Altered Hyaluronan Biosynthesis and Cancer Progression: an Immunological Perspective

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Abstract: Hyaluronan is a glycosaminglycan present in practically all tissues as an important component of the extracellular matrix. In spite of its apparent simple chemical structure, hyaluronan is a molecule with multiple and complex physiogical and pathological functions. Hyaluronan is able to regulate a variety of biological processes such as cellular growth, migration, differentiation and inflammation, not only in normal but also in cancer tissues. Besides, increasing evidence suggests hyaluronan as a potent modulator of immune responses which supports a potential role of this molecule in cancer immunotherapy.

Keywords: Extracellular matrix, hyaluronan, CD44, immune system, dendritic cells, cancer.

1. INTRODUCTION

Hyaluronan, a member of the glycosaminoglycan family, is a uniformly repetitive and linear molecule composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine [1]. In spite of its apparent simple chemical structure, hyaluronan was shown to regulate a variety of biological processes such as cell growth, adhesion, migration and differentiation, and to have multiple and complex physiological functions in wound healing, inflammation [2] and immune responses [3]. Hyaluronan effects seem to be dependent on its molecular weight and concentration, as well as on the type of tissue under study and on its cellular localization [2]. This review discusses the different roles of hyaluronan in both physiological and pathological conditions, with special emphasis on cancer progression and on its therapeutic potential through immunostimulatory mechanisms.

2. STRUCTURE

Hyaluronan is a large and linear glycosaminglycan (GAG) composed of repeated disaccharide units of glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) joined by β(1→3) and β(1→4) linkages (Fig. **1**) [1]. The presence of one carboxyl group per disaccharide unit results in a negative charge at neutral pH [4]. Each disaccharide has a molecular mass of approximately 400 daltons (Da) and an average length of 1 nm. The number of disaccharides units in a hyaluronan molecule can reach up to 25,000. Therefore, a completed hyaluronan molecule is about 10^7 Da with a total extension of 25 μm [5]. Thus, as a consequence of its random-coil structure, its large size and its high capacity to interact with water molecules, hyaluronan-enriched solutions present high viscosity and elasticity. Furthermore, these properties provide this molecule with space filling as well as lubricating and filtering functions [5]. In addition to its ability to interact with other proteoglycans from ECM hyaluronan has the ability to regulate cell activities such as proliferation, adhesion, and migration among others [1, 6].

Hyaluronan is a member of the GAG family but has some differences with other members of the GAG group, which can be summarized as follows: i) its polysaccharide chains are long and lack from sulfate groups or epimerized uronic acid residues, and ii) its synthesis takes place at the inner side of the plasma membrane instead of inside the endoplasmic reticulum and Golgi apparatus [1].

Hyaluronan is present in practically all tissues as an important extracellular matrix (ECM) component. High concentrations of hyaluronan are found in the synovial fluid, vitreous humor, skin and umbilical cord [1]. Hyaluronan is generally present as a molecule of high molecular weight (HMW-HA) ranging from $0.5x10^6$ to 10^7 Da, although a low molecular weight hyaluronan (LMW-HA) form ranging from 10^4 to $0.5x10^6$ Da can also be found. LMW-HA is associated with certain pathological conditions such as inflammation and cancer and it was shown to promote angiogenesis and to stimulate the production of inflammatory cytokines [7]. Smaller fragments ($\leq 10^4$), known as oligomers, share some biological activities with LMW-HA. In addition, some indirect effects have been also described for LMW-HA or HMW-HA [8].

Biosynthesis and Degradation

Hyaluronan biosynthesis is carried out by hyaluronan acid synthases (HAS) at the inner face of the plasma membrane. Thereafter, the growing polymers are extruded through the membrane into the extracellular space [9]. There are three HAS isozymes (HAS-1, HAS-2 and HAS-3) that add GlcA and GlcNAc to the growing chain of hyaluronan polymers using UDP-GlcA and UDP-GlcNAc as substrates.

