Expression of Hyaluronan Synthases and Hyaluronan in Malignant Mesothelioma Cells

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Abstract. Background: Hyaluronan is one of the main components of the extracellular matrix. It is synthesized at the cell plasma membrane by specific hyaluronan synthases (HAS). Although a large number of studies have described hyaluronan in pleural effusion from malignant mesothelioma, the source of hyaluronan in malignant mesothelioma has been subject to controversy. Materials and Methods: The mRNA expression of all three HAS in malignant mesothelioma cells was studied using RT-PCR. The hyaluronan production in culture medium of malignant mesothelioma cells was also examined using high-performance liquid chromatography (HPLC). Results: We found that 9/10 malignant mesothelioma cell lines and one primary culture of malignant mesothelioma cells expressed HAS-1, while 10/10 malignant mesothelioma cell lines and one primary culture of malignant mesothelioma cells expressed HAS-2 and HAS-3. In addition, we demonstrated hyaluronan in the culture medium of 6 out of 10 malignant mesothelioma cell lines and one primary culture of malignant mesothelioma cells. Conclusion: Our results show that malignant mesothelioma cells express all three HAS isoforms and synthesize hyaluronan. The expression of HAS isoforms and hyaluronan in malignant mesothelioma cells in cultures and previous observations by other investigators indicate that these cells are, at least in part, responsible for hyaluronan synthesis in vivo.

Hyaluronan (HA, previously termed hyaluronic acid or hyaluronate) is a non-sulphated glycosaminoglycan (GAG), which is widely distributed in the extracellular matrix (ECM) (1). Hyaluronan plays an essential physiological role in processes such as cell growth, differentiation, adhesion and cell migration (2). Hyaluronan is also involved in the invasive and metastatic behaviour of tumor (3, 4). In contrast to other GAGs, which are synthesized intracellularly, hyaluronan is produced at the surface of plasma membrane by specific hyaluronan synthases (HAS). Currently, three HAS isoforms (HAS-1, HAS-2 and HAS-3) have been identified in mammalian cells. Each HAS isoform possesses the ability to synthesize hyaluronan molecules of different size (5-7).

Malignant mesothelioma is a highly aggressive diffuse tumor arising from mesothelial-lined surfaces, most often the pleural cavities (8). High concentrations of hyaluronan in pleural fluid from patients with malignant mesothelioma have been demonstrated in a large number of studies and its presence has been ascribed diagnostic importance in this tumor (9-11). However, the source of hyaluronan in patients with malignant mesothelioma has been subject to controversy. Some studies revealed that malignant mesothelioma synthesized hyaluronan (12, 13), whereas other studies proposed that malignant mesothelioma cells release growth factors that stimulate surrounding normal mesothelial cells and fibroblasts to synthesize hyaluronan (14, 15).

In order to further investigate potential molecular mechanisms responsible for the production of hyaluronan in malignant mesothelioma cells, we examined these cells for the expression of all three HAS isoforms. Further, we assayed the culture medium of malignant mesothelioma cell lines and primary culture of malignant mesothelioma cells for the presence of hyaluronan.

Materials and Methods

Reagents. RPMI 1640, FCS, penicillin, streptomycin and L-glutamine were obtained from Life Technologies (Paisley, UK).

Cells and culture conditions. Primary culture of malignant mesothelioma cells was obtained from pleural fluid of a patient with malignant mesothelioma. Ten human malignant mesothelioma cell lines were used: STAV-FCS, STAV-AB (13), ZL5, ZL34, SPC 212 (16, 17), M9K, M10K, M14K, M28K and M38K (18). Human
breast cancer cell line MCF-7 was purchased from American Type Cells Collection (ATCC). The primary culture of malignant mesothelioma cells and all malignant mesothelioma cell lines were cultured in RPMI 1640 supplemented with 5% FCS and 2 mM L-glutamine except for STAV-AB, which was cultured in RPMI 1640 supplemented with 10% human AB serum. MCF-7 was cultured in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. The cells were maintained in a humidified atmosphere of 5% CO₂ in air at 37°C.

RNA isolation, cDNA synthesis, primers and RT-PCR.
Total RNA was isolated from cells, using the guanidinium isothiocyanate method as previously described (19), and treated with DNase. cDNA synthesis was performed using a first-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions. For cDNA synthesis, 5 µg of total RNA were taken and Not- (T) 18 primers were used for cDNA generation. The HAS and -β-actin primers used are presented in Table I (20-22). To confirm the absence of any genomic DNA contamination, cDNA syntheses without reverse transcriptase were used for RT-PCR (data not shown). Normal mesothelial cells (for HAS-1) and MCF-7 (for HAS-2 and HAS-3) were used as positive control. Amplification without template was used as negative control. The reverse transcript (1 µl in a final volume of 25 µl) was subjected to PCR under the following conditions. For the HAS-1 primers, one cycle consisted of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, with a total of 35 cycles being performed. For the HAS-2 primers, PCR consisted of initial denaturation at 94°C for 2 min, followed by 35 cycles where each cycle consisted of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The PCR was finalized by elongation for 2 min at 72°C. For the HAS-3 primers, one cycle consisted of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min. The PCR was finalized by elongation for 2 min at 72°C. For the β-actin primers, one cycle consisted of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, with a total of 25 cycles being performed. PCR products were visualized by ethidium bromide staining of 1.5% agarose gel. Integrity of RNA and cDNA synthesis was monitored by amplification of β-actin mRNA.

