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EJ-*ras* oncogene transfection of endothelial cells upregulates the expression of syndecan-4 and downregulates heparan sulfate sulfotransferases and epimerase

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Abstract

The EC rabbit endothelial cell line was transfected with the EJ-*ras* oncogene (EJ-*ras* EC). EJ-*ras* EC cells display over expression of the *Ras* oncogene, morphological changes and deregulation of the cell cycle, becoming more densely populated and serum-independent. In addition, EJ-*ras*-transfectant cells show higher levels of the syndecan-4 mRNA. In addition to the increase in the core protein, a parallel increase in the glycosylation of the syndecan-4 protein, a proteoglycan that bears heparan sulfate chains, also occurs. This increase is observed both for the heparan sulfate proteoglycan synthesized by the cells and for that secreted to the culture medium. This enhancement in heparan sulfate synthesis was observed through metabolic labeling of the cells, immunoprecipitation of syndecan-4 and heparitinases treatment. Furthermore, the EJ-*ras*-transfectant cells do not exhibit decreased synthesis of heparan sulfate during the G_1-S phase transition, as observed for the parental cell line. Also, heparan sulfate synthesis is not stimulated by PMA as displayed by parental endothelial cells. Significant structural changes of heparan sulfate, such as decreased *O*-sulfation, were observed in the EJ-*ras*-transfected cells. Decreases in the mRNA levels of some enzymes (glucuronosyl C-5 epimerase, iduronosyl-2-*O*-sulfotransferase, glucosaminyl-6-*O*-sulfotransferase-1 and *N*-deacetylase/*N*-sulfotransferase-1), involved in the biosynthetic pathway of heparan sulfate, were also observed. The results suggest that overexpression of the EJ-*ras* oncogene alters the cell cycle, through signal transduction cascades, upregulates the expression of syndecan-4, and downregulates enzymes involved in the heparan sulfate biosynthesis related to chain modification, leading to the structural changes of the heparan sulfate syndecan-4 proteoglycan in endothelial cells.

Keywords: EJ-*ras* oncogene; Sulfotransferases; Phorbol ester; Syndecan-4; Heparan sulfate structure

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; ΔGlcA-GlcNAc,6S or ΔIdoA-GlcNAc,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-glucose 6-sulfate; ΔGlcA-GlcNS or ΔIdoA-GlcNS, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-sulfamino-D-glucose; ΔIdoA-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-sulfamino-D-glucose 6-sulfate; ΔIdoA,2S-GlcNS, O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)-(1→4)-2-sulfamino-D-glucose; ΔIdoA,2S-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)-(1→4)-2-sulfamino-D-glucose 6-sulfate; EC, endothelial cell; EJ-*ras* EC, endothelial cell transfected with EJ-*ras* oncogene; PMA, phorbol 12-myristate-13-acetate.

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1. Introduction

Previous studies have shown that oncogenic *Ras* proteins [1] induce numerous alterations in processes that affect cellular structure and organization, such as cytoskeletal reorganization [2,3], pinocytosis [2], cell swelling [3], alterations in calcium metabolism [4] and changes in cell surface glycosylation [5,6]. Malignant transformation is frequently accompanied by aberrant glycosylation of proteins and lipids, which could contribute to certain properties of transformed cells, such as abnormal adhesion to the extracellular matrix or to other cells, with significant consequences in their invasive and metastatic potential [7,8].

Compelling evidence ascribes a pivotal role to endothelial cells in the orchestration of tumor angiogenesis and metastatic growth, suggesting antiangiogenic therapy as an attractive approach for anticancer treatment. In this context, activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway has been found to be fundamental in transducing extracellular stimuli that modulate a number of cellular processes including cell proliferation, migration and invasion [9].

Experimental evidences suggest that heparan sulfate proteoglycan (HSPG) play a role in cell spreading, cellular recognition, cellular adhesion and growth control [10–18]. In addition, several reports describe high affinity association of heparin-like molecules with growth factors [19–22], implying that heparan sulfate effects on cell growth are likely to be mediated by growth factors [21–29].

