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Comparative evaluation of cytotoxic and anti-metastatic function of microbial chondroitin sulfate and animal-originated commercial chondroitin sulfate in cancer cells

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ABSTRACT

Cancer has the second-highest mortality rate worldwide after cardiovascular disease. In addition, cervical and breast cancer are two of the leading causes of cancer-related deaths among women. The tumor microenvironment, which consists of cells that form blood vessels, proteins, fibroblasts, and immune cells, is a therapeutic target for cancer therapy. As part of the extracellular matrix (ECM), glycosaminoglycan Chondroitin Sulfate (CS) is related to diverse aspects of tumor growth and metastasis depending on the CS sulfate pattern. This study analyzed the roles of Microbial CS and Commercial CS in tumor growth and metastasis using HeLa cervical cancer cells, MDA-MB-231 metastatic breast cancer cells, and normal fibroblasts. In addition, the role of CS types in wound healing was also assessed comparatively. Microbial CS was more cytotoxic in MDA-MB-231 cells than HeLa compared to Commercial CS. Although both CS reduced cell viability in normal cells, the selective index of Microbial CS in MDA-MB-213 cells was higher than its commercial counterpart. In addition, the role of CS types in wound healing was also assessed comparatively. Both types of CS decreased the cell migration in MDA-MB-231 breast cancer cells, but HeLa cells were more sensitive to Microbial CS than Commercial CS to heal the wound. The wound healing of NIH3T3 cells after Microbial CS was similarly high to the healing after Commercial CS. This preliminary study shows that microbial CS produced by biotechnological methods from a recombinant source created by our team can be an effective therapeutic agent in various types of cancer.

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Introduction

Cancer is defined as an uncontrolled and rapidly occurring abnormality in the cell structure due to genetic or epigenetic changes. Cancer has an increased mortality rate and is the second-highest reason for death in the world after cardiovascular diseases (1). The number of cancer cases worldwide in 2015 was 17.5 million, and 8.7 million resulted in death (2). In addition, 606,520 cancer deaths resulted from 1,806,590 new cancer cases in the United States of America in 2020. (1). Breast, lung, and colorectal cancer are women's most common cancer types, accounting for 50 % of all new cancer diagnoses. Among these cancers, breast cancer alone constitutes 30 % of female cancers and is the primary reason for cancer death for women worldwide. There has been a significant increase in breast cancer rates in recent years. Breast cancer cases have risen by 33% between 2005 and 2015 (2-4). Moreover, cervical cancer remains the second-highest reason for cancer-related death in women aged 20-39 (1). Methods including surgery, radiation, chemotherapy, and immunotherapy are commonly used individually or in combination for cancer treatment.

It is reported that various factors such as chronic low-grade inflammation are effective in the growth and progression of tumor cells (5, 6). However, the inflammation-dependent initiation of tumor growth factors is due to an increase in chemokines and cytokines. Tumor cells synthesize chemokines and macrophages that are related to tumor cells. Proinflammatory cytokines (IL-1, IL-6, IL-12, IL-23, and tumor necrosis factor X) synthesized by M1 macrophages rise in the early phases of tumor growth. Cytokines such as IL-4 and IL-10, synthesized by M2 macrophages, also have antitumor activity in the early phases of tumor growth (5, 7, 8). The activity of growth factors and cytokines is due to the effect of these molecules on the expression of other growth factors and cytokines, the extracellular matrix (ECM) components, and the various enzymes responsible for the ECM process (5).

The tumor microenvironment formed by cancer cells gives information about the effectiveness of the treatment against cancer cells. The tumor environment is composed of healthy cells, which are fibroblasts, immune cells, and cells that form blood vessels. Proteins produced by cancer cells are also found in this environment. The tumor microenvironment is mostly considered to be targeted by drugs as the components of this structure can promote cell invasion and metastasis (9-12). Chondroitin Sulfate (CS) is found in tumor stroma and promotes meaningful interactions in tumor microenvironments (13, 14). In a study, the elimination of CS by Chondroitinase ABC injected into the tumor did not impact the growth of the existing tumor but increased lung metastasis and caused the development of secondary tumors (13). Since the severe

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side effects and/or unspecific mechanisms of chemotherapeutics limit the success of the therapy, patients/medicals tend to use natural compounds to improve the outcome of the therapy. Although the role of CS, which is an anionic linear polysaccharide, in tumor growth and metastasis is controversial, recent studies support that CS can be used as an anticancer agent (13, 15, 16). CS consists of repetitious disaccharide units of D-*N*-acetyl-galactosamine and D-glucuronic acid (→4GlcAβ1→3GalNAcβ1→), varying according to the number and location of sulfate groups (Figure 1). CS is an essential building block of the ECM. Moreover, it is found in the ECM of many tissues, such as cartilage, skin, bone, and tendon (17). Differences in the origins and production techniques of CS affect the purity rate, and accordingly, its efficacy in treatment may vary (18, 19). The molecular weight of animal-originated CS referred to as Commercial CS, varies from species to species and averages around 50-100 kDa. Microbial CS has a lower molecular weight (10-30 kDa) than its animal-originated counterparts. Therefore, Microbial CS provides a more elevated absorption after oral intake and is sufficient for medical uses (20).

Sulfate groups are generally located at C-2 and/or C-3 of glucuronic acid and C-4 and/or C-6 of N-acetyl-galactosamine, and enable certain connections with various compounds, such as growth factors, chemokines, cytokines, lipoproteins, and adhesion proteins (22-24). CS binds to serine (Ser) in the amino acid chains that constitute the protein via a tetrasaccharide bond consisting of two galactose, xylose, and glucuronic acid (23). As a result of the biosynthesis of CS, the formation of certain Chondroitin Sulfate Proteoglycans (CSPGs) such as aggrecan (the primary Proteoglycan of cartilages), versican (the major Proteoglycan of connective tissues), decorin, and biglycan formation occur. Animal-originated CS generally has CS-A and CS-C structures and does not show double sulfation, while marine-originated CS exists in CS-A, CS-C, and CS-E forms. The Microbial CS we produced is in CS-A and CS-C forms, like animal-originated CS. Nuclear Magnetic Resonance (NMR) confirmed this structure of Microbial CS (25).

CS has been proven to have immunomodulatory activity. CS may decrease oxidative stress and biosynthesis of several proinflammatory molecules (26-29)**.** Nevertheless, the CS-mediated effect on inflammation is cell-specific and may vary depending on the location of the sulfate in the glycosaminoglycan structure. Considering that macrophages provide protumor and antitumor balance, the activity of CS on macrophages also affects tumor development and metastasis. The CS-mediated effect on inflammatory responses in the tumor environment is complicated. Moreover, the sulfation design of CS is critical in cancer cell growth and metastasis (30). Since bovine Hyal enzymes, one of the ECM processing enzymes regulated in the tumor niche, produce CS-C actively stimulating human monocytes, the proinflammatory cytokine IL-12 has been released from monocytes (31, 32). Therefore, the balance between CS-C accumulation and degradation in the tumor microenvironment influences tumor-associated inflammation. It is important to note that CS-C promotes the anti-inflammatory action of IL-10 in the tumor environment. Therefore, it prevents the initiation of a tumor or promotes the spread of an existing tumor. In addition, GAGs in the ECM interact with chemokines and inhibit the proteolysis of these cytokines. GAGs facilitate the migration of leukocytes and help recruit these cells to areas of tissue damage (33). Considering the effectiveness of CS as an anti-cancer agent on cancer progression and metastasis, in this work, the cytotoxic and anti-metastatic effects of Microbial CS, which we produced biotechnologically, and Commercial CS on HeLa cervical cancer cells and metastatic breast cancer cells were evaluated comparatively (25, 34). The response of cancer cells against CS types was also compared to normal fibroblast cells. This study revealed that the origin of chondroitin sulfate could have significantly different effects on cancer cells as anti-proliferative in MDA-MB-231 cells and anti-metastatic in both MDA-MB-231 breast cancer cells and HeLa cervical cancer cells, but more anti-metastatic in HeLa cells than MDA-MB-231 cells. The molecular mechanisms defining the specific functions can be related to the histological characteristics of cancer origin and genomic/epigenomic characteristics of the cells examined. The candidate / relevant mechanisms, therefore, need to be elucidated.

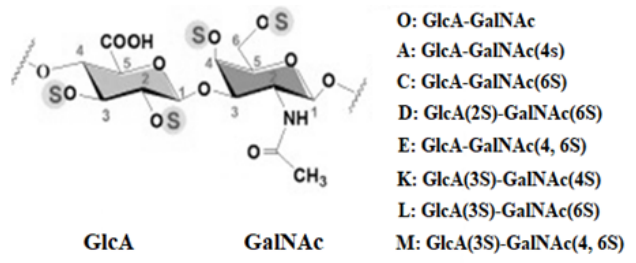


Figure 1. The structural formula of repeating the disaccharide unit of CS and its types according to the position of sulfate groups. Reprinted from reference (21).

Materials and Methods

Strains

E. coli (C2987), needed for producing Microbial CS, was purchased from NEB (New England Biolabs). Following, plasmids pETM6_PACF (Mattheos Koffas, Rensselaer Polytechnic Institute, NY), carrying *kfA*, *kfoC*, and *kfoF* genes (for chondroitin synthesis), and pUC8:15 carrying the *Vitreoscilla* hemoglobin gene-*vgb* were transformed into *E. coli* for the production of Microbial CS. The recombinant bacterium *E.coli* pETM6-PACF- *vgb* strain having the *Vitreoscilla* hemoglobin gene and the genes responsible for capsular chondroitin synthesis was used.

Media

Luria-Bertani (LB) medium was prepared to inoculate *E. coli* pETM6-PACF- *vgb,* the origin of Microbial CS. 80 mg/L of Ampicillin (Amp) was added to the LB medium to eliminate contamination risks. For LB preparation, 5 g/L yeast extract, 10 g/L NaCl, and 10 g/L tryptone were used. 1.5% agar was used to obtain the LB agar medium.

Commercial Chondroitin Sulfate

Animal-originated CS, referred to as Commercial CS, was purchased from Merck (Sigma-Aldrich, CAS#: 39455-18-0, Darmstadt, Germany). Commercial CS with an MW value of approximately 50-100 kDa was used to compare with MCS.

Microbial Chondroitin Production

The non-pathogenic *E. coli* strain, created by transforming plasmid pETM6-PACF-*vgb*, was inoculated on an LB-amp agar plate and incubated at 36 °C overnight. A few colonies from the plate were transferred into a 2-liter LB-amp broth medium and incubated for two days (48 h) at 36 °C with shaking at 140 rpm. The incubated broths were centrifuged to purify capsular chondroitin, and the cell pellet was collected to obtain microbial biomass. The cell pellets were resuspended in distilled water and then autoclaved for 15 minutes. The supernatant was obtained by centrifugation at 9000 rpm for 10 mins two times. After that, it was precipitated with 80 % volume of ethanol cooled at +4 °C to acquire intracellular and extracellular chondroitin. Precipitated supernatant stored at -20°C. Afterward, centrifugation at 9000 rpm for 10 mins was carried out to collect the pellet. The pellet was dissolved in digestion buffer, which consists of 100 mM Tris, 10 mM CaCl, and 50 mM MgCl at pH 7.5. 1 mg/l of DNAse (Sigma-Aldrich) was added to the sampling and incubated at 36°C for 1 hour. Subsequently, 2.5 mg/mL of Protease K (Sigma-Aldrich) was added to the sample to obtain purified chondroitin, and then the sample was placed in an incubator at 56 °C for 2 hours. After that, the second precipitation was performed with 80 % volume of cooled ethanol at +4 °C, and the pellet was obtained. Consequently, the samples were dissolved in distilled water and dried under a vacuum to acquire solid microbial chondroitin.

Microbial Chondroitin Sulfation Method

First of all, 6 g of produced microbial chondroitin was resuspended in 100 mL of DMF (Dimethyl Formamide) and then cooled to 4 °C. Next, 15 grams of pyridine sulfotrioxide was added to the sample. 500 mL of NaCl saturated acetic acid was added to the sample and precipitated at 20-22 °C (room temperature). The mixture was dried under vacuum by lyophilizing. Subsequently, this mixture was resuspended in 200 mL of distilled water and neutralized with 1 N NaOH. After that, the mixture was heated to 40 °C, and 0.2-0.3 N 60 mL NaOH was added, and then it waited 2-3 hours at 40 °C. The mixture was neutralized by adding 1 N HCl acid. This mixture was then filtered using a membrane with less than 10 µS conductivity. As a final step, the solution is dried by lyophilization. The produced Microbial CS was verified by analysis by NMR (Nuclear magnetic resonance) Spectroscopy (Bruker Advance III HD 600 MHz spectrometer) (34).

