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Tumor specific low pH environments enhance the cytotoxicity of lovastatin and cantharidin

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ABSTRACT

In tumor cell masses, the extracellular pH decreases below 6.5. The effect of external acidic pH on the efficacy of 24 chemical compounds including molecular-targeted inhibitors and anti-tumor reagents was investigated in human cancer cells. Lovastatin showed no cytotoxicity in mesothelioma or pancreatic carcinoma cells at concentrations up to 10 μM and pH around 7.4, but 10 μM lovastatin decreased the survival of these cells below 40% at acidic pH. Lovastatin inhibits HMG-CoA reductase, resulting in a decrease in the levels of cholesterol and prenylated proteins. An inhibitor of the former pathway showed pH-independent cytotoxic activity, whereas an inhibitor of the latter pathway had stronger activity at acidic pH. The inhibitory efficacy of cantharidin also increased at acidic pH. On the other hands, no pH dependency or slightly impaired efficacy at low pH conditions was observed in other 20 reagents, and especially, the activity of aphidicolin was suppressed under acidic conditions. These results suggested that screening under acidic conditions would be useful for developing new chemotherapeutic reagents.

1. Introduction

In mammals, blood and tissues are usually maintained within a narrow pH range around 7.4, mainly through the regulation of respiration and renal acid extrusion. Each organ functions normally at this slightly alkaline pH, and organ functions are strongly affected by the disruption of pH homeostasis because all organs contain a large number of enzymes with pH sensitive catalytic activity. In the central regions of solid tumors, the extracellular pH decreases below 6.5 as a consequence of lactate accumulation, which is caused by the hypoxic conditions produced by a lack of sufficient vascularization [1,2] or an increase in tumor specific aerobic glycolysis combined with impaired mitochondrial oxidative phosphorylation [3].

Impaired efficacy of paclitaxel, mitoxantrone, and topotecan was reported at pH 6.5 as compared with their efficacy at pH 7.4 in murine EMT6 and human MGH-U-1 cells [4], and acidic conditions induced daunorubicin resistance by increasing the activity of p-glycoprotein via p38 activation [5]. In contrast, there have been few studies of drugs that show increased activity under acidic environments. Our group previously found that different signal transduction pathways function under acidic environments [6,7], and that CTIB, an Ikappa B beta variant, acted as a critical transcription factor at pH 6.3 but not at pH 7.4 [8,9]. These previous findings led us to assume that different metabolic pathways are activated under acidic conditions. In addition to these molecules, numerous enzymes may work preferentially under low pH conditions, and chemical compounds that inhibit these enzymes could be good candidates for new chemotherapeutic reagents.

Malignant pleural mesothelioma is an aggressive tumor related to asbestos exposure, and its prognosis is very poor [10]. Mesothelioma shows resistance against many chemotherapeutic reagents [11]. Pancreatic tumors are also resistant to a variety of chemotherapies, and this may be

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responsible for their poor prognosis [12]. Gemcitabine is the current standard treatments for advanced and metastatic pancreatic cancer therapy, but shows little survival benefit either alone or in combination with other anti-tumor drugs [13,14].

We assumed that a new strategy for investigating drug efficacy under acidic environments was necessary for the further development of chemotherapeutics against these intractable tumors and examined the pH-dependent efficacy of anti-tumor drugs and molecular-targeted agents in tumor cells such as HeLa, mesothelioma, and pancreatic tumor cells. To mimic the Warburg effect [3], cancer cells were cultured at acidic pH without hypoxia in this study, and our data showed that the inhibitory effects of some reagents increased dramatically under acidic conditions, suggesting that our new method could be useful for developing new types of anti-cancer chemotherapeutics.

2. Materials and methods

2.1. Cells and reagents

HeLa cells derived from a human cervical carcinoma were cultured at 37 °C under 5% CO_2 in DMEM containing 24 mM $NaHCO_3$ (Sigma), 10 μg/ml gentamicin, 5 μg/ml fungizone, and 10% fetal bovine serum. The human pleural mesothelioma cell lines NCI-H2452 (ATCC CRL 5946) and NCI-H2052 (ATCC CRL 5915) and the human pancreatic carcinoma cell lines PANC-1 (ATCC CRL 1469) and BxPC-3 (ATCC CRL 1687) were cultured in RPMI-1640 instead of DMEM under the same conditions.