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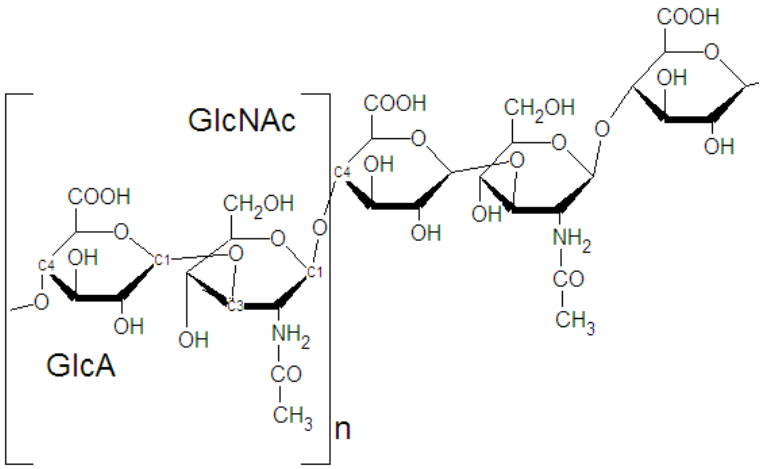

Fig. (1). Hyaluronan molecule is composed of n (~2500) repetitions of glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) joined by β(1→3) and β(1→4).

The principal differences between these isozymes are the catalytic activity and the size of the synthesized hyaluronan. Thus, HAS-3 produces predominantly LMW-HA and it is thought to be more active than HAS-1 and HAS-2, while these two later forms produce longer hyaluronan molecules [2].

The hyaluronan turnover appears to be important for the maintenance of tissue homeostasis and it is calculated that almost 30% of the hyaluronan is daily replaced by newly formed molecules in humans. Nevertheless, hyaluronan catabolic rate varies in different tissues [7]. Degradation of hyaluronan occurs by the coordinated action of an endoglycosidase, hyaluronidase (Hyal) and two exoglycosidases (β-glucuronidase and β-N-acetyl glucosaminidase). Hyal degrades hyaluronan polysaccharides into oligosaccharides, which are further digested into GlcA and GlcNAc by β-glucuronidase and β-N-acetyl-D-hexosaminidase, respectively [10]. In humans, six hyaluronidase genes have been identified so far: *HYAL1*, *HYAL2*, *HYAL3*, *HYAL4*, *HYAL-P1* and the sperm-specific *HYAL PH20* [10]. From them, both Hyal-1 and Hyal-2 account for the most hyaluronan degradation in somatic tissues and, together with PH-20, are the best characterized hyaluronidases. While Hyal-1 highly specifically degrades hyaluronan into oligosaccharides, Hyal-2 is able to degrade HMW-HA into fragments of intermediate size (20 kDa), and PH-20 was shown to be necessary for ovum fertilization [10]. It has been recently postulated that Hyal-2, present at the cell surface, starts hyaluronan catabolism by generating intermediate fragments of 50–100 saccharides, which enter early endosomes. As they become lysosomes, the fragments are degraded into tetrasaccharides by lysosomal Hyal-1 [4].

Removal of hyaluronan occurs by endocytic uptake within the tissue, especially in lymph nodes and liver. Importantly, the presence of reactive oxygen species enhances hyaluronan turnover. Internalization and degradation of hyaluronan is triggered by its binding to CD44 [11] and/or lymphatic vessel endothelial receptor-1 (LYVE-1). LYVE-1, the most efficient receptor for hyaluronan internalization [12], is highly expressed in lymphatic endothelium and has the ability to form large molecular complexes composed of several protein subunits [12].

Hyaluronan Receptors

Biological functions of hyaluronan are mediated by its molecular interaction with different cell surface receptors such as CD44, Receptor for Hyaluronan-Mediated Motility (RHAMM) and other HA-binding proteins [13]. CD44 is a cell surface glycoprotein encoded by a single gene, although there are a number of isoforms expressed in a number of cells and tissues as a result of alternative splicing [14]. It has been shown that some splice variants such as CD44v-9 and CD44v-6 are involved in tumor metastasis [15, 16]. However, and since it is well known that there is high heterogeneity in the expression of the different CD44 variants among human tumors, their expression pattern cannot be always correlated with malignant progression [17]. The expression of CD44 is not always indicative of its capacity to bind hyaluronan. CD44 shows different states of activation: i) an active form that binds hyaluronan constitutively, ii) an inducible form that binds hyaluronan after stimulation with inducing factors (e.g., cytokines, phorbol ester or mAbs), and iii) an inactive form which is unable to bind hyaluronan. Because of its ubiquitous expression, an external stimulus is necessary for CD44 activation which is considered to be a protective mechanism [14, 18]. The CD44 amino terminal sequence shares nearly 35% sequence homology with other hyaluronan binding proteins such as LYVE-1, aggrecan, versican, brevican, neurocan and tumor necrosis factor inducible protein-6 (TSG-6) [13, 14]. Importantly, all of these proteins present a hyaluronan-binding domain, termed “link module”, which contains disulfide-bonded loops [13, 14]. In addition, there are other proteins, such as RHAMM, inter-α-trypsin inhibitor heavy chain (SHAP) and endocytic receptor for HA (HARE), found to be expressed in the liver, spleen, and lymph nodes, which lack the “link module” but might also interact with hyaluronan [13,19]. Recently, Toll-like receptors (TLR) 2 and 4 have been reported to bind small fragments of hyaluronan, having an important role in innate immunity and dendritic cells (DCs) activation [20-22].

3. PHYSIOLOGICAL FUNCTIONS

Despite its simple chemical composition, hyaluronan possesses diverse and complex functions both in physiological and pathological conditions (Fig. **2**) [6]. Besides being a major component of the ECM, hyaluronan expression can also be found in different cell types such as in oocytes prior to ovulation or in proliferating/migrating cells during morphogenesis and tissue remodeling [23]. As an ECM component, hyaluronan has a structural function. Its biophysical properties provide hyaluronan with functions that influence the hydration and biomechanical structure of different tissues, especially those of the vitreous humor in the eye, the synovial joint fluid and the dermis [1]. In addition, hyaluronan interaction with other ECM molecules such as versican or aggrecan was shown to be essential for the assembly and structure of certain tissues [13]. For example, increased levels of aggrecan, shown to be immobilized upon hyaluronan interaction, in presence of collagen, provides the tensile, osmotic and adequate biochemical properties of articular cartilage [24].

Hyaluronan interactions with CD44 and RHAMM receptors influence cell behavior through the activation of Rac, PI3K (phosphoinositide 3-kinase) and Ras signalings affecting cell motility, cellular transformation and survival [25-27]. It has been observed that hyaluronan induces migration and facilitates transformation of cardiac endothelial cells to mesenchymal cells through Ras, since expression of a dominant-negative form of the Ras protein in those cells was shown to significantly reduce this process and their migratory properties [28]. It has been demonstrated that hyaluronan-activated PI3K signaling promotes the survival of several tumor cell types such as lung, colorectal and mammary carcinoma by inducing anchorage-independent growth [29]; similar effects were observed in other cell types such as lymphoma by modulating Akt phosphorylation [30].

During morphogenesis, hyaluronan synthesis is up-regulated prior to mitosis which in turn facilitates the detachment and motility of cells in a hydrated pericellular matrix and promotes their interaction with other extracellular molecules such as versican [23]. These events rely on hyaluronan own physical properties as well as on signaling events triggered by hyaluronan-CD44 and/or hyaluronan-RHAMM interaction [23,31]. It is worth noting that hyaluronan synthesis is tightly regulated during cell cycle: there is a peak of synthesis during mitosis which is followed by its biosynthesis inhibition subsequently leading to cell cycle arrest. In addition, hyaluronan was shown to play an important role during embryogenesis since it was found to promote proliferation and migration of undifferentiated cells from stem cell niches to sites of organ development [26]. For example, in muscle differentiation hyaluronan likely induces myoblasts proliferation through inhibiting their further differentiation and thereby avoiding the appearance of morphological abnormalities [32], as shown for limb development [33]. Besides, it has been observed that *Has 2-/-* mice have

