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Stromal transforming growth factor-beta 1 is crucial for reinforcing the invasive potential of low invasive cancer

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ARTICLE INFO

Article history:
Accepted 28 March 2014

Keywords:
Transforming growth factor β1
Podoplanin
Invasion
Stromal
Fibroblast
Xenograft

ABSTRACT

Objective: Tumour cells alter the characteristics of the adjacent stroma to create a supportive microenvironment during cancer progression. *In vitro* and *in vivo* experiments were carried out to verify the role of stromal TGF-β1 in reinforcing of the invasive potential in low invasive cancer.

Materials and methods: Isolated NF or CAF was co-cultured with low invasive HSC-2 cells to evaluate whether stromal TGF-β1 induced PDPN expression by Transwell invasion and influenced tumour growth in orthotopic xenografts.

Results: Stimulation by TGF-β1 promoted PDPN expression and Transwell invasion through SMAD signalling as well as activation of Src, P38 mitogen activated protein kinase and extracellular regulated kinase 1/2. PDPN induction was TβRII-dependent. Tumour growth of HSC-2 OSCC in a mouse xenograft was intensified in the tumour CAF microenvironment.

Conclusions: Stromal TGF-β1 signalling promoted PDPN expression in cancer cells, thereby enhancing tumour growth and leading to a more invasive phenotype.

1. Introduction

Aggressive cancer is intimately associated with increased recurrence and mortality and invariably kills the host if left untreated.[1] Molecular analyses of cancer cells in various stages of progression have revealed that alterations in tumour suppressor genes and oncogenes accumulate during tumour progression and correlate with the clinical aggressiveness of cancer.[2] Comparative analyses of gene expression profiles between metastatic and non-metastatic cells have revealed that various genes are differentially expressed in association with the metastatic potential of cancer cells. Carcinomas are malignant neoplasms derived from epithelial cells surrounded by stroma that interact with cancer cells to modulate cell growth, migration, invasion, and tissue-specific gene expression. Recent studies have shown that interactions between tumour cells and their adjacent microenvironment are

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http://dx.doi.org/10.1016/j.archoralbio.2014.03.017

intimately associated with tumour growth and the development of metastasis.[3] Therefore, cancer-associated stromal cells may support primary tumour growth by altering the tumour microenvironment, thereby leading to invasion and metastasis. However, despite extensive research, the details of the biological mechanisms by which cancer cells acquire motility and invasiveness are largely unknown. Cancer-associated fibroblasts (CAFs) are a source of various paracrine and autocrine growth factors that regulate growth of surrounding cells as well as themselves.[4] Different types of growth factors and cytokines secreted by the surrounding stromal cells and the signalling pathways induced by cell–cell interactions play important roles in tumorigenesis and metastasis.[5] Tumour cells alter the characteristics of the adjacent stroma to create a supportive microenvironment during cancer progression. Thus, there is an increasing interest to study CAFs as drug targets for anticancer therapies.

Tumour invasion pattern can be morphologically classified into tumour budding (isolated tumour cells or clusters of <5 cells at the invasive tumour front) and collective invasion (typically invade as a cohesive multicellular unit). The tumour budding is correlated with changes in the expression and function of adhesive (*e.g.* cadherins) and regulatory proteins (*e.g.* Snail family members, transforming growth factor β). During this so-called epithelial-mesenchymal transition (EMT), cells lose epithelial markers, such as E-cadherin, and gain the expression of mesenchymal markers, such as N-cadherin and vimentin.[6] However, a collective invasion of large cell cluster maintains the expression of epithelial adhesion structures but can nonetheless invade into the neighbouring stromal tissues. Interestingly, podoplanin (PDPN) that is known as a lymphatic marker has been reported to be expressed at the invasive front of tumours and to mediate pathways leading to collective cell migration and invasion *in vivo* and *in vitro*.[7] PDPN has the presumed function of promoting the detachment of tumour cells and the associated crucial steps towards metastasis.[7] PDPN increases in tumours, and its expression is associated with tumour malignancy.[8] Restricted expression of PDPN, often in a one-cell layer at the invasive front of tumours, leads to the question whether factors in the surrounding tissue influence PDPN expression.

