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Blockade of the ERK pathway enhances the therapeutic efficacy of the histone deacetylase inhibitor MS-275 in human tumor xenograft models

Toshiaki Sakamoto [a], Kei-ichi Ozaki [a], Kohsuke Fujio [a], Shu-hei Kajikawa [a], Shin-ichi Uesato [b], Kazushi Watanabe [c], Susumu Tanimura [a], Takehiko Koji [d], Michiaki Kohno [a,c,e,*]

[a] Laboratory of Cell Regulation, Department of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8521, Japan
[b] Department of Biotechnology, Faculty of Engineering, Kansai University, Osaka 564-8680, Japan
[c] Proubase Technology Inc., Kanagawa 211-0063, Japan
[d] Department of Histology and Cell Biology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8523, Japan
[e] Kyoto University Graduate School of Pharmaceutical Sciences, Kyoto 606-8501, Japan

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ABSTRACT

The ERK pathway is up-regulated in various human cancers and represents a prime target for mechanism-based approaches to cancer treatment. Specific blockade of the ERK pathway alone induces mostly cytostatic rather than pro-apoptotic effects, however, resulting in a limited therapeutic efficacy of the ERK kinase (MEK) inhibitors. We previously showed that MEK inhibitors markedly enhance the ability of histone deacetylase (HDAC) inhibitors to induce apoptosis in tumor cells with constitutive ERK pathway activation in vitro. To evaluate the therapeutic efficacy of such drug combinations, we administered the MEK inhibitor PD184352 or AZD6244 together with the HDAC inhibitor MS-275 in nude mice harboring HT-29 or H1650 xenografts. Co-administration of the MEK inhibitor markedly sensitized the human xenografts to MS-275 cytotoxicity. A dose of MS-275 that alone showed only moderate cytotoxicity thus suppressed the growth of tumor xenografts almost completely as well as induced a marked reduction in tumor cellularity when administered with PD184352 or AZD6244. The combination of the two types of inhibitor also induced marked oxidative stress, which appeared to result in DNA damage and massive cell death, specifically in the tumor xenografts. The enhanced therapeutic efficacy of the drug combination was achieved by a relatively transient blockade of the ERK pathway. Administration of both MEK and HDAC inhibitors represents a promising chemotherapeutic strategy with improved safety for cancer patients.

1. Introduction

Aberrant activation of the extracellular signal-regulated kinase (ERK) signaling pathway contributes to the pathogenesis of many types of human cancer [1,2]. In particular, activating mutations of the epidermal growth factor receptor (EGFR), Ras, and Raf, all of which result in activation of MEK (ERK kinase) isoforms 1 and 2 (MEK1/2) and ERK isoforms 1 and 2 (ERK1/2), have been detected in various human cancers [3–5]. The ERK pathway thus represents a promising target for the development of anticancer drugs, and highly selective small-molecule inhibitors of MEK1/2, including PD184352, PD0325901, and AZD6244, have been developed [6].

We have shown that specific blockade of the ERK pathway by MEK inhibitors markedly suppressed not only the proliferation but also the invasiveness of tumor cells with aberrant activation of this pathway [7,8]. However, blockade of the ERK pathway by itself was largely cytostatic, rather than cytotoxic, resulting in only a moderate induction of apoptosis in these tumor cells [7]. Thus, although PD184352 or AZD6244 totally suppressed the proliferation of T24 bladder carcinoma cells in culture [9] or that of HT-29 colon adenocarcinoma or BxPC3 pancreatic cancer xenografts in vivo [10], respectively, these tumor cells remained viable and resumed proliferation after removal of the inhibitor or cessation of drug administration. Recent clinical studies of MEK inhibitors in patients with advanced cancers have shown that, although PD184352 or AZD6244 achieved target inhibition at well-tolerated doses, these drugs alone exhibited insufficient antitumor activity [11,12]. Efficient induction of apoptotic cell death is essential for the development of effective cancer chemotherapy.

Optimal use of molecularly targeted therapies lies in combination treatment, either with classic cytotoxic drugs or with other targeted therapies [6,13]. In this regard, specific interruption of the cytoprotective ERK pathway by MEK inhibitors has been

* Corresponding author. Address: Laboratory of Cell Regulation, Department of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.
E-mail address: kohnom@nagasaki-u.ac.jp (M. Kohno).

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proposed as a means to enhance the lethal actions of cytotoxic anticancer agents through a shift in the balance between pro- and anti-apoptotic signaling [14]. Consistent with this notion, MEK inhibitors have been shown to enhance the induction of apoptosis by several anticancer agents, including microtubule inhibitors, in human tumor cells in culture [9] as well as in human tumor xenografts in nude mice [15–17].

We have recently shown that blockade of the ERK pathway by PD184352 markedly enhanced the induction of apoptosis by histone deacetylase (HDAC) inhibitors in a variety of solid tumor cells with aberrant ERK pathway activation in vitro, an effect that appeared to be attributable to the increased accumulation of reactive oxygen species (ROS) [18]. Furthermore, such enhanced cell death induction by the combination of a MEK inhibitor and an HDAC inhibitor was apparent even in non-small cell lung cancer and chronic myelogenous leukemia cells exhibiting resistance to EGFR or Abl tyrosine kinase inhibitors, respectively [19]. We now show that blockade of the ERK pathway with a MEK inhibitor resulted in marked potentiation of the therapeutic efficacy of the HDAC inhibitor MS-275 in human tumor xenograft models.

