Protein Tyrosine Kinase and Estrogen Receptor-dependent Pathways Regulate the Synthesis and Distribution of Glycosaminoglycans/Proteoglycans Produced by Two Human Colon Cancer Cell Lines

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Abstract. The soy isoflavone genistein can affect cell metabolism by specifically inhibiting protein tyrosine kinase (PTK) and/or interacting with the estrogen receptors (ERs). Glycosaminoglycans (GAG)/proteoglycans (PG) may participate in tumor development and progression. The synthesis of GAG by two human colon cancer cell lines, HT-29 and SW-1116, and the effects of genistein on their production and distribution between culture medium and cell membrane were studied. The mitogenic activity of genistein on both cell lines growth was also examined. Metabolic labeling, sensitive high pressure liquid chromatography (HPLC) techniques and fluorometric cell proliferation assays were utilized. The results demonstrate that both estrogen receptor β-positive (ERβ+) cancer cell lines produced hyaluronan (HA), both extracellular and membrane-associated galactosaminoglycans (GalAG) and heparan sulfate (HS), with the HT-29 cells producing all GAG fractions at significantly higher rates. The observed dose-dependent inhibitory effect of genistein on the synthesis of both secreted and cell-associated GAG/PG by the SW-1116 cells, as well as on their growth, was suggestive of a PTK mechanism. On the other hand, the synthesis of GAGs/PGs by HT-29 cells in the presence of genistein was dependent on their type and localization which implies the active participation of the ERs, which was further supported by the observed growth stimulation at low concentrations of genistein.

Abbreviations: (GAGs) glycosaminoglycans; (PGs) proteoglycans.

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Colorectal carcinoma is one of the leading causes of cancer death in both sexes in Western countries (1) and the third most common type of cancer in the U.S.A. (2). Previous studies have shown that genomic instability (3), as well as environmental factors such as diet and hormones, can be correlated to the risk of colon cancer (4). The prognosis for colonic malignancies is usually poor, if not diagnosed at an early stage, as this cancer type is characterized by a high metastatic potential and recurrence rate (5). Tumor invasion and metastasis formation are a major cause of treatment failure and death in cancer patients (6) and are correlated with the ability of cancer cells to degrade the surrounding extracellular matrix (ECM) and basal membrane (7, 8). The ECM is a complex network mainly consisting of fibrillar collagen, fibronectin, laminin, vitronectin and proteoglycans (PG), the content of which affects various cellular functions (9).

Proteoglycans (PGs) are macromolecules composed of linear sulfated glycosaminoglycan (GAG) chains covalently attached to a protein core and they can be located in the ECM, associated with the cell membrane or in intracellular granules (10). GAG/PG are able to interact with growth factors and/or growth factor receptors and through these interactions may affect various cellular functions such as proliferation, differentiation or migration (11). Furthermore, quantitative and qualitative changes in GAGs/PGs production have been suggested to have a role in the development of some types of cancer, such as osteosarcoma, melanoma, breast carcinoma and colon adenocarcinoma (9).

The major soy isoflavone genistein is an active growth inhibitor for many tumor types including colon cancer, as has been previously demonstrated in both in vitro and in vivo studies (12, 13). Generally, its actions have been associated with a G2/M cell cycle arrest (14), although genistein has also
been suggested to induce arrest at the G0/G1 phase in some cell lines (15). On the other hand, at low concentrations (1-2 μM) genistein may also increase colon cancer cell proliferation (16). Moreover, previous reports have demonstrated that, in addition to proliferation, genistein affects the biosynthesis of GAG/PG in various human cancer cell lines (17, 18) and that changes in PG/GAG levels may be correlated to cell proliferation rates (19).

Several possible mechanisms of genistein action in tumor cells have been proposed. Thus it has been demonstrated that genistein specifically inhibits protein tyrosine kinases (PTKs) (20), interacts with the estrogen receptors (ERs), (21), has antioxidant activities and inhibits DNA topoisomerase II (16).

The possible effects of genistein on the synthesis of GAGs/PGs by human colon cancer cell lines have not, to our knowledge, been examined. The purpose of this study therefore was to characterize the types of GAG synthesized by the two colon carcinoma cell lines, to examine the effects of genistein on the synthesis and distribution of GAG/PG between the culture medium and the cell membrane as well as to investigate the effects of genistein on their proliferation.

