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TGF-beta1 causes epithelial-mesenchymal transition in HaCaT derivatives, but induces expression of COX-2 and migration only in benign, not in malignant keratinocytes

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ABSTRACT

Background: Transforming growth factor β (TGF-β) acts as a tumor promoter by inducing epithelial-mesenchymal transition (EMT), which leads to a motile phenotype, enabling invasion and metastasis of cancer cells. Cancer-related inflammation, mediated by prostaglandins, has been proposed as a critical mechanism in conversion of benign cells to malignant.
Objective: Induction of cyclooxygenase 2 (COX-2), producer of prostaglandins, is thought to be a prerequisite for TGF-β-induced EMT in benign cells. We used HaCaT derivatives, representative of skin cancer progression, to investigate TGF-β1 mediated EMT response, and the role of COX-2 in it.
Methods: Effect of TGF-β1 was investigated by analyzing cell proliferation, morphology and protein expression. Chemotaxis and scratch-wound assays were used to study migration.
Results: TGF-β1 caused proliferation arrest of benign and malignant HaCaT cells, and changed the epithelial morphology of benign and low-grade malignant cells, but not metastatic cells, to mesenchymal spindle-shape. Epithelial junction proteins ZO-1 and E-cadherin were downregulated in all cell lines in response to TGF-β1, but mesenchymal markers were not induced, suggesting a partial EMT response. COX-2 and migration were induced only in benign HaCaT derivatives. Malignant derivatives did not induce COX-2 in response to TGF-β 1 treatment, thus emphasizing the role of inflammation in EMT response of benign cells.
Conclusions: TGF-β1 operates via distinct mechanisms in inducing EMT and metastasis, and supporting this we show that TGF-β1 induces COX-2 and promotes the migration of benign cells, but does not further augment the migration of malignant cells, indicating their resistance to TGF-β1 in the context of motility.

1. Introduction

Epithelial-mesenchymal transition (EMT) is a biological process that allows tightly organized epithelial cells to acquire a motile mesenchymal phenotype. Recently it has been suggested that EMT should be classified into three different subtypes, as it occurs in various biological and pathological settings. Type 1 EMT is associated with embryonal and organ development, type 2 with wound healing, tissue regeneration and fibrosis, and type 3 with cancer progression and metastasis [1]. In the multi-step process of cancer progression, invasion through the basement membrane is a crucial event, and EMT has been proposed as the critical mechanism [2]. A key event in breaking the polarity is the loss of epithelial junctions, mediated by downregulation of tight and adherens junction proteins, zonula occludens 1 (ZO-1) and E-cadherin, respectively. At the same time mesenchymal proteins, such as vimentin and fibronectin, are upregulated, facilitating the migratory phenotype [3]. Cytoskeletal rearrangement is critical for migration of epithelial cells, often observed as formation of filamentous actin stress fibers [4]. The HaCaT keratinocyte cell panel represents different stages of skin carcinogenesis from spontaneously immortalized non-malignant cells (HC) to *H-ras* transformed benign A5 cells, to low-grade malignant HaCaT II-4 cells forming locally invasive highly differentiated squamous cell carcinomas (SCC), and to high-grade malignant, metastasizing RT3 cells [5,6]. Transforming growth factor β (TGF-β) is a regulatory cytokine that has paradoxical roles in cancer. It suppresses tumor progression of normal and premalignant cells, but when cancer cells lose their response to TGF-β, it induces differentiation into an invasive phenotype and is thus a potent inducer of EMT [7]. TGF-β1 has been shown to induce EMT in immortal and malignant HaCaT cells through mitogen-activated protein kinase (MAPK) and Smad (transcriptional regulator of TGF-β) signaling pathways. However, the immortal HC cells initiated EMT only when the Ras pathway

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was activated in these cells by epidermal growth factor (EGF) [8]. In a study by Wilkins-Port et al. [9], TGF-β1 together with EGF potentiated the invasion of II-4 cells through collagen type I. The collagenolytic phenotype was mediated by increased expression of matrix metalloproteinase 10 (MMP-10).

Studies using non-tumorigenic intestinal [10] and mammary [11] epithelial cells have shown that TGF-β1 induces expression of cyclooxygenase 2 (COX-2) through the p38 MAPK dependent pathway and causes inactivation of Smad signaling. COX-2 is expressed aberrantly in a variety of carcinomas, leading to increased conversion of its metabolic product prostaglandin E_2 [12]. Prostaglandins are executors of inflammation, but are also linked to cancer progression, and PGE_2 has been shown to promote proliferation, survival, angiogenesis, migration and invasion [13]. EMT mediated through upregulation of COX-2 has been shown to be PGE_2 dependent [11].

We have previously shown that co-culturing HaCaT derivatives with nemotic fibroblasts (for review, see [14]) does not cause induction of COX-2 or EMT phenotype in HaCaT cells [15]. Instead, as we recently reported, nemosis increases proliferation and augments motility of the HaCaT derivatives [16]. Therefore the purpose of the present study was to investigate the EMT response of the HaCaT cell panel induced by TGF-β1 and the role of COX-2 in this process. The effect of TGF-β1 on HaCaT derivatives was investigated by analyzing changes in cell proliferation, expression of known EMT markers and migratory response.

