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Mini Review

Study designs to investigate Nox1 acceleration of neoplastic progression in immortalized human epithelial cells by selection of differentiation resistant cells ☆

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ABSTRACT

To investigate the role of NADPH oxidase homolog Nox1 at an early step of cell transformation, we utilized human gingival mucosal keratinocytes immortalized by E6/E7 of human papillomavirus (HPV) type 16 (GM16) to generate progenitor cell lines either by chronic ethanol exposure or overexpression with Nox1. Among several cobblestone epithelial cell lines obtained, two distinctive spindle cell lines – FIB and NuB1 cells were more progressively transformed exhibiting tubulogenesis and anchorage-independent growth associated with increased invasiveness. These spindle cells acquired molecular markers of epithelial mesenchymal transition (EMT) including mesenchymal vimentin and simple cytokeratins (CK) 8 and 18 as well as myogenic alpha-smooth muscle actin and caldesmon. By overexpression and knockdown experiments, we showed that Nox1 on a post-translational level regulated the stability of CK18 in an ROS-, phosphorylation- and PKCepilon-dependent manner. PKCepilon may thus be used as a therapeutic target for EMT inhibition. Taken together, Nox1 accelerates neoplastic progression by regulating structural intermediate filaments leading to EMT of immortalized human gingival epithelial cells.

Contents

Abbreviations: AIG, anchorage-independent growth; CK, cytokeratin; EGF, epidermal growth factor; EMT, epithelial mesenchymal transition; GM, gingival mucosal; HPV, human papillomavirus; IAP, inhibitor of apoptosis protein; iNOS, inducible nitric oxide synthase; MEF2, myocyte enhancing factor 2; MMP, matrix metalloproteinases; Nox, NAD(P)H oxidase; PMA, 12-O- tetradecanoylphorbol-13-acetate; ROS, reactive oxygen species

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Introduction

This mini-review illustrates our research approach to investigate the role of NADPH oxidase homolog 1 (Nox1) in neoplastic progression of human epithelial cells. It is known that a direct tumorigenic conversion of human epithelial cells to cancer phenotype is very difficult, and impossible to achieve in tissue cultures. Our experimental designs described below have provided new insights that Nox1 plays a role in regulation of epithelial mesenchymal transition (EMT) on the molecular and functional levels.

Role of ROS and NADPH oxidases in epithelial cancers

The implication for reactive oxygen species (ROS) to play a role in epithleial cancers has long been recognized based on the fact that human cancer cells produce high levels of H_2O_2 [1], and superoxide radical mediates neoplastic transformation of mouse keratinocytes [2]. Data in the last 14 years have shown that the sources of ROS in non-phagocytic epithelial cells are the homologs of gp91*phox*, the catalytic subunit of the respiratory burst oxidase of phagocytes, so-called Nox consisting of Nox1, Nox3, Nox4 and Nox5 (while gp91*phox* is Nox2) [3,4]. These Nox proteins are ~63–65 kDa in size and show 20–60% sequence identity with one another. The first sequence of Nox cDNA from 3 laboratories was all cloned from normal human colon initially called Mox1 (mitogenic oxidase1) and later renamed as Nox1 [5–7].

Nox1, 2, 4, and 5 mRNAs are expressed in a variety of human cancer cell lines indicating an association with cancer development [3]. The role of Nox1 in cancer was first shown by growth stimulation and tumorigenic conversion of rodent NIH3T3 fibroblasts upon Nox1 overexpression [5]. This tumorigenic phenotype was reversed by overexpression of catalase indicating that H_2O_2 generated secondarily Nox1 serves as transformation signals [8]. Nox1 acting as a mitogenic oxidase was further confirmed by data showing Nox1 as a regulator of cyclin D1 [9], and that Nox1 produces H_2O_2 via Rac1 upon epidermal-growth-factor (EGF) receptor ligation [10].

The role of Nox1 in human epithelial cancers was first reported by showing that overexpression of Nox1 increased tumorigenic potentials of DU-145 human prostate cancer cell line [11]. While Nox1 mRNA is present in both primary and transformed human colonic epithelial cells [12], Nox1 mRNA is detected only in human colon tumors but not in ovarian, lymph, breast, prostate, lung, brain, and melanoma tumors [13]. Nox1 is strongly expressed in gastric adenocarcinomas, colonic adenomas and well differentiated adenocarcinomas [14], and breast and ovarian tumors [15]. However, a study in human colon reported that Nox1 was constitutively expressed in colonic epithelium but was not associated with colonic tumorigenesis [16]. These data did not support the role of Nox1 in cancer but rather in inflammation as Nox1 protein is also present in lymphocytes in inflammatory bowel disease.