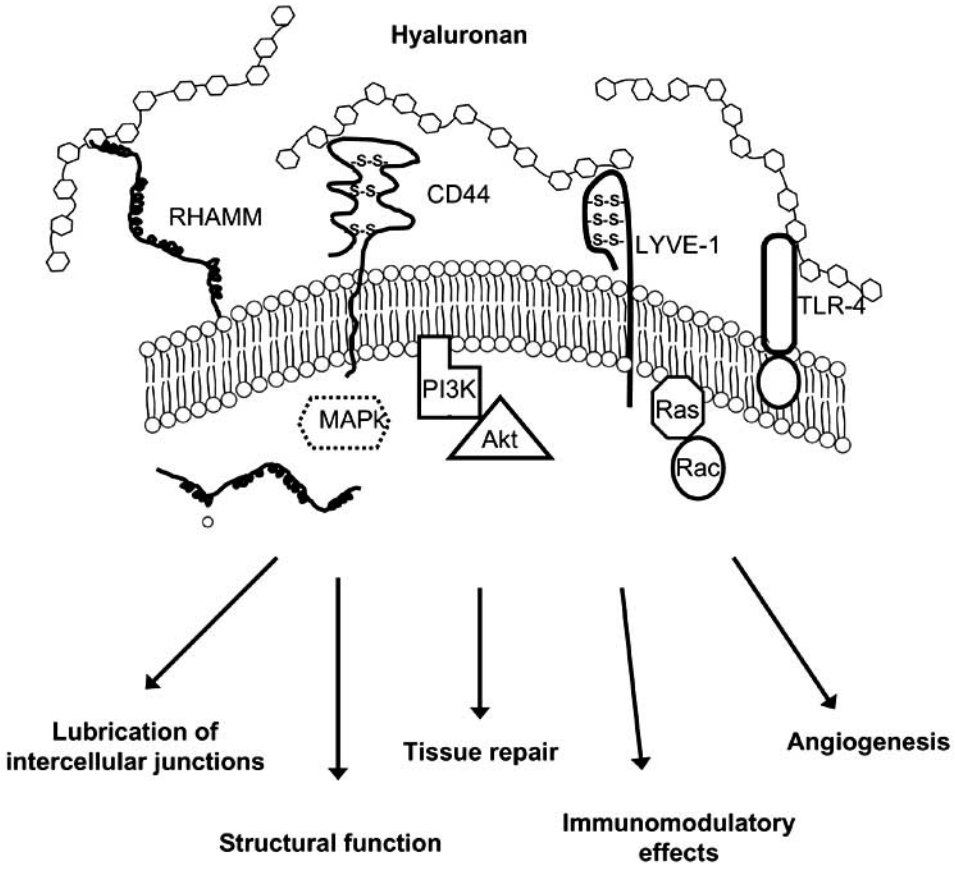

Fig. (2). Hyaluronan interaction with some cellular receptors such as CD44, RHAMM and TLR-4 mediates several physiological functions involved in tissue remodeling, angiogenesis and modulation of immune response.