A SCADS inhibitor kit was kindly donated by the Screening Committee for anti-cancer drugs, and this organization was supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science, and Technology of Japan. Mitomycin C (Sigma), lovastatin (Wako, Osaka, Japan), cantharidin (Calbiochem), and aphidicolin (Wako, Tokyo, Japan) were purchased from the above mentioned manufactures. Cell counting kit 8 was purchased from DOJINDO (Kumamoto, Japan).

2.2. Media for cell proliferation and cytotoxicity assays under different pH conditions

To minimize the change in the pH of the medium during the cell proliferation and cytotoxicity assays, 10 mM PIPES [piperazine-*N*,*N*′-bis (2-ethanesulfonic acid)] was added to the pH 6.7 medium and 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was added to the pH 7.7 medium instead of $NaHCO_3$. The culture medium pH was adjusted by the addition of NaOH.

2.3. Effects of SCADS inhibitors on cell proliferation at different pH values

Cells suspended in DMEM (for HeLa) or RPMI-1640 (for other cells) medium were placed in 24 well plates (3×10^3 cells/well) and incubated for 24 h without CO_2 supply. The media were then exchanged for different pH media containing Good's buffers and one of the inhibitors after counting the number of cells, and then the cells were incubated for 120 h at 37 °C without CO_2 supply. Cell proliferation was determined by counting the number of cells stained with trypan blue.

2.4. Cytotoxicity assay in different pH media

The cells were suspended in DMEM (for HeLa) or RPMI-1640 (for other cells) medium at pH 7.7, and 50 μl of the cell suspension were placed in 96 well plates at 3×10^3 cells/well. Following incubation for 24 h at 37 °C without CO_2 supply, 100 μl of pH 6.1 media containing one of the inhibitors were added to the wells to create acidic conditions, and the pH of the resulting mixture was 6.7. For incubation at pH 7.7, 100 μl of pH 7.7 media with or without inhibitor were added to the wells. Cell cytotoxicity was determined with a cell counting kit 8 after incubation for 72 or 96 h without CO_2 supply.

2.5. Immunoblot with anti-PARP (poly(ADP-ribose)polymerase) antibody

NCI-H2052 cells were incubated with simvastatin at pH 7.7 or 6.7 for 48 h. Then, the cells were harvested, washed with PBS three times, and then lysed with TNE buffer (1% NP-40, 20 mM EDTA, 50 mM Tris–HCl (pH 7.8), 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, and 0.2 μM leupeptin) on ice for 20 min. The lysates were centrifuged at 12,000×*g* for 5 min to remove cell debris. The resulting supernatant (10 μg protein) was subjected to SDS–PAGE, and immunoblotting was carried out with rabbit anti-PARP antibody (Cell signaling).

3. Results

3.1. pH-dependent inhibition of HeLa cell proliferation

The SCADS inhibitor kit 1 contains 93 chemical compounds including molecular-targeted inhibitors and anti-tumor reagents (for more details, see http://gantoku-shien.jfcr.or.jp). The effects of the inhibitors in the SCADS inhibitor kit 1 on the proliferation of HeLa cells were first investigated in pH 7.7 and 6.7 media containing 1 μM of one of the reagents. HeLa cells proliferated in the pH 7.7 medium in the absence of reagents, and the medium pH had decreased to 7.4 after 120 h culture. Only 22 compounds inhibited HeLa cell proliferation at pH 7.7 or 6.7, and we used these inhibitors for the subsequent studies. One μM lovastatin had no effect on proliferation in the pH 7.7 medium (Fig. 1A). HeLa cells proliferated in the pH 6.7 medium in the absence of reagents although the increase in cell number was only 2.5-fold during 120 h culture, and the medium pH decreased to 6.6. In contrast to its effects in the alkaline medium, 1 μM of lovastatin decreased the cell number by nearly half in the pH 6.7 medium (Fig. 1A).

One μM cantharidin decreased the surviving cell number by more than 90% in the pH 6.7 medium; whereas, the cells proliferated in the pH 7.7 medium in the presence or absence of 1 μM cantharidin (Fig. 1B). Aphidicolin induced cell death in the pH 7.7 medium; whereas, the cell number increased slightly in the pH 6.7 medium in the presence of aphidicolin (Fig. 1D). Five other reagents showed low efficacy at acidic pH (Table 1), although their pH-dependency was weak.

Mitomycin C prohibited cell proliferation similarly at both pH values (Fig. 1C). In addition to mitomycin C, 13 other inhibitors showed identical inhibition of HeLa cell proliferation at both pH values (Table 1). No other inhibitors in the SCADS inhibitor kit 1 inhibited HeLa cell proliferation at