Materials and Methods

Materials. Fetal bovine serum (FBS) and RPMI-1640 medium were from Gibco-Invitrogen (Paisley, UK) whereas penicillin, streptomycin, amphotericin B and L-glutamine were all obtained from Biochrom KG (Berlin, Germany). D-glucosamine hydrochloride-[6-3H (N)] was provided by DuPont (Billirica, USA). The precipitation of PGs was achieved by the addition of 4 vol of 95 % (v/v) ethanol containing 2.5 % (w/v) sodium acetate. To ensure complete precipitation of the PGs, 40 μl CSA (0.2 mg/L) was added as a carrier. The solutions were left for 30 minutes at 4°C and centrifuged in a Beckman microfuge (10,000 xg for 10 minutes). The precipitates of PGs were digested with the proteolytic enzyme papain at 65°C for 60 minutes using 2 units/ml in 100 mM phosphate buffer pH 7.0 containing 5 mM Na2 EDTA and 5 mM L-cysteine hydrochloride (22). The liberated GAGs were precipitated by the addition of 10 vol 1% (w/v) cetylpyridium chloride (CPC) and the solution was left for 60 minutes at room temperature. Following centrifugation at 10,000 xg for 10 minutes, the pellets obtained were dissolved in 500 μl of 95 % (v/v) ethanol containing 2.5% (w/v) sodium acetate. The precipitates were then washed with ethanol and left to dry.

In order to identify the HA and GalAGs (chondroitin sulfate, dermatan sulfate), the GAG preparation was dissolved in water and digested with an equi-unit mixture (0.2 units/mL) of chondroitinases ABC, AC II and chondro-4- and -6-sulfatases (25). The removal of the chondroitinase-resistant GAGs (heparan sulphate (HS), keratan sulphate (KS)) was carried out by ethanol precipitation, and aliquots from the supernatant were analysed by ion-suppression high-pressure liquid chromatography (HPLC) for hyaluronan (HA)- and galactosaminoglycan (GalAG)-derived Δ-disaccharides using radiochemical detection (26) as well as by reversed polarity high pressure capillary electrophoresis (HPCE) (23).

Cell cultures. The HT-29 and SW-1116 human colon adenocarcinoma and MCF-7 breast cancer cell lines were obtained from ATCC. The HT-29 and SW-1116 cells were grown in a humidified atmosphere at 37°C, 5% (v/v) CO2 in RPMI-1640 medium supplemented with 10% FBS and 0.5% gentamicin. The MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 2 g/l sodium bicarbonate, 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.01 mg/ml bovine insulin. The cells were cultured in 75 mm2 tissue culture flasks and the culture medium was changed every other day. Confluent cultures were first washed with phosphate-buffered saline (PBS) and then harvested by trypsinisation with 0.23% (w/v) trypsin in PBS containing 0.1% (w/v) Na2 EDTA for 15 minutes at 37°C and 1 vol. of medium was added to terminate enzymic activity. Centrifugation at 200 xg for 15 min was used for the collection of the cells and they were counted utilizing Hank’s balance solution, using a Coulter particle counter (Hialeah, FL, USA). Prior to RNA and protein extraction the cells were serum deprived for 24 and 48 hours, respectively.

The effects of genistein on GAG synthesis were examined by treating the cells for 48 hours with 10 or 30 μg/ml of genistein in serum free medium. The metabolic labeling of GAGs was performed by supplementing the cultures with D-glucosamine hydrochloride-[6-3H(N)] (10 μCi/ml) for 16 hours before the end point of the experiment (22).

Fractionation and determination of GAGs. Cell-associated PGs were extracted with 50mM Tris-HCl pH 8.0 containing 1% (v/v) Triton X-100 and 0.1% (w/v) NaCl and phenylmethylsulphonyl fluoride proteinase inhibitor, benzamidine hydrochloride and hexanoic acid at a final concentration of 2, 5 and 50 mM, respectively (23). The extraction was carried out for 2 h under mild shaking at 4°C, using 1 ml of the extraction solution per 106 cells. The culture medium was concentrated to 1:100 of its original volume on a YM-10 Amicon membrane, Millipore (Billirica, USA). The precipitation of PGs was achieved by the addition of 4 vol of 95 % (v/v) ethanol containing 2.5 % (w/v) sodium acetate. To ensure complete precipitation of the PGs, 40 μl CSA (0.2 mg/L) was added as a carrier. The solutions were left for 30 minutes at 4°C and centrifuged in a Beckman microfuge (10,000 xg for 10 minutes). The precipitates of PGs were digested with the proteolytic enzyme papain at 65°C for 60 minutes using 2 units/ml in 100 mM phosphate buffer pH 7.0 containing 5 mM Na2 EDTA and 5 mM L-cysteine hydrochloride (22). The liberated GAGs were precipitated by the addition of 10 vol 1% (w/v) cetylpyridium chloride (CPC) and the solution was left for 60 minutes at room temperature. Following centrifugation at 10,000 xg for 10 minutes, the pellets obtained were dissolved in 500 μl of 60 (v/v) propanol-1 containing 4% (w/v) CPC (24). Re-precipitation of the liberated GAGs was carried out by the addition of 6 vol of 95 % (v/v) ethanol containing 2.5% (w/v) sodium acetate. The precipitates were then washed with ethanol and left to dry.
RNA isolation and polymerase chain reactions (PCR). Total ribonucleic acid was isolated with the TRIzol method Gibevo-Invitrogen (Paisley, UK) according to the manufacturer's instructions. One μg of total RNA was used for cDNA synthesis using the ThermoScript™ reverse transcription-PCR (RT-PCR) System (Invitrogen, Paisley, UK). Specific oligonucleotide primer pairs were designed for ERα (F: AATCCAGATAATCGACCGCA, R: GTATTCACATCTCCCTC), ERβ (F: GTCCATGCA GTATCACATC, R: GCCGTACCCTCCTACACGA) and GAPDH (F: GGAGTGAAGGTAAGCGGATCA, R: GTCATT GATGGCAAATATCCACT) using published literature or sequence information contained in the National Center for Biotechnology InformativeGenBank database with OLIGO 4.0 software. Reactions were performed in 20 μl aliquots using the Platinum Taq DNA polymerase ThermoScript™ RT-PCR System (Invitrogen) according to the manufacturer's instructions.