We have previously reported that fetal calf serum (FCS) and phorbol 12-myristate-13-acetate (PMA) specifically stimulate the synthesis of syndecan-4, a HSPG of endothelial cells, an effect that is likely to be mediated by protein kinase C activation since staurosporine abolishes the effect. The increased synthesis of HSPG is not related to protein kinase A pathway, since forskolin, a potent activator of this pathway and 8-bromo adenosine 3′,5′-cyclic monophosphate, a modulator of adenylate cyclase, have no effect [30]. Specifically, a syndecan-4 cytoplasmic domain interacts with PKCα [30–33] and activates its kinase activity in the presence of PIP_2 in vitro [31,34]. Syndecan-4 directly also interacts with PKCα in vivo, regulating localization of PKCα in the cytoskeleton. In addition, syndecan-4 stabilizes and activates the activity of PKCα, resulting in a sustained PKC activity [35]. Recently, Murakami et al. [34] have shown the involvement of PKCα and δ in the phosphorylation of syndecan-4 in endothelial cells.

The involvement of RAS in signal transduction pathways is well documented [36–38]. The mechanism by which *ras* proto-oncogene products regulate cell growth and differentiation is not completely understood. The *ras*-encoded protein, $p21^{ras}$, displays both a GTP binding and a GTPase activity. However, the effector systems for G^{ras} regulatory proteins in mammalian cells still remain unknown. The *ras* proto-oncogenes are rendered active oncogenes, capable of malignant transformation, by point mutations that usually impair the $p21^{ras}$ GTPase activity [39].

The cell cycle requirements for peptide growth factors have been demonstrated in cells rendered quiescent by growth factor deprivation [40,41]. It has been shown [42] that transfectants exhibiting high EJ-*ras* expression do not require competence growth factors and are tumorigenic. On the other hand, low EJ-*ras* expression is not sufficient either to deregulate the cell cycle or to override the cell requirement for growth factors [42].

These and other results led us to investigate the correlation between cell proliferation and proteoglycan expression in endothelial cells transfected with EJ-*ras* oncogene. We now report that overexpression of the EJ-*ras* oncogene in endothelial cells modifies the cell cycle, the expression of syndecan-4 and the sulfation pattern of heparan sulfate chains.

2. Material and methods

2.1. Substrates, enzymes and materials

Heparan sulfate from bovine pancreas was a gift from Dr. P Bianchini (Opocrin Research Laboratories, Modena, Italy). Chondroitin 4-sulfate, dermatan sulfate, chondroitinases AC and ABC were purchased from Seikagaku Kogyo (Tokyo, Japan). Heparitinases I and II were prepared from induced *Flavobacterium heparinum* bacteria using previously described methods [43,44]. Ethylenediamine (1,2-diaminoethane) and propylenediamine (1,3-diaminopropane) were purchased from Aldrich Co. (Milwaukee, WI). [^{3}H]methyl thymidine (85 Ci/mmol) was purchased from Amersham Co. (Buckinghamshire, UK) and carrier free [^{35}S]sulfuric acid from IPEN (São Paulo, SP, Brazil). Maxatase, a protease from *Sporobacillus*, was purchased from Biocon do Brasil Industrial Ltd. (Rio de Janeiro, RJ, Brazil). Mouse monoclonal anti-syndecan-4 IgG was raised against a synthetic peptide containing the first N-terminal 29 aa sequence of syndecan-4 from an established rabbit endothelial cell line [45]. This antibody cross-reacts with human syndecan-4 [46].

2.2. Cell culture

The EC endothelial cell line derived from rabbit aorta [47] was maintained in F12 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FCS (Cultilab, Campinas, SP, Brazil) at 37 °C, 2.5% CO_2. Serum-starved cultures were obtained by maintaining the cells in the presence of F12 containing 0.2% FCS, for 48 h at 37 °C, 2.5% CO_2 before stimulus with 10% FCS or 100 ng/ml PMA.

2.3. DNA-mediated transfection

The previously described DNA-calcium phosphate co-precipitation [48] was used, with slight modifications [49]. Briefly, EC endothelial cells (1 week after thawing) were plated, at 4×10^5 cells/60 mm diameter dish, 12–15 h before co-transfection with 0.1–0.5 μg pX343 plasmid DNA (genetic marker) plus 2.5–20 μg of the EJ-*ras* oncogene construct (pEJ 6.6 kbp from Dr. R.A. Weinberg's Laboratory, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA;