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Nek6 suppresses the premature senescence of human cancer cells induced by camptothecin and doxorubicin treatment

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ABSTRACT

Cellular senescence plays an important role in tumor suppression. The mitotic kinase Nek6 has recently been shown to be overexpressed in various cancers and has been implicated in tumorigenesis. Previously, we reported that the down-regulation of Nek6 expression was required for p53-induced senescence. In this study, we examined the effect of Nek6 overexpression on the premature senescence of cancer cells induced by the anticancer drugs camptothecin (CPT) and doxorubicin (DOX). We found that CPT- and DOX-induced morphology changes and increases in senescence-associated β-galactosidase staining were significantly inhibited in EJ human bladder cancer cells and H1299 human lung cancer cells overexpressing HA-Nek6. DOX-induced G2/M cell cycle arrest and the reduction in cyclin B and cdc2 levels after DOX treatment were significantly reduced by Nek6 overexpression. In addition, an increase in the intracellular levels of ROS in response to DOX was also inhibited in cells overexpressing Nek6. These results suggest that the increased expression of Nek6 renders cancer cells resistant to premature senescence, and targeting Nek6 could be an efficient strategy for cancer treatment.

1. Introduction

The proliferation of normal cells is restricted after a limited number of cell divisions after cells enter an irreversible arrest stage, known as replicative senescence. Generally, the progressive shortening of telomeres plays a critical role in the induction of replicative senescence [1,2]. Recently, another type of senescence, termed premature senescence or accelerated senescence, has been reported. Premature senescence is induced as a result of various cellular stresses, such as oncogene activation, oxidative stress, and DNA damage, and is independent of telomere shortening [3,4]. Premature senescence is accompanied by physiological and phenotypic changes similar to replicative senescence, including growth arrest, enlarged and flattened morphology, and the appearance of senescence-associated β-galactosidase activity (SA β-gal) at pH 6.0 [3,4]. Premature senescence is thought to act as an important internal barrier against tumorigenesis by eliminating premalignant cells [5]. Bypassing the senescence process is therefore an essential prerequisite for cancer development.

A number of previous studies have shown that various cancer cells undergo premature senescence after treatment with ionizing radiation and anticancer drugs, such as camptothecin (CPT) and doxorubicin (DOX), suggesting that premature senescence is a major cellular response of cancer cells to anticancer drugs [6]. The premature senescence of cancer cells has also been reported *in vivo*. In breast cancer and lung cancer patients, premature senescence was observed in tumor samples obtained from patients treated with neoadjuvant chemotherapy, whereas tumors from untreated patients did not show premature senescence [7,8]. Schmitt et al. [9] reported that the tumor response following anticancer drug chemotherapy correlated to the amount of premature senescence in a transgenic murine lymphoma model. These results indicate that premature senescence is an important determinant for cancer chemotherapy. Therefore, the identification of the regulatory proteins of premature senescence is important to understanding how cancer cells acquire resistance to anticancer drug chemotherapy and to develop efficient strategies for cancer treatment.

Nek6 is a serine/threonine kinase belonging to the Nek (NIMA-related kinase) family, which plays an important role in mitotic cell cycle progression [10]. In addition to its role in normal cell cycle progression, we have identified Nek6 as a direct target of the DNA damage checkpoint. We previously showed that the downregulation of Nek6 activity was essential for G2/M cell cycle arrest after DNA damage [11]. The upregulation of Nek6 protein expression and kinase activity has recently been reported in a variety of malignant cancers, such as breast, colon, lung, and gastric cancers [12–14]. Furthermore, it has been shown that the overexpression of Nek6 increases the anchorage-independent growth of cancer cells and that the knockdown of

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Nek6 results in the reduction of tumors in the nude mouse xenograft model [12,13]. These studies collectively suggest that Nek6 plays a role in tumorigenesis, but the underlying mechanism is not fully understand.

Recently, we have shown that Nek6 expression levels are decreased during premature senescence, and the ectopic expression of Nek6 inhibits p53-induced premature senescence of human cancer cells [15]. In this study, we examined the effect of Nek6 overexpression on the anticancer drug-induced premature senescence of cancer cells. We found that Nek6 overexpression significantly reduced the phenotypic changes characteristic of cellular senescence after CPT and DOX treatment in EJ and H1299 cells, suggesting that Nek6 also suppresses the premature senescence of human cancer cells induced by anticancer drugs.

2. Materials and methods

2.1. Cell lines and treatments

EJ-vector control cells (EJ-vec), EJ-Nek6 cells, H1299-vector control cells (H1299-vec), and H1299-Nek6 cells were generated using a retrovirus control vector or a retrovirus encoding wild-type HA-tagged Nek6 as previously described [15]. EJ and H1299 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO).

Camptothecin (CPT) and doxorubicin (DOX) were purchased from Sigma. To induce premature senescence, cells were treated with 30 nM of CPT or DOX for 16 h and incubated with drug-free culture medium for the indicated time.