Proliferation assays. Exponentially growing cells from non-confluent cultures were harvested and seeded in a flat-bottom 96-well black plate (Corning, USA) at a density of 10x10³ HT-29 or SW-1116 cells per well in 0.2 ml of RPMI (10% FCS). Prior to all growth assays, the cells were serum starved for 24 h and the treatment was performed in 0% FCS medium. Genistein was added at concentrations of 1, 10 and 30 μg/ml of culture medium and the cells were incubated for 48 h. After treatment, the number of living cells was determined using the fluorometric CyQUANT cell proliferation Assay Kit (Molecular Probes-Invitrogen) according to the manufacturer's instructions.

Statistical analysis. The grade of dependence of GAG synthesis on genistein was evaluated by the use of the t-test and the one-way completely randomized variance analysis (ANOVA) using the Microcal Origin (version 5.0) software. The independence/ dependence between characteristics was regarded as significant at p≤0.05.

Results

Synthesis and distribution of GAG. The analyses of the metabolically [3H]-labelled GAG/PG synthesized by the colon HT-29 and SW-1116 cell lines demonstrated that they produce HA, GalAG and HS (Figure 1A-E). As presented in Table I, HT-29 cells produced 34% higher amounts of total GAG as compared to SW-1116 cells indicating that the two colon cancer cell lines had different biosynthetic patterns. Specifically the HT-29 cells synthesized up to 300% more HA and at the cell membrane retained 250% higher amounts of GalAG as compared to the SW-1116 cells (Table I). Furthermore, major differences were noted in the distribution pattern of the HS fraction between the HT-29 and SW-1116 cells. HS was mainly retained membrane by the HT-29 cells (57%) whereas the SW-1116 cells secreted and retained approximately equal amounts of this GAG type in the cell. On the other hand, GalAG were mainly secretory products in both the HT-29 (62%) and SW-1116 (84%) cells, whereas both cell lines secreted into the medium more than 93% of the HA produced.

Table I. Levels and distribution of GAGs between the culture medium and the cell layer in the human colon cancer HT-29 and SW-1116 cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Fraction</th>
<th>HA</th>
<th>GalAGs</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>Medium</td>
<td>14373 (&gt;93)</td>
<td>11330 (62)</td>
<td>80950 (43)</td>
</tr>
<tr>
<td></td>
<td>Cell fraction</td>
<td>4383 (38)</td>
<td>106610 (57)</td>
<td></td>
</tr>
<tr>
<td>SW-1116</td>
<td>Medium</td>
<td>4494 (&gt;93)</td>
<td>11090 (84)</td>
<td>61790 (49)</td>
</tr>
<tr>
<td></td>
<td>Cell fraction</td>
<td>1766 (16)</td>
<td>64641 (51)</td>
<td></td>
</tr>
</tbody>
</table>

*Levels are expressed as [3H]-incorporated radioactivity in GAGs; *The percent distribution of GAG is given in parenthesis.

Effects of genistein on the synthesis of secreted and cell-associated GAG/PG. Genistein affected the GAGs/PG synthesis of the HT-29 cells in a manner specific to the GAG type and distribution. Thus, when genistein was present at a concentration of 10 μg/ml, the HT-29 cells significantly increased the synthesis of exported HS, whereas the synthesis of HA and GalAG secreted to the medium was not affected (Figure 1A-E). The cell-associated HS production was down-regulated at the same concentration of genistein. Interestingly, when genistein was present at 30 μg/ml, the synthesis of secreted HS was significantly suppressed in comparison to that with 10 μg/ml genistein (Figure 1D), whereas the synthesis of total GalAG was increased (Figure 1B and C).