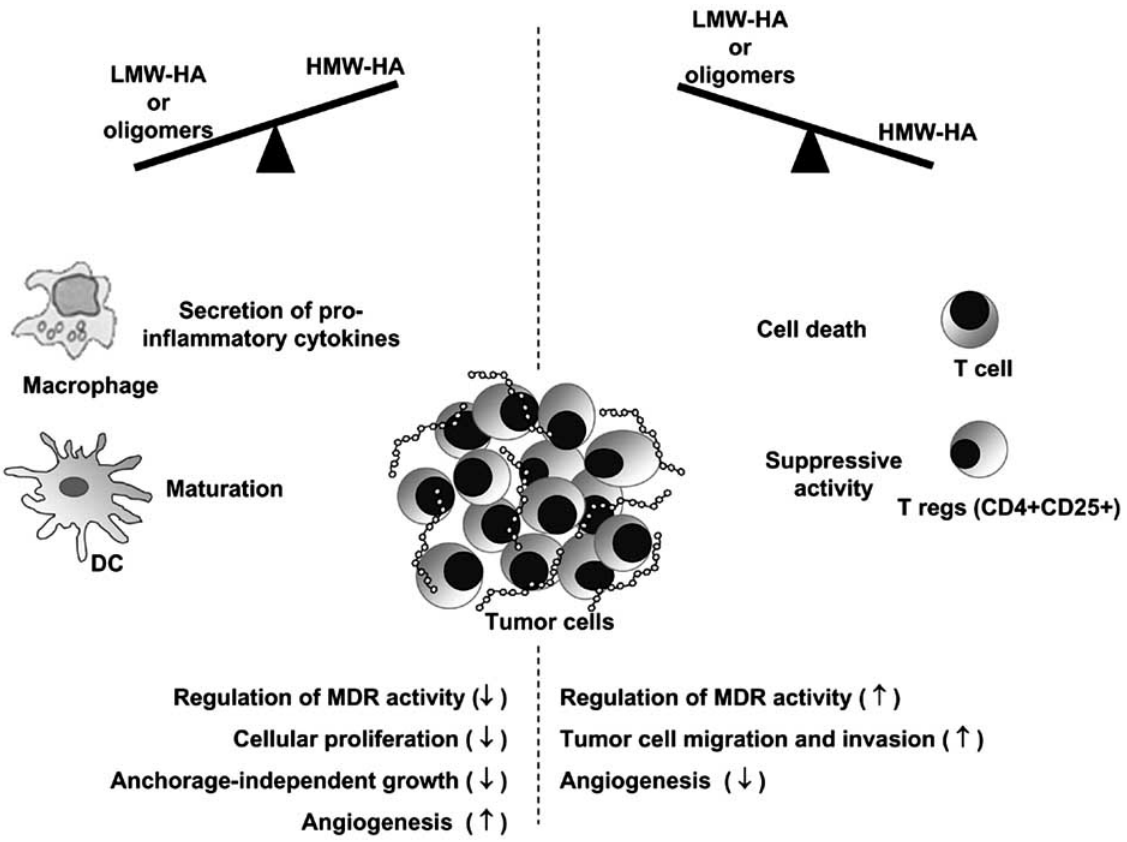

Fig. (3). The balance between HMW-HA and LMW-HA or oligomers determines the role of this molecule in tumor progression and immune response.

severe alterations during morphogenesis, which lead to mid-gestation death due to structural defects in heart and vascular structures [28].

Hyaluronan has a crucial role in angiogenesis during wound healing since it maintains the permeation of luminal capillaries [34]. Hyaluronan is present in the blood vessel wall in different amounts and distribution depending on the type and age of the vessel [34]. Although native hyaluronan is considered to have anti-angiogenic properties, the fragments generated during tissue injury and wound healing may stimulate endothelial cell proliferation, migration and new vessels formation [35, 36]. In physiological conditions, these fragments are removed and replaced by native hyaluronan, thus inhibiting further proangiogenic processes [35, 37], whereas abnormal hyaluronan turnover during vascular diseases and tumor development might promote angiogenesis [34, 36].

The multiple effects of hyaluronan described above have led to the use of hyaluronan in different clinical applications. The most extended medical application of hyaluronan is within the context of osteoarthritis as sodium hyaluronate (NaHA). It has been reported that high molecular weight (> 500,000 Da) NaHA improves joint mobility and reduces pain [38, 39]. Intra-articular injection of NaHA has the ability to stimulate the expression of the inhibitor of metalloproteinase 1 (TIMP1), to inhibit the degradation of aggrecan and to decrease nitric oxide production, thus resulting in a significant suppression of articular cartilage erosion [40]. Exogenous administration of hyaluronan can stimulate endogenous hyaluronan production and the growth and function of chondrocytes [40], resulting in a beneficial effect in patients with osteoarthritis and rheumatoid arthritis [41].

Another interesting field of application of hyaluronan is ophthalmology. Hyaluronan has been successfully used to avoid corneal edema in cataract surgery [42] and it is believed that hyaluronan protects the endothelial layer of the cornea and other tissues from physical damage associated with surgical procedure. Moreover, several works have shown that hyaluronan plays an important role in corneal epithelial wound healing [42,43].

The physical properties of hyaluronan (i.e. viscosity, moisture, water holding ability and capacity to create volume) allow its use of this molecule in cosmetic preparations and as biological material filler in esthetic surgery and dermatology, with the aim of removing wrinkles and preventing skin aging [44].

4. HYALURONAN AND CANCER. A ROLE FOR HYALURONAN IN CANCER PROGRESSION

Tumors are formed by not only tumor cells but also fibroblasts, endothelial cells and tumor-associated macrophages and they can be influenced by several matricellular chance proteins by components [45, 46]. Therefore, there is a complex cross-talk between cancer cells and their microenvironment. Thus, strong evidence indicates that tumor microenvironment can regulate the capacity of tumors to grow and

metastasize [45, 46]. It is known that hyaluronan is implicated in tumor progression, although its mechanisms are not well defined yet [47]. In many types of tumors, such as breast, ovarian, prostate, pancreas, colon and gastric cancer, the biosynthesis of hyaluronan in the extracellular matrix is altered, both a high hyaluronan concentration and an altered Has/Hyal activity are frequently found in tumor tissues [47, 48]. In some cases, high levels of hyaluronan are associated with malignant progression. Overexpression of the HAS enzymes results in an excessive production of hyaluronan and an enhanced tumorigenesis of colorectal and mammary carcinoma, melanoma and mesothelioma [49-51]. In contrast, it has been observed that HAS-2 overexpression ameliorates tumorigenesis of glioma cells [52]. Besides, Hyals are overexpressed in prostate cancer, bladder carcinoma and head and neck cancer, promoting tumor invasion [53-55].

On the other hand, an excessive degradation of hyaluronan or an upregulation of Hyal activity results in the generation of hyaluronan fragments or oligomers, which are able to attenuate or inhibit cancer cell signaling induced by the native polymer [29] (Fig. **3**). Moreover, oligomers can directly modulate cancer cell survival and apoptosis [30].

The role of hyaluronan in tumor progression is complex and depends critically on its molecular weight [56] but also on its capacity to interact with other proteins (e.g. TSG-6, SHAP) [57]. In addition, the concentration and cellular localization (intra or extracellular) of hyaluronan might also influence its effect on cancer biology [31, 58-59].

Several reports have shown that hyaluronan facilitates tumorigenesis through its interaction with different receptors (e.g. CD44, RHAMM, LYVE-1 and TLR-2, TLR-4) [58]. The mechanisms implied include: i) stimulation of proliferation and resistance to apoptosis of tumor cells by activation of survival signals such as MAP kinase, and PI3K/AKT pathways [25, 60-62]; ii) stimulation of cell migration and invasion through the expression of metalloproteinases and cytoskeletal rearrangement of proteins such as ankirin and ERM (erzin/radix/moesin) [63, 64]; iii) modulation of multidrug-resistance genes such as MDR or MRP [65]: while hyaluronan enhances MDR activity, its fragments or hyaluronan oligomers have the contrary effect, i.e. they sensitize breast and lymphoma cancer cells to different chemotherapeutic drugs [66, 67]; and iv) promotion of angiogenesis through stimulation of endothelial cell proliferation and migration, by induction of FAK and ERK signals [36, 37].