2.2. Senescence associated (SA)-β-galactosidase staining

Cells were fixed in 0.25% glutaraldehyde, and SA-β-galactosidase staining was performed at pH 6.0 as described previously [16]. After staining, cells were imaged with a microscope using a CCD camera. At least 300 cells in several fields were examined, and SA β-gal-positive cells were counted. These experiments were repeated three times, and the results are presented as mean values with standard deviations.

2.3. Cell cycle analysis

To determine the cell cycle distribution, 1×10^6 cells were seeded in a 100-mm dish. After DOX treatment, cells were trypsinized at the indicated time points and fixed in 70% ethanol. Subsequently, cells were stained with propidium iodide (PI), and flow cytometry analysis was performed using an EPICS XL cytometer and WINCYCLE software (Beckman Coulter, Inc., Brea, CA). A total of 10,000 events were analyzed for each sample, and the experiment was repeated at least three times.

2.4. Western blot analysis and antibodies

Cells were lysed in RIPA buffer and subjected to western blot analysis as described previously [17]. Antibodies for cyclin B and cdc2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Actin levels were monitored as an internal loading control using anti-actin antibodies (Sigma).

2.5. Measurement of ROS level

To measure the intracellular levels of ROS, cells were stained with 50 μM of 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma) for 30 min and then harvested. The fluorescent intensities were quantified using an EPICS XL cytometer (Beckman Coulter Inc.) Experiments were performed in triplicate, and the results are presented as mean values with standard deviations.

3. Results

3.1. Nek6 overexpression overrides CPT and DOX-induced premature senescence

To address the effect of Nek6 overexpression on the anticancer drug-induced premature senescence of cancer cells, we compared the premature senescence of EJ human bladder cancer cells and H1299 human lung cancer cells, which express moderate levels of endogenous Nek6, to previously generated EJ and H1299 cells overexpressing HA-tagged Nek6 cells (EJ-Nek6 and H1299-Nek6) [15] after camptothecin (CPT) or doxorubicin (DOX) treatment. When treated with 30 nM CPT or DOX, both EJ- and H1299-vector control cells became flat and showed enlarged morphology, which is a characteristic phenotypic change in premature senescence [3,4]. Moreover, senescence-associated SA β-galactosidase (SA β-gal) staining was greatly increased. These morphological changes and the increase in SA β-gal stained cells confirmed that CPT and DOX treatment efficiently induced the premature senescence of EJ human bladder carcinoma cells and H1299 human lung cancer cells.

However, neither EJ-Nek6 nor H1299-Nek6 cells showed these morphological changes, and most of the cells maintained normal morphology following CPT and DOX treatment (Fig. 1A and B, left panel). Moreover, the CPT- and DOX-induced increase in the proportion of SA β-gal stained cells was significantly reduced in both EJ-Nek6 and H1299-Nek6 cells (Fig. 1A and B, right panel). These results suggest that Nek6 overexpression suppresses the anticancer drug-induced premature senescence in human cancer cells.

3.2. Overexpression of Nek6 inhibits DOX-induced cell cycle arrest

Cell cycle arrest and the loss of cell proliferation potential are two of the most obvious characteristics during the onset of premature senescence [5]. To investigate the mechanism through which Nek6 overexpression affects anticancer drug-induced premature senescence, we examined changes in cell cycle distribution after DOX treatment in both Nek6-overexpressing cells and vector control cells of EJ and H1299. Consistent with a previous study [18,19], EJ- and H1299-vector control cells were mainly arrested in G2/M cell cycle phase after DOX treatment. The proportion of cells in G2/M phase was increased from 12.6% to 46.7% in EJ-vector control cells and from 12.3% to 37.4% in H1299-vector control cells 4 days after DOX treatment (Fig. 2A and B). In contrast, the percentage of cells in S phase was decreased after DOX treatment. However, EJ- and H1299-Nek6 cells did not show an increase in the proportion of cells in G2/M phase after DOX treatment and maintained the proportion of cells in S phase 4 days after DOX treatment (Fig. 2A and B). These results suggest that Nek6 overexpression in cancer cells overrides DOX-induced G2/M cell cycle arrest and maintains the proliferative potential, even after DOX-treatment.

3.3. DOX-induced decrease of cyclin B and cdc2 is suppressed by Nek6

Cyclin B and cdc2/cdk1 are essential regulators of cell cycle G2/M phase progression and have been shown to be key regulators of premature senescence after genotoxic treatment [20]. To investigate the mechanism by which Nek6 overexpression inhibits DOX-induced cell cycle arrest, we examined the protein levels of cyclin B and cdc2 after DOX treatment using western blot analysis. In EJ- and H1299-vector control cells, the cyclin B level was profoundly decreased 2 days after DOX treatment, and the cdc2