Analysis of the hyaluronan production.
Analysis of hyaluronan production was performed as described elsewhere (23). Briefly, the cell-free medium was collected after 48 h of culture from both primary culture of malignant mesothelioma cells and malignant mesothelioma cell lines. One ml of culture medium was concentrated in Centricon 10 tubes (Amicon, Inc., Beverly, MA, USA) to a final volume of 100 µl and washed with 2 ml sodium acetate (0.5 M). The glycosaminoglycans were precipitated with 4 volumes of ethanol. The precipitate was recovered after centrifugation (10,000 x g for 5 min) and incubated with 100 µl of the digestion mixture at 37°C overnight. Following centrifugation (10,000 x g for 15 min) in Microcon 3 tubes, 20 µl of the digest was directly taken for high-performance liquid chromatography (HPLC) analysis. The peaks obtained at 164 nm were compared with external standards. The experiments were repeated 2 times.

Results

Expression of HAS-1, -2 and -3 mRNA in malignant mesothelioma cells by RT-PCR.
In order to investigate if specific HAS is responsible for synthesizing hyaluronan in malignant mesothelioma cells, we examined the mRNA expression of all three HAS in these cells by RT-PCR. As shown in Figure 1, all malignant mesothelioma cell lines and primary culture of malignant mesothelioma cells expressed HAS-1, except for ZLS cells. HAS-2 and HAS-3 were also expressed by all malignant mesothelioma cell lines and primary culture of malignant mesothelioma cells.
Hyaluronan production in culture medium of malignant mesothelioma cells. In order to further investigate the hyaluronan production in culture medium of malignant mesothelioma cells, we performed analysis of hyaluronan content by HPLC. As shown in Table II, hyaluronan was secreted from 6 out of 10 malignant mesothelioma cell lines and one primary culture of malignant mesothelioma cells to give chemically detectable amounts of this molecule. However, 4 out of 10 malignant mesothelioma cell lines did not produce detectable amounts of hyaluronan. The experiments were repeated 2 times.

Discussion

An association between hyaluronan and malignant mesothelioma was observed as early as 1939 (24). Since then, a large number of studies have investigated the functional, diagnostic and prognostic importance of hyaluronan in this tumor (9-11, 25-27). Up to 70% of malignant pleural mesothelioma patients are associated with elevated hyaluronan levels in pleural effusions or serum (26). High levels of hyaluronan in pleural effusions appear to be related to the epithelial differentiation and longer survival of malignant mesothelioma (27, 28). Even though, the source of hyaluronan in patients with malignant mesothelioma has been subject to controversy. An increasing concentration of newly synthesized hyaluronan was demonstrated in cultured malignant mesothelioma cells (13, 29). Hyaluronan was also demonstrated on human malignant mesothelioma cells growing in nude mice xenografts (12). However, other studies proposed that normal human mesothelial cells rather than their malignant counterparts were the source of hyaluronan in malignant mesothelioma and that secretion of hyaluronan into pleural fluids was induced by growth factors (14, 15).

HAS are enzymes responsible for the synthesis of hyaluronan in plasma membrane. Among the three HAS isoforms, previous studies showed that only a few malignant mesothelioma cell lines express HAS-1 or HAS-3, respectively (20, 30). The expression profiles of all three HAS isoforms in malignant mesothelioma cells has not been thoroughly investigated. In the present study, we demonstrated the mRNA expression of all three HAS isoforms (except for ZL5 which did not express HAS-1) in ten malignant mesothelioma cell lines and one primary culture of malignant mesothelioma cells. The production of hyaluronan in the cell cultures was detected in most of the malignant mesothelioma cell lines exhibiting the expression of HAS mRNA. One possible explanation could be that the method used for examination of hyaluronan did not have enough sensitivity to demonstrate low concentrations of this molecule. Our results show that malignant mesothelioma in culture synthesize hyaluronan and that these cells are at least in part the source of this molecule in malignant mesothelioma in vivo.

Malignant mesothelioma is characterized by a highly invasive diffuse tumor. Our previous studies have shown that different factors such as integrins, growth factors, matrix metalloproteases and extracellular matrix components are involved in the invasive and metastatic behavior of malignant mesothelioma (31-35). Recent studies also indicate that CD44, which is the principal receptor of hyaluronan, is expressed in malignant mesothelioma cells and that hyaluronan has the capacity to stimulate proliferation and migration of malignant mesothelioma cells through interaction with hyaluronan receptor (36, 37). In addition, several studies revealed that hyaluronan production and interaction with CD44 promote invasion and/or metastasis in different malignancies such as breast cancer, glioma, gastric cancer and ovarian cancer (38-41). Our results suggest that the expression of HAS isoforms could contribute to the production of hyaluronan and correlate with the highly invasive behavior of malignant mesothelioma.
Taken together, our results show that malignant mesothelioma cells not only express all three HAS, but also synthesize detectable amounts of hyaluronan in cell cultures. These data may further contribute to our understanding of various aspects of malignant mesothelioma interaction with different components of the ECM and to the possible role of these interactions for the invasive behavior of this tumor.

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References


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