression of the pro-apoptotic genes Bax and Bad, with the most intense increase being observed in the HA-Ju 1000 μg/mL sample. Meanwhile, a significant reduction in the antiapoptotic gene was observed only after stimulation with HA-Ju 1000 μg/mL for 24 hours (Figure 5).

Figure 5.
Relative fold expression of mRNA expression of pro- and anti-apoptotic mitochondrial markers in SAOS-2 cells after stimulation with HA-Fu and HA-Ju (250 and 1000 μg/mL) for 24 h.

The results were reported for 18 s and for the control group (non-stimulated cells) and expressed as mean values ± SD as a result of three independent experiments. The statistical analysis was performed by applying the one-way ANOVA method and Dunnett’s post-test (*** p < 0.01, **** p < 0.0001)

Bcl-2 proteins act as anti-apoptotic agents by inhibiting the expression of pro-apoptotic factors such as Bax and Bad, thus protecting the cell against apoptosis. However, in tumour cells, an increase in Bcl-2 activity is associated with a poor prognosis and resistance to cell death. In contrast, some external stimuli can cause the expression of pro-apoptotic genes (Bax and Bad) to increase, thus enhancing tumour cells’ response to treatment [15]. As far as we know, hyaluronic acid alone has not been evaluated in relation to its impact on Bcl-2 protein levels. Instead, Dou and colleagues evaluated the effect of nanoparticles containing hyaluronic acid and methotrexate on lung cancer cells - A549, observing an increase of pro-apoptotic genes, Bak1 [6]. Gold nanoparticles with hyaluronic acid also demonstrated a similar effect on gene expression by reducing the expression of Bcl-2 protein and increasing the expression of Bax, a pro-apoptotic gene [18]. In a similar study, liposomes containing hyaluronic acid and ursolic acid were shown to inhibit the expression of the anti-apoptotic gene Bcl-2 in A549 cells [19].
Conclusions

The current study examined the cytotoxic potential of two types of hyaluronic acid at the level of osteosarcoma cells - SAOS-2. According to the results of the study, both types of hyaluronic acid are cytotoxic, causing a decrease in cell viability and changes in cellular morphology in a dose-dependent manner. The nuclei also showed changes characteristic of cellular apoptosis, including the condensation of chromatin and the appearance of apoptotic bodies. In addition to these findings, RT-PCR analysis indicated that hyaluronic acid increases the expression of pro-apoptotic genes (Bax and Bad) while decreasing the expression of anti-apoptotic genes (Bcl-2). In conclusion, the results suggest an antitumor potential of hyaluronic acid, but additional studies are needed to elucidate the biological mechanisms and the safety profile.

Conflict of interest

The authors declare no conflict of interest.

References


