BBA - Molecular Cell Research 1865 (2018) 1878–1890

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Neuroendocrine differentiation contributes to radioresistance development and metastatic potential increase in non-small cell lung cancer

Rongying Zhu[a,b,1], Xiaodong Yang[b,1], Xiang Xue[b], Mingjing Shen[b], Feng Chen[a], Xiaodong Chen[a], Ying Tsai[a], Peter C. Keng[a], Yongbing Chen[b], Soo Ok Lee[a,*], Yuhchyau Chen[a,*]

[a] Department of Radiation Oncology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA
[b] Department of Cardiothoracic Surgery, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215004, PR China

ARTICLE INFO

Keywords:
NSCLC
NED
Radioresistance
IL-6
cAMP
MEK/Erk

ABSTRACT

Radiation treatment induces neuroendocrine differentiation (NED) in non-small cell lung cancer (NSCLC) A549 and H157 cells, so higher NE-like features in radioresistant A549 (A549R26-1) and H157 (H157R24-1) cells are observed than in parental cells. We detected higher NED marker expressions in A549R26-1 cell-derived tumors than in A549 cell-derived tumors. In mechanism studies, we found that NED induction in A549R26-1 and H157R24-1 cells was accompanied by increased intracellular cAMP and IL-6 levels. Treatment of radioresistant lung cancer cells with the inhibitor (SQ22536) of adenylate cyclase (AC) which is the enzyme responsible for the cAMP production, or the neutralizing antibody (Ab) of IL-6, resulted in decreased NE-like features in radioresistant lung cancer cells. In addition, we found MEK/Erk is the signaling pathway that triggers the cAMP- and IL-6-mediated NED induction in radioresistant lung cancer cells. Also, we found that MEK/Erk signaling pathway inhibition decreased NED in radioresistant cells. Radioresistant lung cancer cells exhibiting high NE-like features also showed higher radioresistance and higher metastatic potential than parental cells. When we inhibited cAMP-, or IL-6-mediated pathways, or the downstream MEK/Erk signaling pathway, radiosensitivity of radioresistant lung cancer cells was significantly increased and their metastatic potential was significantly reduced. In *in vivo* mouse studies, reducing NED by treating mice with the MEK/Erk inhibitor increased radiosensitivity. Immunohistochemical staining of tumor tissues lowered expressions of the NED/epithelial-mesenchymal transition (EMT)/metastatic markers when mice were treated with the MEK/Erk inhibitor.

1. Introduction

Neuroendocrine differentiation (NED) is defined as morphological changes and positive staining with NED markers such as neuroamines, neuropeptides, neuron specific enolase (NSE) [1], chromogranin A (CgA) [1,2], synaptophysin (Syn) [3], Pro-opiomelanocortin (POMC, the precursor of the stress hormone adrenocorticotrophic hormone [ACTH]), and CD56 (also called neural cell adhesion molecule, NCAM). NED has been observed in solid tumors, including lung cancer [4,5], colorectal cancer [6], breast cancer [7], prostate cancer (PCa) [8,9], and pancreatic cancer [10]. In lung tumors, approximately 25% to 33% of NED has been observed [4,5]. Essentially, all small-cell lung cancer (SCLC) and carcinoid tumors show distinct histological features of NE cells and stain positive for NE markers. However, NED is not limited to SCLC. NED has been observed in large-cell NE carcinoma (LCNEC) [11], and in about 10 to 20% of NSCLC including adenocarcinomas and squamous cell carcinomas [12,13].

