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Autophagy regulated by prolyl isomerase Pin1 and phospho-Ser-GSK3αβ involved in protection of oral squamous cell carcinoma against cadmium toxicity

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ABSTRACT

Prolyl isomerase Pin1 plays an important role in cell proliferation and is overexpressed in many human tumors. However, its role in autophagy induction remains undefined. Here we show that Pin1 regulates cell survival via autophagy in cadmium (Cd)-exposed oral squamous cell carcinoma (OSCC). OSCC exposure to Cd induced autophagy, as demonstrated by the formation of green fluorescent punctae in transfected cells expressing GFP-conjugated microtubule-associated protein light chain 3 (LC3) and by LC3 flux in the presence of autophagy inhibitors. Suppression of Atg5 enhanced Cd-induced apoptosis, indicating that autophagy is involved in cell protection. In dose–response experiments, cleavage of procaspase-3, PARP-1, and LC3-II was induced by Cd with an IC_{50} of 45 μM. Expression of Pin1 was decreased at or above the Cd IC_{50} value and was inversely correlated with the level of phospho(p)-Ser-GSK3αβ. Genetic or pharmacologic inhibition of Pin1 suppressed Cd-induced autophagy, but increased p-Akt-mediated p-Ser-GSK3αβ; this was reversed by overexpression of Pin1. However, suppression of GSK3αβ inhibited Cd-induced autophagy and induced apoptosis, which could be reversed by overexpression of GSK3β. The PI3K inhibitor Ly294002 blocked p-Akt-mediated increases in p-Ser-GSK3αβ and autophagy and induced apoptosis. Therefore, p-Ser-GSK3αβ can directly regulate Cd-induced autophagy, although its function is suppressed by Pin1. Collectively, the present results indicate that targeting Pin1 and GSK3αβ at the same time could be an effective therapeutic tool for Cd-induced carcinogenesis.

1. Introduction

Cadmium is a ubiquitous contaminant in the environment, derived from industrial and agricultural usage. Human exposure to Cd occurs via smoking and intake of contaminated material in the diet. Oral cancers, including mouth, tongue, lip, and throat cancer, make up 3–4% of all cancers, and their worldwide incidence is increasing annually [1]. Most patients with oral cancer are tobacco users [2]. Tobacco smoke contains many kinds of carcinogens, including Cd. Smoking can damage epithelial cells in the lining of the oral cavity and oropharynx and thus is one of the main risk factors for oral squamous cell carcinoma (OSCC). Indeed, Cd is known to interfere with DNA repair systems, which can induce the development of cancer [1]. However, the mechanism underlying Cd-induced carcinogenesis in OSCC remains unknown.

Pin1, a peptidyl prolyl isomerase, binds to phosphorylated serine or threonine residues that precede prolines (p-Ser/Thr-Pro) in many proteins, and induces posttranslational modifications that are essential for controlling the biological functions of target proteins, affecting protein stability, protein interactions, and intracellular localization [3,4]. Pin1 plays a critical role in regulating cell-cycle progression, cell proliferation, cell metabolism, and tumor development and is overexpressed in a number of human cancers [5]. Pin1 overexpression has been reported in OSCC, and its level is correlated with the expression of β-catenin and cyclin D1 [6,7]. Because Pin1 inhibition might suppress cell proliferation and subsequent tumorigenesis, it may be a useful target, as well as a marker in the pathological diagnosis of OSCC. Pin1 has been found to play an inconsistent role in apoptosis. It interacts with survivin and inhibits activation of caspase-3 and caspase-9 [8]. In contrast, Pin1

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enhanced JNK1-mediated apoptosis in response to oxidative stress [9]. Upon genotoxic stress, Pin1 binds to p53, which promotes mitochondria-dependent apoptosis [10]. However, the involvement of Pin1 in Cd toxicity in Pin1-overexpressing oral cancer cell lines, as well as its underlying molecular mechanisms, remains undetermined.

Glycogen synthase kinase 3 (GSK3), a ubiquitously expressed serine/threonine protein kinase, plays a role in a variety of signaling pathways, including that of Wnt. It has several substrates, including β-catenin, tau, cyclin D1, and glycogen synthase, and promotes or suppresses growth in different types of cancers [11]. The activity of GSK3αβ is regulated by site-specific phosphorylation of tyrosine 276/216 residues. Its inactivation is regulated by phosphorylation at serine 9/21 residues, under the control of several protein kinases, including Akt, PKA, and PKC [12], as well as by p38MAPK [13]. OSCC cell lines express a high basal level of p-Ser-GSK3αβ, which acts as a cell cycle regulator by preventing cyclin D1 and cyclin E phosphorylation [14,15]. Although, GSK3αβ activation is critical for cell survival [16], it is associated with apoptosis [17,18] and autophagy [19–21]. Therefore, GSK3αβ can act as either a tumor promoter or tumor suppressor. However, its functions in OSCC are still unclear.

Interactions between Pin1 and GSK3αβ have been investigated in neuronal pathology, in which Pin1 inhibits GSK3αβ via binding to its pT330-Pro motif [22]. Pin1 affects the epithelial–mesenchymal transition by regulating Akt-mediated GSK3αβ inactivation [23], indicating that Pin1 may play a critical role in serine phosphorylation of GSK3αβ. However the involvement of Pin1 in GSK3αβ–mediated autophagy has not been investigated. Recently, we found that Cd toxicity was associated with a p-Ser-GSK3αβ autophagy-signaling pathway [21,24]. Thus, the aim of this study was to determine the involvement of Pin1 in Cd-induced-autophagy signaling in OSCC. Here we show the molecular mechanisms by which Pin1 regulates Cd-induced autophagy, through competition with p-Ser-GSK3αβ, which is involved in cell survival.

2. Materials and methods

2.1. Cell culture and chemicals

Human oral squamous carcinoma cells (YD8) were maintained in DMEM (Gibco, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a 5% CO_2–95% air humidified incubator. Cadmium, bafilomycin A1, PiB (Diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[lmn][3,8] phenanthroline-2,7-diacetate) and MTT [3-(4,5-dimetylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma–Aldrich (Munich, Germany). SB216763 was from Santa Cruz (CA, USA). Other chemicals used were of the purest grade available from Sigma (St. Louis, MO).

2.2. Cytotoxicity assays

Cell viabilities were determined using MTT. In brief, cells were suspended in complete media, at a concentration of 1×10^5 cells/ml, and 200 μl of cell suspensions were seeded onto 48-well plates and cultured overnight. After treatment with chemicals, cells were incubated with MTT (0.5 mg/ml) for 4 h, and the formazan crystals were dissolved in DMSO and measured at 540 nm. Cell viabilities are expressed relative to those of untreated controls.

2.3. Transfection and gene silencing

Cells were transduced with control siRNA and siRNAs for GSK3β, GSK3α, Atg5, and Pin1, or Myc-tagged human Pin1, hemagglutinin (HA)-tagged human GSK3β, and pcDNA3.1, as previously described [21] and [24].

2.4. Immunoblot analysis

Immunoblot analysis was performed as described previously [25]. Antibodies were as follows: anti-LC3B, cleaved caspase-3, HA-tag, p-Ser GSK3αβ, and Atg5 were obtained from Cell Signaling (Beverly, MA, USA). GSK3αβ was purchased from Millipore (Temecula, CA). Anti-procaspase-3, Pin1, β-catenin, p62, Myc, and β-actin were Santa Cruz Biotechnology.

2.5. Statistical analysis

All experiments were repeated at least three times, and values are expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Induction of autophagy and apoptosis in Cd-exposed YD8 cells

To examine Cd sensitivity, YD8 cells were treated with increasing concentrations of Cd for 24 h and then subjected to MTT assays. The IC_{50} value for Cd was 45 μM (Fig. 1A). To examine whether sensitivity to Cd was related to apoptosis and autophagy, we assessed procaspase-3 and poly ADP ribose polymerase-1 (PARP-1) cleavage, which was detected in cells treated with Cd concentrations above 45 μM. Cleaved, lipidated LC3 (LC3-II) was induced above 45 μM Cd, as was a decrease in sequestosome-1 (p62), an autophagy adaptor molecule (Fig. 1B). The induction of autophagy in Cd-exposed YD8 cells was further demonstrated by transfection with green fluorescent protein (GFP)-LC3B plasmid DNA. In untreated cells, GFP fluorescence was diffused evenly throughout the cytoplasm; Cd treatment induced GFP punctae, indicating recruitment of LC3-II to the autophagosome (Fig. 1C). Autophagy induction was further confirmed by the observation of autophagic flux using bafilomycin A1 (BaF1) and chloroquine (CQ). Both autophagy inhibitors caused marked accumulation of membrane-bound LC3-II forms, with decreasing p62 (Fig. 1D). To address the role of autophagy, cells were transfected with siRNA directed against autophagy protein 5 (Atg5) to block autophagic vesicle formation, and silencing of ATG5 was confirmed (Fig. 1E). Knockdown of ATG5 markedly suppressed the Cd-induced LC3-II level, increased cleaved caspase-3, and enhanced PARP-1 cleavage (Fig. 1F). These data indicate that Cd toxicity in YD8 cells may be due in part to apoptotic cell death. Conversely, autophagy may provide cell protection via inhibition of Cd-induced apoptosis.

3.2. Modulation of p-Ser-GSK3αβ by p-Akt and Pin1 expression in response to Cd

It has been reported that Cd-induced autophagy is regulated by GSK3αβ [19,21,24]. Furthermore, p-Ser-GSK3αβ and Pin1 are strongly expressed in OSCC [11,7]. We thus examined the expression of both proteins in YD8 cells treated with increasing concentrations of Cd for 18 h, or with the Cd IC_{50} concentration (45 μM) for different lengths of time. The level of p-Ser-GSK3αβ increased in a concentration- and time-dependent manner, whereas Pin1 began to decrease in Cd-treated cells after 12 h β-catenin, a target of Pin1 as well as of p-Ser-GSK3αβ, increased in a concentration- and time-dependent manner, indicating that it may act downstream of p-Ser-GSK3αβ, but not of Pin1 (Fig. 2A and B). To address the relationship