2. Materials and methods

2.1. Reagents and antibodies

PD184352 [8], AZD6244 [10], and MS-275 [20] were synthesized as described previously. Cremophore EL was obtained from Sigma–Aldrich (St. Louis, MO). Antibodies to ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), those to diphosphorylated ERK1/2 were from Sigma–Aldrich, those to acetyl–histone H3 (Lys^9) were from Merck–Millipore (Billerica, MA), those to histone H3 were from Active Motif (Carlsbad, CA), and those to 8-hydroxy-2′-deoxyguanosine (8-OHdG) were from Japan Institute for the Control of Aging (Shizuoka, Japan).

2.2. Animals and tumor cell implantation

The human tumor cell lines HT-29 (colon adenocarcinoma) and H1650 (lung adenocarcinoma), obtained from American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. These tumor cells (2×10^6) were injected subcutaneously into the right flank of 5- to 6-week-old female BALB/c nu/nu mice (CLEA Japan, Tokyo). After the resulting tumors had achieved a size of $\sim$200 mm^3, mice were randomly assigned to balanced groups of five to seven animals. PD184352, AZD6244, and MS-275 were each suspended in an 8/1/1 (v/v/v) mixture of phosphate-buffered sa-line/ethanol/Cremophore EL. Mice were treated every 3 or 4 days (twice per week) with PD184352 (200 mg/kg), AZD6244 (50 mg/kg), or vehicle by oral administration (twice per day with an interval of 6 h) as well as with MS-275 (10–40 mg/kg) or vehicle by oral administration (once per day, 1 h after the first administration of PD184352 or AZD6244). Tumor volume was measured with digital calipers and calculated according to the formula: (longest diameter) $\times$ (shortest diameter)^2/2. Body weight, tumor volume, and toxicities were noted every 2–4 days for the duration of the experiment.

2.3. Immunoblot analysis

Tumor extracts were prepared by mechanical homogenization of excised tumors in a hypotonic cell lysis buffer on ice, fractionated by SDS–polyacrylamide gel electrophoresis, and subjected to immunoblot analysis as described [17]. Immune complexes were detected with an enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ).

2.4. Immunohistochemical analysis

HT-29 or H1650 xenografts were harvested, fixed in buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. After removal of paraffin and rehydration, tissue sections were incubated consecutively with primary antibodies and horseradish peroxidase-conjugated secondary antibodies and were then stained with 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin and examined with a microscope equipped with Axiovision software (Carl Zeiss, Jena, Germany) [17]. Apoptotic cells were detected with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay as described [21].

2.5. Statistical analysis

Data are presented as means ± SD. Differences between means were analyzed with the two-tailed Student's *t* test or two-way analysis of variance (ANOVA). A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Blockade of the ERK pathway by a MEK inhibitor enhances induction of apoptosis by MS-275 in tumor xenograft models

Nude mice harboring subcutaneous HT-29 or H1650 tumors ($\sim$200 mm^3), in which the ERK pathway is activated as a result of mutation of B-Raf or EGFR, respectively, were treated orally with PD184352 at a dose of 200 mg/kg [17] or AZD6244 at 50 mg/kg [10], respectively. Immunoblot analysis of tumor extracts revealed that a single dose of these MEK inhibitors almost completely suppressed the phosphorylation of ERK1/2 for 6 h, after which the phosphorylation level gradually returned to control levels by 12 h (Fig. 1A). These results indicated that administration of PD184352 or AZD6244 every 6 h would be required for continuous suppression of ERK1/2 activation in these tumors in nude mice.

For examination of the effects of the HDAC inhibitor MS-275 in tumor xenografts, mice bearing subcutaneous HT-29 tumors were treated orally with MS-275 at a dose of 20 or 40 mg/kg. Whereas the acetylation of histone H3 at Lys^9 was virtually undetectable in untreated tumors, treatment with MS-275 increased the acetylation of histone H3 in a dose-dependent manner and this effect was apparent more than 24 h after drug administration (Fig. 1B).

To examine the potential of AZD6244 to enhance the induction of apoptosis by MS-275 in vivo, we treated mice bearing subcutaneous H1650 tumors ($\sim$200 mm^3) with AZD6244 (50 mg/kg, orally) or vehicle twice, with an interval of 6 h between the two administrations, and with MS-275 (40 mg/kg, orally) at 1 h after the first AZD6244 treatment. Immunoblot analysis of tumor extracts as well as immunostaining of tumor sections with antibodies to phosphorylated ERK1/2 revealed that ERK1/2 phosphorylation was suppressed completely for up to 12 h after the initial AZD6244 administration and that it had returned to control levels by 24–36 h (Fig. 1C). Co-administration of MS-275 did not interfere with the AZD6244-induced inhibition of ERK1/2 phosphorylation, and co-administration of AZD6244 did not interfere with the MS-275-induced increase in the acetylation of histone H3 (Supplementary Fig. 1).

TUNEL staining of tumor sections for cells undergoing apoptotic death revealed that AZD6244 treatment alone did not increase the number of apoptotic cells (Fig. 1D), consistent with results obtained in vitro [18]. MS-275 treatment alone slightly increased the number of TUNEL-positive cells in H1650 xenografts by 24 h, and this effect was markedly enhanced by co-administration of