The significance of NED in most solid tumors is not clear except in PCa. The NE carcinoma phenotype in PCa has been studied quite extensively, and has been linked to resistance to inhibition of androgen receptor (AR) signaling, aggressive tumor characteristics, and dismal prognosis [14–16]. SCLC, which has the NE phenotype, also exhibits

Abbreviations: NE, neuroendocrine; NED, neuroendocrine differentiation; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; LCNEC, large-cell neuroendocrine carcinoma; PCa, prostate cancer; NSE, neuron specific enolase; CgA, choromogranin A; Syn, synaptophysin; EMT, epithelial-mesenchymal transition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cAMP, cyclic adenosine monophosphate; CRE, cAMP response element; CREB, CRE binding protein; IBMX, 1-isobutyl-methylxanthine; Erk, extracellular signaling kinase; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay

* Corresponding authors at: Department of Radiation Oncology, James P. Wilmot Cancer Center, University of Rochester Medical Center, 601 Elmwood Ave., Box 647, Rochester, NY 14642, USA.
E-mail addresses: Soook_Lee@urmc.rochester.edu (S.O. Lee), Yuhchyau_Chen@urmc.rochester.edu (Y. Chen).
[1] These authors contributed equally.

https://doi.org/10.1016/j.bbamcr.2018.09.005
Received 2 February 2018; Received in revised form 11 September 2018; Accepted 13 September 2018
Available online 15 September 2018

aggressive behavior, rapid growth, early spread to distant sites, exquisite sensitivity to chemotherapy and radiation, and lower survival [17]. On the other hand, prognostic relevance and clinical significance of NED in NSCLC is controversial [18]. Some studies suggest that NED can be a predictor of responses to chemotherapy or radiotherapy (RT) in NSCLC [19,20], and others suggest that NED in NSCLC is not of prognostic significance [21]. It was postulated that NED represents an intermediary transition between NSCLC and SCLC [22]. Recently, Feng et al. [13] examined the correlation between NE features and the prognosis of NSCLC, and showed that tumors with high levels of NE features had worse disease free survival and overall survival.

Whether therapy resistance and NED is correlated depends on the cancer cell type. While it was suggested that NED is involved in chemoresistance in PCa [23,24], the SCLC that show high levels of NED characteristics are known to be extremely chemosensitive and radiosensitive [25]. Whether NED of NSCLC is associated with therapy resistance and is a step towards more malignant behavior has not been resolved.

In this study, we discovered that NED is increased upon radiation treatment in lung cancer cells, and high levels of NED are consistently observed in radioresistant lung cancer cells than in parental cells. We then found the radioresistant cells that exhibited NE-like features have higher metastatic potential than their parental cells. We investigated whether NED is correlated with the development of therapy resistance and whether the radiation-induced NED is essential in increasing the metastatic potential of NSCLC cells. We also investigated the mechanism by which NED is induced in radioresistant NSCLC cells. As we have previously reported on the cAMP and downstream MEK/Erk involvement of NED in NSCLC and observation of higher expressions of Erk signaling in excised large-cell neuroendocrine carcinoma (LCNEC) tumors of lung cancer patients [26], our mechanism studies were now focused on revealing a correlation of these signaling pathways with NED in NSCLC.

2. Methods and materials

2.1. Cell culture

A549 and H157 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 containing 10% FBS. All cells were maintained in a humidified 5% CO_2 environment at 37 °C. In testing NED induction, cells were treated with 0.5 mM cAMP (A9501, Sigma, St Louis, MO)/0.5 mM IBMX (15879, Sigma), 10 ng/mL rhIL-6 (200-06, Peprotech, Rocky Hills, NJ), or 10 ng/mL rhEGF (AF-100-15, Peprotech). For inhibitor studies, cells were treated with 5 μM SQ22536 (S153, Sigma), 10 μM U0126 (U120, Sigma), or 0.1 μg/mL IL-6 Ab (P620, Thermo-Fisher Scientific, Waltham, MA) (vehicle or control IgG were used as control).

2.2. Development of radioresistant cell lines

Parental A549 (A549P) and H157 (H157P) cells were repeatedly treated with 2–6 Gy every week for 4–5 weeks. After receiving a cumulative total of 26 Gy (for A549P cells) and 24 Gy (for H157 cells), the surviving cells were plated at low densities. Ten to eleven colonies were picked, expanded, and the radioresistance of each sub-line was tested. Radioresistant cells were also cultured in RPMI 1640 containing 10% FBS. All cells were maintained in a humidified 5% CO_2 environment at 37 °C.