On the other hand, hyaluronan might exert an antitumoral effect when it is exogenously administered. Different studies have shown that hyaluronan antitumoral effects are dependent on its concentration and molecular weight [68]. For example, intratumoral injection of HMW-HA at high concentrations was shown to inhibit tumor growth in a human breast cancer cells xenotransplant model [69]. In this work, the authors speculated that high hyaluronan concentrations might disrupt endogenous hyaluronan binding, thus resulting in tumor regression; nevertheless, definitive proof for the involvement of such mechanisms is still lacking [69]. In another study, treatment with LMW-HA or oligomers was found to result in antitumoral effects, through induction of myeloid differentiation, in an acute myeloid leukemia cell model [70]. Oligomers were also shown to inhibit tumor growth in murine mammary carcinoma, human lung cancer and colorectal carcinoma models [29, 56]. Moreover, we have recently found that LMW-HA was able to significantly reduce murine colorectal carcinoma growth *in vitro* and *in vivo* [71]. One of the mechanisms involved in the observed LMW-HA antitumoral effects relies on the stimulation of the immune system through the activation of dendritic cells and the T-cell responses [71]. We have also developed a preventive model of treatment in mice with subcutaneous colorectal carcinoma (using CT26 cells). Repetitive intravenous injection of LMW-HA significantly decreased tumor growth and increased survival without significant toxicity (Fig. **4**).

4.1. Tumor Immune Modulation

Failure of the immune system to recognize and destroy tumor cells may lead to the escape from the immune system control [72]. In the last decade, some researchers have demonstrated that hyaluronan in its different forms is a potent modulator of the immune system [73]. A number of studies have shown that hyaluronan is capable of modulating adhesion, migration and activation of immune cells expressing hyaluronan receptors (CD44, RAHMM, and TLR) [8]. Naïve T lymphocytes express CD44 in an inactive form that is unable to bind hyaluronan. During T cell activation, CD44 expression increases in these cells as well as its binding to hyaluronan [74]. Accordingly, it has been shown that T cells synthesize hyaluronan which is critical for IL-2-mediated proliferation [75]. Some authors consider that either LMW-HA or hyaluronan fragments can act as a danger signal by promoting Ag-specific T cell response [21]. These observations underscore an important role for hyaluronan in T cell biology.

The interaction between CD44 and TLR with hyaluronan fragments, but not with native hyaluronan, induces the expression of inflammatory mediators in murine and human macrophages such as macrophage inflammatory protein-1 (MIP-1), monocyte chemoattractant protein-1 (MCP-1) RANTES, nitric-oxide synthase, interleukin-1β, IL-6, IL-8 and TNF-α [76-78]. Conversely, it has been observed that native hyaluronan induces apoptosis of PMA or CD3-activated T cells [79]. Besides, it has also been shown that HMW-HA stimulates regulatory T cells (CD4+CD25+) [80]. Only few studies have shown immunomodulatory mechanisms of hyaluronan during tumor development. One may speculate that the high levels of hyaluronan, frequently found around tumors, could regulate the immune system and, depending on the predominant hyaluronan molecular weight, the result could be either immune activation or suppression. The armamentarium for immunotherapy protocols has been improved by the identification of dendritc cells (DCs) as protagonists of antigen presentation [81]. DCs are the most relevant professional antigen-presenting cells (APC) and are central in the interface between innate and adaptive immune response. While mature DCs are able to induce antitumoral immunity, antigen presentation by immature DCs results in tolerance [82]. Therefore, they are considered as key tools in different immunotherapy protocols for cancer treatment. A number of clinical trials using DCs have been carried out over the last decade [83]. However, long lasting tumor-