Setting up model human epithelial cells to study Nox1 functions

Transformation and tumorigenic conversion of mouse cells, such as, keratinocytes [2] and fibroblasts [5] could be accomplished in one step by overexpression of an oncogene or oncogene-like such as Nox1. Human epithelial cells are highly resistant to transformation even after treatment with carcinogens [17,18]. Tumorigenic conversion and transformation of human epithelial cells can be achieved in a multi-step fashion, whereby immortalization is the critical step to obtain tumorigenicity [19,20]. This accounts for the difficulties to perform molecular studies on tumorigenic conversion of human cells, and in fact many studies utilize carcinoma cell lines. In the latter case, the mechanisms for an early stage of cell transformation cannot be studied. Therefore, we had taken a step in choosing a non-cancer immortalized cell line to study the role of Nox1 on phenotypic changes associated with preneoplastic progression beyond immortalization.

It is known that HPV16E6 is able to immortalize human keratinocytes [21], and confers resistance against radiation stress [22]. In our laboratory, we were able to successfully immortalize human gingival mucosal (GM) keratinocytes with human papillomavirus (HPV) type 16 E6/E7 oncogenes to generate a so-called GM16 cell line [23]. Immortalization by HPV has been shown to be prerequisite for progression of cell transformation elicited by subsequent exposure to a carcinogen, such as, benzo(*a*)pyrene [24], asbestos [25], cisplatin [26], and cigarette smoke condensate [27].

About the same time as the discovery of Nox1 [5], an earlier biochemical characterization of ROS generation by human epithelial cells was already described in human skin keratinocyte HaCaT cells during growth-factor activation [28] and overexpression of Ras [29]. In 2004, we accordingly have reported that HaCaT cells express Nox1, Nox2, and Nox4 at mRNA levels, and that Nox1 appears to be the major gp91*phox* homolog expressed on the protein level [30]. HaCaT cells carry genetic abnormalities from spontaneous immortalization and were identified as pretransformed. HaCaT as an immortalized cell line proliferate in standard DMEM. We showed that HaCaT cells expressed Nox1 protein more than GM16 keratinocytes which proliferate only in low-calcium keratinocyte growth medium (KGM) [30].

Selection of preneoplastic human epithelial cells by differentiation resistance

Defective terminal differentiation in cell cultures is regarded as a consistent and selectable character of neoplastic human keratinocytes [31], and considered as an initiation of carcinogenesis [32]. In experiments using cultured cells, resistance against calcium-induced terminal differentiation has been used for selection of preneoplastic cells [33,34]. Indeed, preneoplastic cells induced by Ras [33], HPV16E6 [34], or treatment of 12-O- tetradecanoylphorbol-13-acetate (PMA) [35], exhibit an inhibition of terminal differentiation. Therefore, we utilize a criterion in selecting cells that are resistance against calcium-induced differentiation in our experiment.

Ethanol increases neoplastic progression of GM16 to cells expressing Nox1

As HPV16-immortalized human epithelial cells have been used to study tumorigenic potential of carcinogens [24–27], we were interested in whether ethanol could further neoplastic progression of HPV16-immortalized gingival mucosal epithelial keraitnocytes. In our first study, we exposed GM16 cells to 30 mM ethanol in KGM a closed small incubator with once per week ethanol replenishment for 9 weeks [23]. Many cells were initially died off when cultured medium was changed from KGM to standard high-calcium DMEM containing 10% serum without ethanol. Few selectable cells persisted and finally proliferated again to confluent cultures. Upon further 15 passaging in DMEM, mixed population of cobblestone and elongated progenitor cells were obtained. By differential trypsinization, two distinct cell populations were obtained named as EPI and FIB cell lines with epithelium-like cobblestone and elongated morphology, respectively (Fig. 1A). These EPI and FIB cells selected to survive ethanol-dependent stress and proliferating well in DMEM were considered more transformed than the parental GM16 cells.

FIB cells were fibroblast-like cells showing EMT also exhibited anchorage-independent growth (AIG), and thus considered as