Cell Culture

Cells used in this study were MDA-MB-231 metastatic breast cancer cells (human) (Cat No HTB26, VA, US), HeLa cervical cancer cells (human) (Cat No CCL-2), and NIH3T3 embryonic fibroblast cells (mouse) (Cat No CRL1658) were purchased American Type Cell Collection (ATCC) and were cultured in RPMI (Wisent Inc., Cat No 350-000-CL, Quebec, Canada), EMEM (Wisent, Cat No 320-026-CL) and DMEM (Wisent Cat No 319-005-CL) basal media. Media for NIH3T3 cells included 10% bovine calf serum (Sigma -Aldrich, Cat No 12133C), however, media for MDA-MB-231 and HeLa cells contain 10% fetal bovine serum (Capricorn Scientific GmbH, Cat No FBS11-A, Germany). All media was also supplemented with 1% penicillin-streptomycin antibiotics (Wisent, Cat No 450-201-EL) and cultured at 37 °C with 5 % CO_2 humidification. Cells were cultured in 96-well microplates. Each experiment was performed including triplicate treatments.

MTT assay

Final concentrations of Commercial CS and Microbial CS were prepared as, 4 mg/ml, 1 mg/ml, 0.25 mg/ml, 0.00625 mg/ml, and 0.004 mg/ml for MTT assay, and cells were cultured with those for 3 days. After 3 days, media with or without Commercial CS and Microbial CS were removed. 190 µl culture media and 10 µl MTT (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) (Sigma, Cat No M2003) were added to each well and incubated at 37°C for 2h. Subsequently, media including MTT was removed, followed by the treatment of cells with DMSO (dimethylsulfoxide) for at least 1.5h on a shaker at dark till purplish color was observed. Absorbances were then measured at 570nm with a microplate reader. OD (optical density) values were used to comparatively compute the cell viability of treated cells and untreated counterparts. The logarithmic graph created plots for log concentrations vs cell viabilities (%), and IC50 values were calculated according to the plots. Selective indexes (SI) were calculated according to the formula given below.

$$Selectivity\ Index\ (SI) = IC50\ value\ for\ normal\ cells / IC50\ value\ for\ cancer\ cells$$

Scratch assay (Wound healing assay)

Final concentrations of Commercial CS and Microbial CS were prepared as 4, 1, 0.25, 0.00625, and 0.004 mg/ml for wound healing assay**,** and cells were treated with compounds for 24h, 48h, 72h, 96h, and/or 120h. Wounds were created using a ruler and a 100µl pipette tip. Media was then replaced with media, including Commercial CS or Microbial CS. Right after wound formation and treatments, images of wells were taken by the camera inverted microscope (Axio Vert A1, Zeiss, Germany) and recorded at 0h. Images were also taken at 24h, 48h, 72h, 96, and/or 120h. Images were captured using a 5x objective.

Analyses of Wounds

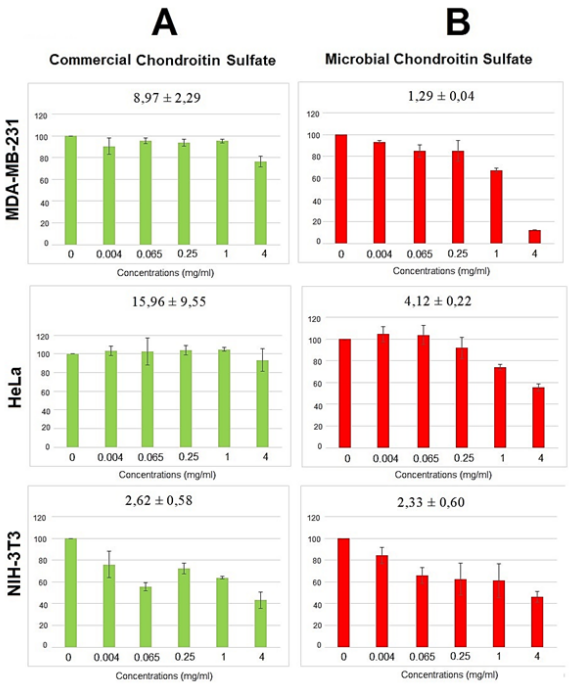
Wounds were analyzed using Image J software (NIH, US). The wound area in each image was selected using the "polygon selection" tool, and selected areas were analyzed using the "measure" tool within the "analyze" menu. Standard errors of the means (s.e.m ±) and bar graphs were created/calculated utilizing SPSS software (Version 23).