In the present study, we investigated the existence of molecular crosstalk between cancer cells and surrounding stromal cells to reinforce the invasive potential of low invasive cancer. Molecular crosstalk between cancer cells and tumour microenvironment components suggests potential targets for new therapeutic approaches.

2. Materials and methods

2.1. Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture, foetal bovine serum (FBS), antibiotic-antimycotic (100×), phosphate-buffered saline (PBS), and 0.25% trypsin–EDTA (1×) were purchased from Gibco BRL Co. (Rockville, MD, USA). Cholera toxin, hydrocortisone, insulin, apo-transferrin, triiodothronine (T3), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SB431542 was purchased from Calbiochem (La Jolla, CA, USA). Recombinant human transforming growth factor (TGF-β1) was obtained from Millipore (Billerica, MA, USA). Human TGF-β1 immunoassay kit and recombinant human Podoplanin Fc chimaera was obtained from R&D System Inc. (Minneapolis, MN). Control siRNA-A was obtained from Santa Cruz (Santa Cruz, CA, USA). The following antibodies were purchased from their respective sources: PDPN (E-1), anti-TβRII (D-2) (Santa Cruz Biotechnology); β-actin and α-smooth muscle actin (clone 1A4, Sigma Chemical); total/phosphor form of SMAD2, Src, P38 mitogen activated protein kinase (MAPK) and extracellular regulated kinase (ERK)1/2 (Cell Signalling Technology, Denver, MA, USA); TGF-β1 (LSBio, LifeSpan BioSciences, Inc., Seattle, WA, USA); vimentin (Clone V9) and PDPN (Clone D2-40) (Dako, Carpentaria, CA, USA); horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science, Little Chalfont, UK); biotinylated anti-mouse/anti-rabbit IgG (H + L) and horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA).

2.2. Cell culture

Oral squamous cell carcinoma (OSCC) cells were cultured in DMEM/F12 (3:1 ratio) medium supplemented with 10% FBS, 1×10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml apo-transferrin, and 2×10^{-11} M T3 in a humidified atmosphere of 5% CO_2 at 37 °C. YD-10B and YD-39 cell lines were obtained from the Oral Cancer Institute at the Yonsei University College of Dentistry, Korea. HSC-2, HSC-3, and Ca 9.22 were gifted by Prof. Takashi Muramatsu, Tokyo Dental College, Japan. These cell lines are oral squamous cell carcinoma (OSCC). Among them, HSC-2 cells showed a relatively low invasive activity through Matrigel-based Transwell as compared with the highly invasive YD-10B, YD-39, HSC-3 and Ca 9.22 (Supplementary Data S1). Cells stimulated with TGF-β1 or PDPN were cultured with media containing 1% FBS for 16 h. Normal gingival fibroblasts (NF) were obtained from a patient with wisdom teeth extraction without any oral mucosal disease. CAF were obtained from a surgical specimen of a patient with OSCC. Informed consent was given by the patient for this study and an approval was given by the Institutional Review Board of Yonsei University College of Dentistry. CAFs were selected from explanted cancer tissues in Versene solution (200 mg EDTA and 1 mg glucose in 1 L PBS buffer). The isolated fibroblasts were maintained in DMEM/F12 (3:1 ratio) complete medium and were characterized by immunohistochemical staining with anti-vimentin (1:100) and anti-α-smooth muscle actin (α-SMA) (1:100). Early passages (<passage 9) of the fibroblasts were subjected to analysis.

2.3. TβRII depletion

A pool of the three duplex siRNAs was synthesized as previously described to prepare downregulated TβRII expressing cells.[9] HSC-2 cells were transfected with siRNA in serum-free Opti-MEM containing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.