2. Materials and methods

2.1. Cell lines and cell culture

The HaCaT cell line panel, representing keratinocyte tumorigenesis, was kindly provided by Prof. Petra Boukamp and Prof. Norbert E. Fusenig (DKFZ, Heidelberg, Germany). All cell lines were cultured at +37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS) (Invitrogen), 0.3 mg/ml glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. For experiments, cells were seeded and grown for 48 h, after which they were serum-starved in medium containing no FCS for 24 h. TGF-β1-containing or control medium was added and cells were cultured further 48 h. HaCaT cells were used until passage number 50.

For morphological analysis 0.75×10^5 cells/well (6-well plate) were seeded and after 48 h of incubation with or without TGF-β1 the cells were imaged with Olympus CXK41 phase contrast microscope and photographed with Olympus DP12 digital camera.

For chemotaxis assay HaCaT cell lines were transduced with pLV-PGK/GFP (kind gift from Professor Seppo Ylä-Herttuala, AIV Institute, Kuopio, Finland) green fluorescent protein-lentivirus. Cells seeded (3×10^5 cells/well) into 6-well plates were transduced with pLV-PGK/GFP virus (titer of 2.8×10^6) in the presence of polybrene (6 μg/ml, Sigma–Aldrich, St. Louis, MO). After overnight incubation, virus-containing medium was removed, and cells were washed and resuspended in normal growth medium for experimentation. The transduction efficiency was over 95% in all cell lines.

2.2. Reagents and antibodies

Recombinant human TGF-β1 was from R&D Systems (Minneapolis, MN) and was used at 2 ng/ml concentration throughout. For immunohistochemistry, rabbit monoclonal anti-Ki-67 antibody was used (Labvision, Fremont, CA). The following primary antibodies were used for immunoblotting and immunofluorescence: mouse monoclonal anti-E-cadherin, mouse monoclonal anti-ZO-1 (both from BD Biosciences, Bedford, MA), mouse monoclonal anti-PCNA, rabbit polyclonal anti-COX-2 (both from Labvision), rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-vimentin [17] and mouse monoclonal anti-fibronectin [18]. Secondary antibodies were: horseradish peroxidase coupled anti-mouse IgG and anti-rabbit IgG (both from Jackson Immunoresearch, Cambridgeshire, UK) for immunoblotting and goat anti-mouse IgG Alexa Fluor 488 (Invitrogen) for immunofluorescence. Rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) was used to stain for F-actin and Hoechst 33342 (Molecular Probes) for nuclei.

2.3. Proliferation assay

The MTT cell proliferation assay (Cayman Chemical Company, Ann Arbor, MI) was used to confirm that TGF-β1 causes proliferation arrest of HaCaT cells. In the assay, performed according to manufacturer's protocol, HaCaT cells were seeded to 96-well plates (20,000 cells/well) and let to plate for 2 h. Culture medium with unattached cells was aspirated and replaced with 100 μl of either control medium or TGF-β1. Cells were incubated for 48 h, after which 10 μl MTT reagent (5 mg/ml) was added and incubated for 4 h at +37 °C in 5% CO_2 incubator. Culture medium was aspirated and 100 μl Crystal Dissolving Solution was added to each well. The absorbance of the dissolved formazan crystals was measured at 540 nm using Multiscan EX microplate reader (Thermo Labsystems, Vantaa, Finland).

2.4. Immunohistochemistry

HaCaT derivatives (5×10^4 cells inside a silicon ring) were grown on SuperFrost Plus adhesion slides (Menzel-Gläzer, Thermo Fisher Scientific, Cheshire, UK) for 24 h, after which medium was replaced with experimental media (ctrl or TGF-β1) and incubated for further 48 h. Ice-cold methanol was used for fixation, after which cells were washed with PBS. Ventana Discovery immunohistochemistry Slide Stainer (Ventana Medical Systems, Tucson, AZ) was used for immunohistochemical staining. Slides were incubated with primary antibody (Ki-67) for 30 min. The staining was performed with the Ventana 3,30-diaminobenzidine tetrahydrochloride (DAB) biotin avidin detection kit. Images were captured with Olympus BX50 microscope using Olympus DP12 digital camera.

2.5. Immunoblotting

Cells (3×10^5 in 6-well plates) were washed twice with PBS and collected in 2× sample buffer (125-mM Tris (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, 10% β-mercaptoethanol, 10% glycerol) and boiled for 10 min. Equal amounts of protein from each sample were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and blocked with 2.5% non-fat powdered milk in TBS (20 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20). Immunoreactive proteins, incubated with appropriate primary and secondary antibodies, were visualized using ECL detection (Pierce, Rockford, IL).

2.6. Immunofluorescence

For immunofluorescence, the HaCaT derivatives (0.75×10^5 in 6-well plates) treated with our without TGF-β1 were fixed with a cytoskeleton-preserving fixative (4% paraformaldehyde, 0.32 mol/l sucrose, 10 mmol/l MES, 138 mmol/l KCl, 3 mmol/l $MgCl_2$, 2 mmol/l EGTA). To diminish background, fixed cells were incubated with NH_4Cl, permeabilized with 0.5% Triton-X-100/PBS and blocked with 0.2% BSA/PBS containing calcium and magnesium. Cells were