Results

Cytotoxicity Assays

Microbial CS was more cytotoxic to cancer cells examined compared to animal-originated Commercial CS. IC50 values of Microbial CS for MDA-MB-231 breast cancer cells and HeLa cervical cancer cells were 1.29 (±0.04) and 4.12 (±0.22), respectively. But IC50 values of Commercial CS for MDA-MB-231 and HeLa cells were 8.97 (±2.29) and 15.96 (±9.55), respectively. However, the cytotoxic response of NIH3T3 cells against Commercial CS or Microbial was quite similar with IC50 values around 2.5 (Figure 2A and B).

All selectivity indexes (SI) were computed at less than 1.0, suggesting that any form of chondroitin (with or without sulfate) did not selectively target the cancer cells rather than normal cells. Even if any type of CS caused a high



Selectivity Index Values (SI = IC50 normal cell / IC50 cancer cell)

Cells	Commercial CS	Microbial CS
MDA-MB-231	0,29	1,81
HeLa	0,16	0,56

Figure 2. Cytotoxic effects of Commercial CS (A) and Microbial CS (B) on HeLa, MDA-MB-231 and NIH-3T3 cells. Selectivity indexes (SI) of Commercial and Microbial CS are in the bottom table.

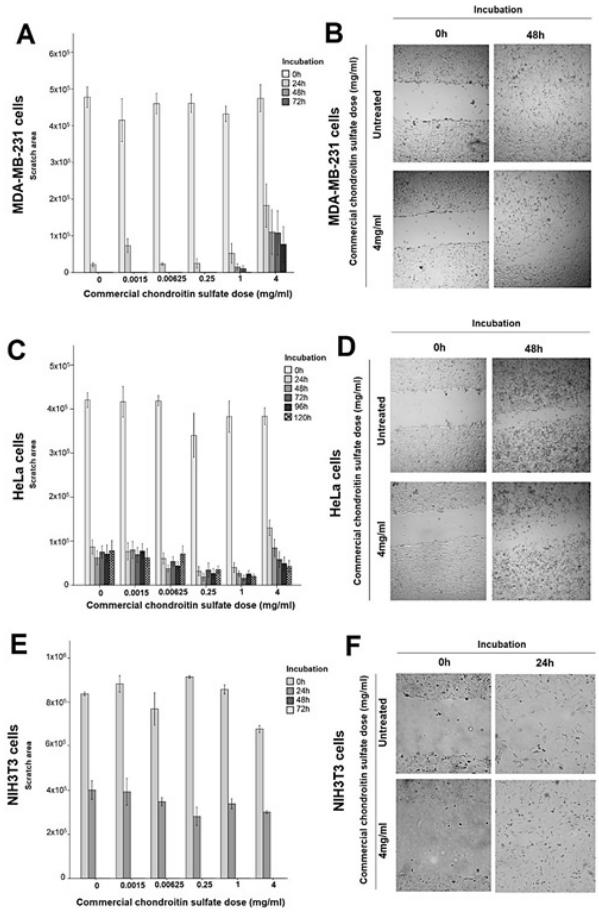


Figure 3. Cell migration impact of Commercial CS on MDA-MB-231 (A, B), HeLa (C, D), and NIH-3T3 (E, F) cells. Bar charts display the scratch space (in optional units) in various amounts (mg/ml) of Commercial CS and under different incubation times (0, 24, 48, and 72 hours). Bar charts are represented as the mean ± standard error. Microscopy images are provided for each chart.

rate of cell death in normal fibroblasts, this rate is less than the death rate in MDA-MB-231 cells. Therefore, the only satisfactory SI value (1.81) was defined for MDA-MB-231 metastatic breast cancer cells (Figure 2, bottom, yellow colored box). These findings can conclude that microbial CS has a specific cytotoxic effect on only one cell line (MDA-MB-231) examined in this study.

Scratch-Wound healing assays

MDA-MB-231 metastatic breast cancer cells after Commercial CS treatment were reverted to unwounded form as untreated counterparts except for 4mg/ml (Figure 3A, B). Wound formation was slowly restored in HeLa cells compared to other cells, and even wounds in untreated cells were not completely healed up to 120h (Figure 3C, D). NIH3T3 cells completely restored wounds up to 48h at each Commercial CS concentration and also after Commercial CS-free culture conditions (untreated control cells) (Figure 3E, F).

MDA-MB-231 cells were observed to have a similar wound healing pattern after Microbial CS with Commercial CS treatment (Figure 4A, B). HeLa cells were more sensitive to Microbial CS than Commercial CS as wound formation was mostly maintained at 1 mg/ml and 4 mg/ml up to 120h (Figure 4C, D), and untreated HeLa cells were again observed not to restore the wounds completely. The wound-healing response of NIH3T3 cells against Microbial CS was similar to the response against Commercial CS, but no significant restoration of wounds was observed after the highest concentration (4mg/ml) (Figure 4E, F). In contrast, wounds were enlarged at 4mg/ml.

Discussion

This study presents comparative analyzes of microbial chondroitin sulfate with its animal counterpart in terms of anti-proliferation and anti-metastasis properties. At this time of distrust against animal-derived drugs worldwide, the trend towards biotechnological drugs obtained from microbial sources, which are vegan and reliable drug potentiometers, is increasing. This study is the first examination revealing such a comparison in breast and cervical cancers. Microbial CS-induced cytotoxicity was detected higher in MDA-MB-231 and HeLa cells than in cells treated with its commercial form. However, the cell viability of NIH3T3 cells also decreased after Commercial CS or Microbial CS. Commercial CS showed reduced selectivity for cytotoxicity of cancer cells, but Microbial CS showed selective cytotoxicity on MDA-MB-231 metastatic breast cancer cells (SI index 1.81).

CS is utilized as an antiviral, anti-infective, antioxidant, and anti-inflammatory agent in medicine, pharmacy, veterinary medicine, and cosmetics. It is also widely used in medical applications as a cell and tissue regenerator (35-37). CS interacts with tumor cells and other cells, such as growth proliferate, growth factor receptors, and cytokines, thus playing an essential role in the growth and metastasis of cancer. In addition, CS is involved in various essential signaling pathways (24, 38). In the studies, it was thought that negatively charged CS chains were effective in invading rapidly proliferating tumor cells. However, various

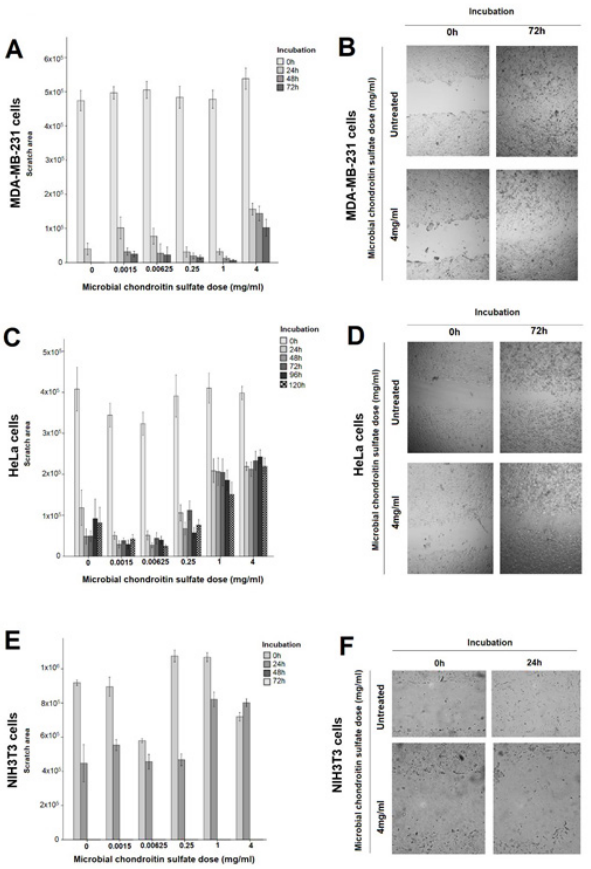


Figure 4. Cell migration impact of Microbial CS on MDA-MB-231 (A, B), HeLa (C, D) and NIH-3T3 (E, F) cells. Bar charts display the scratch space (in optional units) in various amounts (mg/ml) of Commercial CS and under different incubation times (0, 24, 48, and 72 hours). Bar charts are represented as the mean ± standard error. Microscopy images are provided for each chart.

studies suggest CS may be a potent anticancer agent (13, 15, 16).