2.3. Development of siIL-6/sc NSCLC cell lines

To incorporate IL-6 siRNA or scramble (sc) control plasmids into A549 and H157 cells, lentivirus constructs carrying either sc or IL-6 siRNA (pLenti-II vector, Applied Biological Materials Inc) were transfected into 293T cells with a mixture of pLent-II-IL-6 siRNA, psPAX2 (virus-packaging plasmid), and pMD_2G (envelope plasmid) (4:3:2 ratio) using PolyFect Transfection reagent (Qiagen, Germantown, MD). After A549 and H157 cells were virally infected overnight, the culture media containing the virus was replaced with normal culture media, and then maintained under normal cell culture conditions. After sub-culturing cells, the IL-6 knocked down cells were selected by culturing cells in the presence of 2 μg/mL puromycin (540411, Millipore, Billerica, MA) and then maintained in media containing 0.1 μg/mL puromycin.

2.4. In vitro irradiation

For *in vitro* irradiation, cells were plated 24 h before irradiation and 2–6 Gy were applied to the cells using a ^{137}Cs source at a dose rate of 180–205 cGy per minute. Zero to 24 h after irradiation, cells were collected for migration/invasion assays or for qPCR analyses.

2.5. Cell growth assay and cisplatin-cytotoxicity test

For growth assay, cells were plated in 96-well pates (1×10^3/well). Growth of cells was analyzed by adding 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), (M2128, Sigma), dissolving precipitates in DMSO, and by measuring the absorbance at 490 nm. Cell viability was calculated using the formula: OD sample/OD blank control × 100. Triplicate experiments were performed and average values with mean ± SEM were represented. For cisplatin-cytotoxicity test, cells were seeded on 96-well plates (2×10^3 cells/well) and treated with various concentrations of cisplatin for 48 h. MTT test was then performed.

2.6. In vivo xenograft studies

For the animal experiments testing NED marker expressions in tumor tissues of A549 or A549R26-1 cell-derived xenografts, the luciferase tagged H157P and H157R24-1 cells (1×10^6) obtained by transfection of luciferase reporter gene and selection procedures were orthotopically injected (1×10^6 cells in media with Matrigel, 1:1 ratio in volume) into 8-week old female athymic nude mice (NCI) (n = 6 per group). Tumor development was monitored once a week by In Vivo Imaging System (IVIS). Mice were divided into control and test groups. When luminescence reached to 5×10^5 to 1×10^6 radiance (p/s/cm^2/sr), mice in the test group were irradiated (lung site) at 2 Gy for five consecutive days [27] while remainder of the body was shielded from IR using lead blocks. Tumor sizes in irradiated group and control group were monitored twice a week by IVIS for three weeks. At the end of experiments, mice were sacrificed and tumors were obtained. For the experiment testing the radiation and/or MEK inhibitor effects on NED and radioresistance, subcutaneous xenografts were developed by injection of A549R26-1 cells into flanks of athymic mice (n = 20). When tumors developed to 150–200 mm^3, mice were sub-grouped into four (n = 5) groups. The first group of mice were treated with vehicle (10% ethanol/10% cremophor EL/80%, oral gavage), the second group of mice were treated with radiation (2 Gy × 5 days) alone, the third group of mice were treated with the MEK inhibitor (Selumetinib [AZD6244]) (25 mg/kg, oral gavage, 100 μl per day [28,29]), and the fourth group of mice were treated with both radiation and the MEK inhibitor.

All animal studies were performed under the supervision and guidelines of the University of Rochester Medical Center's Animal Care and Use Committee.

2.7. Histology and immunohistochemistry (IHC)

Tissues obtained were fixed in 10% (v/v) formaldehyde in PBS, embedded in paraffin, and cut into 5-μm sections. Tumor tissue sections