The GAG production of the SW-1116 cells was affected in a different manner by genistein. The synthetic rates of HA as well as of both extracellularly-secreted and cell-associated HS were dose-dependently suppressed (Figure 1A, D and E). The production of GalAG destined for export was suppressed only at the higher concentration of genistein (Figure 1B) whereas the synthesis of cell-associated GalAG was not significantly affected (Figure 1C).

Estrogen receptor (ERα and β) expression by HT-29 and SW-1116 cells at the mRNA level. SW1116 and HT-29 cells were negative for ERα mRNA (Figure 2A). MCF-7 cells were used as a positive control (Figure 2A). Both the HT-29 and SW-1116 colon carcinoma cells were found to express ERβ at the mRNA level (Figure 2A).

Effects of genistein on the proliferation of the HT-29 and SW-1116 cells. Treatment of the HT-29 cells with 0.5 μg/ml genistein caused a statistically significant increase in cell growth (Figure 3A). At 1 and 10 μg/ml genistein did not affect their proliferation rates, whereas the higher concentration of 30 μg/ml caused a marginal suppression (16%) (Figure 3A). In contrast, treatment with all tested concentrations of genistein significantly inhibited SW-1116 cell proliferation (Figure 2B).
Discussion

The ability of both colon cancer cell lines to synthesize GAG/PG was significantly affected by the addition of genistein to the cultures. This isoflavonoid has been shown to specifically inhibit protein tyrosine kinases (PTK) with little effect on threonine/serine phosphorylation (20, 29). Therefore, the dose-dependent inhibitory effect of genistein on the synthesis of both secreted and cell-associated GAGs/PGs by the SW-1116 cells may well be attributed to its action on PTK. The PTK-mediated inhibitory effect of genistein on the production of GAG/PG by the SW-1116 cells is in accordance with previous results obtained for both epithelially-differentiated and sarcomatous human malignant mesothelioma (18) as well as for SAOS-2 osteosarcoma cell GAG/PG production (30).
The synthesis of GAG/PG by the HT-29 cells in the presence of genistein, appeared to be dependent on their type and localization. Thus the secretion of HS was significantly stimulated whereas cell-associated HS production was strongly suppressed. GaAG synthesis was modestly stimulated whereas HA production did not appear to be affected by the presence of genistein. The selective effect of genistein on respective GAG fractions may be partially accounted by specific inhibitory effects of genistein on distinct PTK. It has been demonstrated that genistein specifically inhibits insulin-like growth factor receptor, but not epidermal growth factor signaling in the HT-29 colon cancer cell line (31).

Genistein is one of the major phytoestrogens that are structurally similar to estradiol and possesses estrogenic effects (32) mainly perpetrated through binding with ERβ, for which the affinity of genistein is similar (94%) to that of estrogen (29). Different roles for estrogens in colon carcinogenesis have been suggested (33, 34) and estrogen has been reported to both stimulate and suppress colon cancer cell growth (33, 35). Both the HT-29 and SW-1116 colon cell lines express the ERβ mRNA subtype and are negative for ERα mRNA expression (36). Previous reports have shown a stimulation of GAG synthesis by genistein in the ER-positive MCF-7 breast cancer cell line (17). Therefore, the observed selective stimulation of the HT-29 cell GAG/PG synthesis suggests that these cells in vitro specifically respond to an estrogen analog, an effect not duplicated in the SW-1116 cells and possibly reflecting differential in vivo effects of estrogens on heterogeneous colonic cell populations in a tumor lesion. The activation of ER by genistein in HT-29 cells is supported by data obtained in proliferation experiments where it was shown that genistein at low concentrations, and presumably acting as an estrogen agonist, stimulates the growth of these cells, in contrast to the SW-1116 cells which were constantly growth-suppressed at all genistein concentrations.

Conclusion

The results of this study suggest that genistein affects the synthesis of HA, GaAG and HS by the two estrogen receptor positive colon cancer cell lines. In the SW-1116 cell line, genistein had a dose-dependent inhibitory effect on the synthesis of both secreted and cell-associated GAG/PG as well as on cell growth, mediated probably through a PTK pathway. The synthesis of GAGs/PGs by HT-29 cells in the presence of genistein was demonstrated to be dependent on their type and localization, which may partly be explained by an ER-mediated mechanism. ER function in these cells is further supported by the observed stimulation of their growth at low genistein concentrations.

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References


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