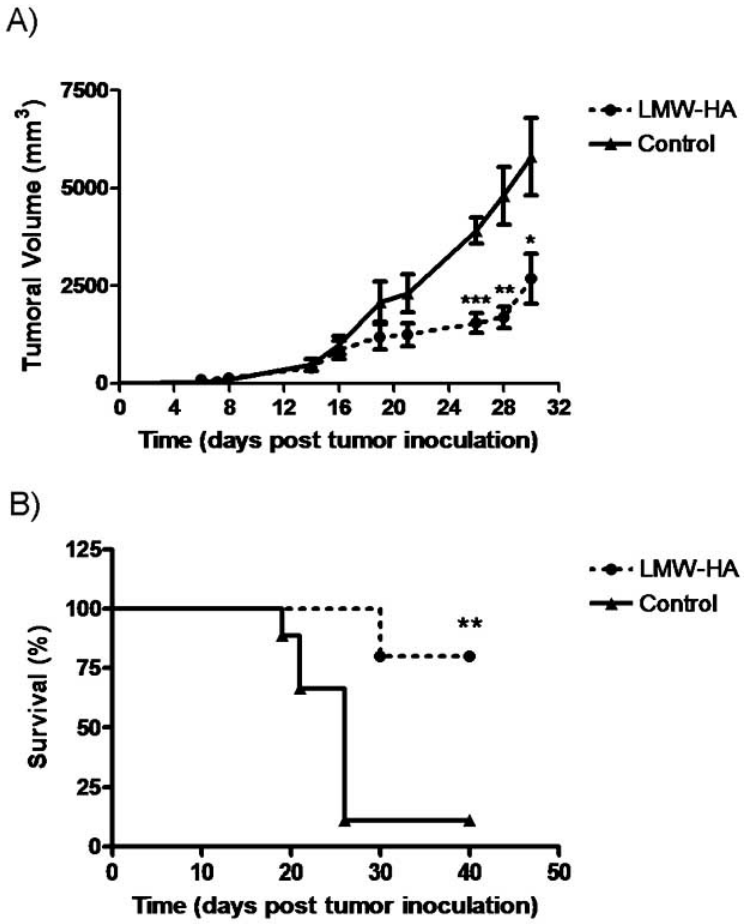

Fig. (4). Effects of i.v. administration of LMW-HA *in vivo* in a preventive colorectal carcinoma model in mice. BALB/c mice were s.c. inoculated with $5x10^5$ CT26 cells and 20 μg/100 μl of either LMW-HA or vehicle alone (control) simultaneously. Inoculation of LMW-HA was repeated each day for five consecutive days. (A) The tumor growth curve is representative of three independent experiments (8 animals per group) (mean ± SD, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (B) Kaplan-Meier 40-day survival curve of mice bearing the CT26 tumor treated with either LMW-HA or vehicle. Log-rank test **$p < 0.01$.

specific immune responses have not been achieved and persistent clinical responses are exceptionally rare [84]. Immunosuppressive factors released by tumor cells can prevent DCs differentiation and activation allowing the tumor to escape from the immune response [84]. Therefore, it is necessary to search for stronger strategies to fight against cancer. One attractive possibility is to find an immune adjuvant or to artificially up-regulate the immune response in order to allow a more effective anticancer effect.

Hyaluronan can modulate DSc maturation [3, 20, 85]. Termmer *et al.* have shown that hyaluronan fragments, but not native hyaluronan, activate DSc by TLR4, but independently of CD44. Addition of hyaluronan oligomers to DCs cultures was found to increase the expression of activation markers such as MCH class II, CD80, CD86 and CD40 and to promote the activation of immature DCs [20, 85]. Moreover, DC synthesized hyaluronan was shown to play an important role in antigen presentation [86]. Consistently, exogenous injection of LMW-HA during immunization with OVA peptide in C57BL/6 OT-II (TCR-transgenic-specific OVA peptide) mice has been found to act as an adjuvant by promoting antigen-specific T cell responses [21]. Thus, an interesting strategy may consist in adding LMW-HA to the cocktail of DCs maturation with the aim of enhancing the induction of specific immune responses against cancer cells upon DCs application *in vivo*.

Anticancer Perspectives

Several reports have shown that exogenous administration of hyaluronan may modulate tumor growth not only by altering the endogenous ECM hyaluronan balance, which can result in direct inhibition of tumor cell proliferation, but also by stimulating the tumor immune system. Therefore, this molecule has a potential to be used as an adjuvant agent for different oncological therapies.

In addition, hyaluronan is a highly conserved molecule, poorly immunogenic and non-toxic. These properties make hyaluronan a safe pharmacological candidate to be used in combination with other immunotherapeutic protocols for cancer treatment.

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