Cancer cells can adhere to extracellular matrix (ECM) and different cell surface molecules such as collagen, selectins, fibronectin, ICAM, CD44, and vascular cell adhesion molecule. Sulfation patterns of CS play a critical role in the interactions mediated by selectins and their ligands. For example, CS-E binds to P- and L-selectin, while CS-A, CS-D, and CS-E bind to the CD44 receptor (24, 39, 40). Thus, the sulfation design of CS is a significant factor in cancer progression associated with selectin expression.

It was found that CS located on the tumor microenvironment and tumor cell surface might play a dual role in cancer progression and metastasis depending on its sulfate pattern. A study found that while enzymatic degradation of CS induced lung metastasis, it did not affect primary tumor growth in a breast cancer animal (13). Moreover, a high presence of CS in malignant cells increased survival in breast cancer patients (41). Cancer growth and metastasis happen because of various biological affairs in inappropriate situations. Abnormal monocyte migration, which ends with angiogenesis, is critical for tumor progression. A study showed that exogenous CS-A could reduce monocyte immigration and thus inhibit tumor angiogenesis (42). In this study, both microbial and commercial CS were shown to lower cell migration in cancer cells. However, MDA-MB-231 cells were determined to have higher metastatic properties than HeLa cells due to faster closure of wound formations. In the study, it was reported that HeLa cells also have metastatic properties (43). Various studies have been conducted to elucidate the metastasis mechanism of MDA-MB-231 breast cancer cells and to target the metastatic inhibition of cells (44-46). In the present study, both Commercial CS and Microbial CS could prevent cancer cells from migrating and restore wounds until 5 days, but normal cells reverted wounds within 2 days. This suggests that normal cells are less resistant to any form of CS so that normal cells can recover more rapidly after treatment.

In light of all this information, it is determined that CS plays a dual role in the tumor environment in accordance with its sulfate pattern. Nevertheless, a deep understanding of the structural formulation of CS and its relationship to GAG function is required for use in anticancer therapy (38). Microbial CS has the same functions as its animal counterpart and is a potent drug that does not carry allergic, prionic, and viral risks due to its source. It is a biocompatible, non-toxic form of GAG with low molecular weight and high kinetic values. In particular, it can be pointed out that because of the anti-inflammatory features of CS, it can be used as a slow-acting anti-inflammatory drug for cancer prevention. CS is involved in the adhesion of fibroblasts and leukocytes to various host cells, which are the source of essential ECM-degrading enzymes and growth factors. Furthermore, CS may be a therapeutic target to reduce infiltration of the cancer niche by host cells by interacting with CD44 (47, 48). Moreover, CS is negatively charged and binds to various cell surface receptors (e.g., HARE and CD44) (47,49). With these properties, CS or CS chain fragments can be applied as drug carriers to increase the efficacy of anticancer therapy (50).

Microbial CS is a natural, biocompatible, non-toxic, therapeutically usable substance. However, Commercial CS mainly originates from animal sources such as sheep, chickens, pigs, sharks, and other fish. Animal-originated CS is problematic because of various risks such as H7N9 avian influenza and bovine spongiform encephalopathy (BSE). In this study, bovine-origin CS was used as Commercial CS. Risk factors of various diseases such as Coronavirus disease (Covid-19), thought to be transmitted to humans through animals as intermediate hosts, cause the reliability of animal sources to be questioned. In addition to the risks of animal sources, synthetic CS preparations have allergic reaction risks and low specificity. The microbial production of CS eliminated all of these risks and the hesitation to use animal products, especially after the Covid-19 pandemic. Considering the serious side effects of using other chemical therapeutics, CS as an anticancer agent can reduce the economic damage caused by the treatment process and protect the patient from the side effects of anticancer drugs. Hospitalization of patients and the use of more anticancer drugs to treat various types of cancer cause billions of dollars to be spent worldwide, thus wasting time and unnecessary effort. Microbial CS can be an effective agent, especially in cancer cases whose pathogenesis is not fully understood. For example, male breast cancer is still poorly understood, and the treatments developed for this type of cancer are limited (51). Hence, by developing anticancer drugs with selective biological targets, problems such as high side effects, low efficacy, and drug resistance in cancer therapies can be solved. Microbial CS as a support for treatment can strengthen the

patient against cancer and reduce the economic damage.

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Conflict of interest
The authors declare that they have no conflict of interest.

Authorship contribution statement
Tuba Unver: Methodology, Investigation, Writing, **Selcen Celik Uzuner:** Methodology, Investigation, Writing, **Ayse Sebnem Erenler:** Conceptualization, Supervision, Writing-Review & Editing.

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