Tumor-Promoting Activity of 48 kDa Molecular Mass Hyaluronic Acid

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Hyaluronic acid (HA), glycosaminoglycan, has long been implicated in malignant transformation and tumor-promoting activity. We found that 48 kDa molecular mass HA in vitro promotes tumor cell growth and tumorigenesis. The increase of cell growth rate in human hepatoma cells (HepG2 cells, cancer cells) and normal human dermal fibroblasts (NHDF cells, normal cells) treated with 48 kDa HA at $2 \times 10^{-4}$ kg/L concentration were 110 $\pm$ 0.9% and 103 $\pm$ 0.5%, respectively. Colony formation activity of 4.8 kDa HA in cultured Balb/3T3 clone A3111 cells was higher than that of 48 kDa HA. However, transforming activity of HA was significantly showed in 48 kDa HA but not in 4.8 kDa HA. These findings suggest that 48 kDa HA has a tumor-promoting activity stronger than 4.8 kDa HA, because the former increase the cell growth of cancer cells than the latter. The increase of cell growth rate in HepG2 cells (cancer cells) and NHDF cells (normal cells) were compared among various molecular masses of HA.

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1. Introduction

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan (GAG) that promotes motility, adhesion, and proliferation in mammalian cells, as mediated by cell-surface HA receptors. Various tumors accumulate HA that suggested to facilitate tumor growth and invasion into the extracellular matrix (ECM) by a hydrodynamic effect, or by altering tumor cell behavior. Hyaluronidase (HAase) degrades HA into small fragments. Decomposing HA into small fragment may result in the release of some cytokines which may promote the tumor growth and angiogenesis. Lokeshwar et al. reported that small fragments HA (3–25 disaccharide units) levels increased in prostate cancer tissues when compared with normal tissues. Moreover, HA and its receptor, RHAMM (receptor for hyaluronic acid mediated motility) are important regulators of cell movement, adhesion and cytoskeletal organization. HA and RHAMM have been implicated in transformation and metastasis, in particular the processes of tumor cell motility and invasion.

In the present study, we survey the growth rate of human hepatoma cells (HepG2 cells) and normal human dermal fibroblasts (NHDF cells), and promoting activity of HA is also confirmed by the in vitro two-stage transformation assay. These results provide the important evidence for a direct relationship between HA and tumorigenesis in tumor cells.

2. Materials and Method

2.1 Materials

AlamarBlue™ agent was purchased from Biosource (Camarillo, CA). Giemsa’s solution was obtained from Merck (Germany). Hyaluronic acids (Fig. 1) (HA: 48 kDa and 800 kDa) were kindly supplied by Seikagaku Industries, Ltd. (Tokyo, Japan). HA of 4.8 kDa was prepared using the method of Cramer and coworkers. All other chemicals of a special grade were used without further purification.

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2.2 Cell cultures

Normal human dermal fibroblasts, NHDF cells (Asahi Techno Glass, Tokyo, Japan), and HepG2 cells (human hepatoma cell line, ATCC No.: HB-8065) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL) and antibiotics [penicillin (100 unit/ml)-streptomycin (1 x 10^{-4} kg/L)]. Balb/3T3 clone A3111 cells (Dr. T. Kuroki, University of Tokyo) were cultured in MEM supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL). The media were changed every two days until the cells became confluent. NHDF and A3111 cells cultured between 4th and 9th passage levels were used for all experiments. Cell cultures were maintained in a humidified 5% CO_2 incubator at 37°C.

2.3 Cell proliferation assay

For measurement of cell proliferation, $8 \times 10^4$ cells (NHDF and HepG2) were seeded into 12-well culture plates. Solutions of various molecular masses of HA then were added to each culture well. After 4 days of HA-treatment, the extent of cell growth was measured by alamarBlue™ assay. Control cells received fresh medium without any additions.

