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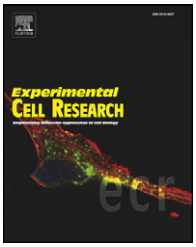

Research Article

Epidermal growth factor receptor reactivation induced by E-prostanoid-3 receptor- and tumor necrosis factor-alpha-converting enzyme-dependent feedback exaggerates interleukin-8 production in airway cancer (NCI-H292) cells

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ABSTRACT

Airway epithelial cancer cells produce increased amounts of the chemokine interleukin-8 (IL-8), inducing pro-tumor responses. Multiple stimuli induce airway epithelial IL-8 production epidermal growth factor receptor (EGFR) dependently, but the mechanisms that exaggerate IL-8 production in airway cancers remain unknown. Here we show that direct activation of EGFR (EGFR-P) by its ligand transforming growth factor (TGF)-alpha induces a second EGFR-P in human airway (NCI-H292) cancer cells but not in normal human bronchial epithelial (NHBE) cells, exaggerating IL-8 production in these cancer cells. The second EGFR-P in NCI-H292 cells was caused by metalloprotease *T*NF-*a*lpha-converting *e*nzyme (TACE)-dependent cleavage of EGFR pro-ligands and was responsible for most of the total IL-8 induced by TGF-alpha. In NCI-H292 cells, TGF-alpha induced cyclooxygenase (COX)-2-dependent prostaglandin (PG)E2 production and release. PGE2 increased the second EGFR-P and IL-8 production via binding to its Gi-protein-coupled E-prostanoid (EP)3 receptor. In NHBE cells, TGF-alpha-induced EGFR-P did not lead to PGE2 production or to a second EGFR-P, and less IL-8 was produced. Thus, we conclude that a positive feedback pathway involving COX-2/PGE2/EP3 receptor-dependent EGFR reactivation exaggerates IL-8 production in NCI-H292 cancer cells but not in NHBE (normal) cells.

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Introduction

Tumor cells produce chemokines that attract leukocytes [1]. The chemokine IL-8 recruits neutrophils to tumors potently [2]. Tumor-associated neutrophils induce "pro-tumor" responses such as cell proliferation, invasiveness and angiogenesis [3–5]. IL-8 can also induce these pro-tumor responses directly via binding to CXCR1 and CXCR2 receptors on epithelial cancer cells [6,7] and on endothelial cells [8].

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Abbreviations: COX, cyclooxygenase; EGFR, epidermal growth factor receptor; EGFR-P, EGFR phosphorylation; EP, E-prostanoid; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; IL, interleukin; NHBE, normal human bronchial epithelial; PG, prostaglandin; TACE, tumor necrosis factor-alpha-converting enzyme; TAPI, tumor necrosis factor-alpha protease inhibitor; TGF, transforming growth factor.

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In airway epithelial cancers, elevated levels of IL-8 have been shown to promote tumor growth, metastasis and angiogenesis [9,10], leading to early relapse and shortened patient survival [11]. Airway epithelial cancer cells are an important source of IL-8 [9], suggesting that airway cancer cells induce pro-tumor responses in part via increased IL-8 production. However, the mechanisms that exaggerate IL-8 production in airway epithelial cancer cells remain unknown.

EGFR activation has been shown to induce IL-8 production in human airway epithelial cancer cells [12,13], in immortalized human bronchial epithelial cells [14], and in NHBE cells [15]. Multiple inhaled stimuli such as cigarette smoke induce metalloprotease TACE-dependent cleavage of EGFR pro-ligands [15,16], resulting in ligand binding to and activation of EGFR and subsequent airway epithelial IL-8 production [12,13,15]. This suggests that the TACE–EGFR surface cascade is a convergent pathway for IL-8 production in airway epithelial cells.

COX-2 synthesizes prostaglandins from arachidonic acid and is highly expressed in many airway epithelial cancers [17] but not in normal airway epithelium. EGFR activation leads to COX-2-dependent PG synthesis in epithelial cancers [18,19], and PGE2 is the most abundant PG detected in cell lines derived from airway cancers [20]. PGE2 effects are mediated by distinct G-protein-coupled EP receptors (EP1–4; [21]) and have been reported to involve metalloprotease-dependent EGFR activation [22]. Therefore, we hypothesized that EGFR activation could stimulate a positive feedback signaling pathway involving the COX-2-dependent synthesis of PGE2 in some airway epithelial cancer cells but not in normal airway cells. This positive feedback could result in PGE2/EP receptor- and TACE-dependent EGFR reactivation, exaggerating IL-8 production in some cancer cells.

To test this hypothesis, we compared IL-8 production in human airway epithelial (NCI-H292) cancer cells and in NHBE (normal) cells. NCI-H292 cells were chosen for these studies because, unlike normal cells, they express COX-2 and produce and release PGE2 COX-2-dependently [23]. Because multiple stimuli have been shown to induce IL-8 production both in airway cancer cells [12,13,15] and in NHBE (normal) cells [15] via metalloprotease-dependent cleavage of pro-TGF-alpha, we selected the potent EGFR ligand TGF-alpha as the stimulus. An important advantage of using TGF-alpha (an EGFR ligand) to stimulate cells is that TGF-alpha binds to and activates EGFR directly, thereby simplifying the analysis of signaling downstream of EGFR. Here we show that EGFR activation stimulates a positive feedback pathway involving COX-2/PGE2/EP3 receptor activation in NCI-H292 cancer cells but not in NHBE cells, exaggerating IL-8 production in these cancer cells.

Materials and methods

Materials

TGF-alpha was purchased from R&D Systems (Minneapolis, MN). AG1478, AG1295, TAPI-1, EGFR-neutralizing antibody (Ab-3), NS-398, pertussis toxin, and dibutyryl cAMP were purchased from Calbiochem (La Jolla, CA). PGE2, PGD2, PGF2-alpha, BWA868C, AL8810, SC19220, AH6809, GW627368, CAY10441, butaprost, and sulprostone were purchased from Cayman Chemical (Ann Arbor, MI). Aprotinin and leupeptin were purchased from Sigma (St. Louis, MO).

Cell culture

Human lung adenocarcinoma (NCI-H292) cells were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 25 mM HEPES at 37 °C in a humidified 5% CO_2 water-jacketed incubator as described previously [24]. The NCI-H292 cell line was derived from a lymph node metastasis of a pulmonary mucoepidermoid carcinoma. NCI-H292 cells have been shown to contain the intermediate filament protein keratin [25], confirming the epithelial origin of these cells. Because cell lines such as NCI-H292 show variability in their responses to stimuli and inhibitors at different passages, all experiments were performed with cells from passages 80 to 90.

Proliferating NHBE cells were purchased from Lonza (Walkersville, MD) and grown in immersed culture in bronchial epithelial growth medium (BEGM; Lonza) according to the recommendations of the supplier. NHBE cells were grown in immersed culture instead of air–liquid interface to maintain culture conditions similar to those for the NCI-H292 cells. NHBE cells have been shown to produce IL-8 in immersed culture [26]. Experiments with NHBE cells were performed with passages 2–4 to limit variable responses.

Confluent cultures were serum-starved (NCI-H292) or incubated with EGF-free BEGM (NHBE) for 2 h before the addition of stimuli. In NCI-H292 cells, COX-2 was present under these conditions (see Fig. 3A). Chemical inhibitors and the EGFR-neutralizing antibody were added 30 min before ("0 h") or at various times after stimulation with TGF-alpha (5 ng/ml). The relatively selective TACE inhibitor TAPI-1 [27] inhibited the second EGFR-P induced by TGF-alpha in NCI-H292 cells dose-dependently (data not shown) and maximally at 30 μM (see Fig. 2C), consistent with previously reported TAPI-1 dose response curves [28]. Prostanoid receptor antagonists were examined at concentrations based on previous reports. In "rescue" experiments, PGs or EP-selective agonists were added 3 h after TGF-alpha stimulation (when endogenous PGE2 levels in the supernatant are maximal and 1 h before the anticipated peak of the second EGFR-P in NCI-H292 cells). Cell lysates and supernatants were harvested at various times after stimulation for measurement of IL-8 (24 h), EGFR-P (up to 6 h), and PGE2 (up to 4 h).

siRNA preparation and transfection of cells

TACE siRNA knockdown and confirmation of specific TACE silencing were performed as described previously [28]. For EP1–4 receptor siRNA knockdown, subconfluent (50–60%) NCI-H292 cells were transfected with EP1 receptor siRNAs (SMARTpool L-005711; Dharmacon, Lafayette, CO), EP2 receptor siRNAs (SMARTpool L-005712; Dharmacon), EP3 receptor siRNAs (SMARTpool L-005713; Dharmacon), EP4 receptor siRNAs (SMARTpool L-005714; Dharmacon) or non-targeting control siRNA (Non-targeting pool D-001810; Dharmacon) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Efficient and selective knockdown of EP1–4 protein expression 72 h after transfection was confirmed by immunoblotting (see Fig. 5A).

Measurement of IL-8, EGFR-P, and PGE2

IL-8 in cell culture supernatants and EGFR-P in cell lysates were measured by sandwich ELISA kits according to the manufacturer's