2.4 Transformation assay

For standard and two-stage cell transformation, $1 \times 10^4$ cells were plated per 60-mm tissue culture dish; 15 dishes were used for each point in all cell transformation experiments. After 24 h, $1 \times 10^{-7}$ kg/L 3-methylcholanthrene (MCA) was added to the MEM medium containing 10% FCS, and 72 h later the culture medium containing 4.8 kDa HA or
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48 kDa HA was changed. The medium was changed twice a week for 6 weeks. The cells were fixed with formaldehyde and stained with 5% Giemsa solution for 12 h. Types of transformed focus were determined under a phase-contrast photomicroscope.

Colony formation was tested in parallel with the transformation assay by seeding 100 cells/60-mm dish, and by treating the cells with the culture medium containing various molecular masses of HA and MCA for 72 h. The cultures were fixed and stained with 3% Giemsa solution, after 10–11 days from cell seeding, and colonies were counted. Each assay was repeated two times. Statistical analysis was carried out by the Wilcoxin Signed Rank Test.

3. Results and Discussion

Results of the cell growth assays are given in Fig. 2. AlamaBlueTM assay indicated that cell proliferation capacities in the NHDF cells and HepG2 cells have different. The growth rate of HepG2 cells was accelerated after co-culture with 48 kDa HA at the concentration of $2 \times 10^{-4}$ kg/L. However, 48 kDa HA had a low effect on growth rate of NHDF cells (Fig. 2(a)). These findings may related with reports that HA fragments by degrading enzyme HAase are intricately associated with cancer angiogenesis and metastasis.15, 16

We surveyed the promoting activity of HA using an in vitro, two-stage transformation assay. Morphology of A3111 cells treated with MCA, 4.8 kDa HA and 48 kDa HA were observed by phase contrast photomicroscope after culture for 6 weeks. Figure 3 shows a photograph of Giemsa staining plates tested by the cell transformation method. As shown in Figs. 3(b) and (c), transformed foci was observed in the dishes of 4.8 kDa HA (b) and 48 kDa HA (c) at MCA concentration of $1 \times 10^{-7}$ kg/L, respectively. When A3111 cells were treated with 48 kDa HA, the number of the transformed foci increased in comparison with 4.8 kDa HA as shown in

![Fig. 3 Images of A3111 cells transformation on the 60-mm tissue culture dish initiated by MCA: (a) MCA of $1 \times 10^{-4}$ kg/L from cell culture 1 day to 4 days, (b) MCA of 0.1 µg/mL from cell culture 1 day to 4 days and HA (4.8 kDa) of $10 \times 10^{-4}$ kg/L from cell culture 7 days to 21 days, (c) MCA of $1 \times 10^{-4}$ kg/L from cell culture 1 day to 4 days and HA (48 kDa) of $10 \times 10^{-4}$ kg/L from cell culture 7 days to 21 days, (d) MCA of 0.1 µg/mL from cell culture 1 day to 4 days and HA (800 kDa) of $10 \times 10^{-4}$ kg/L from cell culture 7 days to 21 days.](image-url)
In vitro transformation experiments showed a significant increase of transformed foci with an initiating dose of $1 \times 10^{-7}$ kg/L of MCA and indicated the possible activity of 48 kDa HA as a tumor promoter. 4, 17, 18) The high molecular mass HA (800 kDa) indicated no transforming activity as shown in Fig. 4(b).

As shown in Fig. 4(a), A3111 colony formation activity of 4.8 kDa HA was higher than that of 48 kDa HA. However, Fig. 2 indicated a slightly higher cell growth in 48 kDa HA than 4.8 kDa HA, in the case of HepG2 cells. These results were considered to be caused by the differences of cell functions between HepG2 cells (cancer cells) and A3111 (normal cells). It seems that the difference of tumor-promoting activity among the different molecular sizes of HA must be considered for the protection of the tumorigenesis, especially at the doubtful site of tumors. 19–21)

In the future, studies to define functional relationship between various molecular masses of HA and tumor cell properties are needed in order to provide insights into regulatory mechanisms involved in cell transformation in the tumor progression.

4. Conclusion

The 48 kDa HA indicated stronger tumor-promoting activity in transformation assay than high molecular mass HA (800 kDa) or low molecular mass HA (4.8 kDa) in vitro. These findings suggest that transformation of A3111 cells can be induced by 48 kDa HA, thus, degradable HA is useful index for early diagnosis of various cancers.

REFERENCES