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Sodium salicylate switches glucose depletion-induced necrosis to autophagy and inhibits high mobility group box protein 1 release in A549 lung adenocarcinoma cells

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Abstract. Sodium salicylate, the active metabolite of aspirin, has been shown to exert anti-inflammatory activities by inhibiting the expression of various pro-inflammatory factors, and has potent anti-cancer effects against a number of human cancers including colon, lung, breast and leukemia. Necrotic cell death is emerging as one of the crucial factors that trigger an inflammatory response since during necrotic death the cell membrane is ruptured and the intracellular constituents including high mobility group box 1 (HMGB1) are released into the extracellular space, thereby activating an inflammatory response. In contrast, autophagic death is regarded as a form of tumour suppressive cell death, as indicated in tumour suppressors such as beclin 1 in autophagic pathways. To better understand the anti-inflammatory properties of sodium salicylate and its effect on necrotic cell death in A549 cells induced by glucose depletion (GD), a common characteristic of the tumour micro-environment, was examined. While GD induced mostly necrotic death in A549 cells, salicylate suppresssed GD-induced necrosis and HMGB1 release. In addition, salicylate shifted the cell death pattern to autophagy by inhibiting GD-induced Cu/Zn superoxide dismutase release and ROS production. These results indicate that the activity of salicylate to prevent necrotic death may contribute to its anti-inflammatory action and suppress tumour development possibly through switching the cell death mode from tumour-promoting necrotic cell death to tumour-suppressive autophagic cell death.

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Introduction

Sodium salicylate, a plant-derived hormone that plays an important role in defenses against herbivorous insects and microbial pathogens, is known to exert anti-inflammatory activity by inhibiting the expression or activation of various pro-inflammatory factors, including cyclooxygenase 2, iNOS and interleukin 1ß (1,2). Sodium salicylate and its acetylated form aspirin are also reported to have potent anti-neoplastic effects against a number of human cancers including colon, lung, breast and leukemia (3,4). The chemopreventive activity of sodium salicylate and aspirin is thought to be linked to their ability to inhibit cell proliferation (5-7), and induce differentiation and apoptosis (8-12). The caspase family, including caspase-3 and -8, has been shown to participate in the initiation and execution of sodium salicylate/aspirin-induced apoptosis in many types of cancer cells (8-12). In addition, sodium salicylate and aspirin are known to induce apoptosis through induction of the non-steroidal anti-inflammatory drug activated gene-1 (NAG-1), a member of the TGF-ß superfamily (13-17). Furthermore, sodium salicylate-induced ROS plays a crucial role as a key mediator of deltapsi(m) collapse, which leads to the release of cytochrome c, is followed by caspase activation and culminates in tumour apoptosis (18,19). Sodium salicylate/aspirin-induced activation of NF-κB signaling may play a part in the apoptotic response. Although sodium salicylate and aspirin are generally considered to inhibit this pathway (20), more recent studies have shown that they can activate NF-κB signaling and stimulate apoptosis in colorectal cancer cell lines (21). The activation of p38 MAPK has also been reported to play a critical role in sodium salicylate/aspirin-induced apoptosis (22,23).

Under circumstances of metabolic or cytotoxic stresses, cells die by apoptosis, autophagy and necrosis (24-26). Apoptosis is a genetically regulated process of cell-suicide

that is characterized by membrane blebbing and DNA degradation and eventually, apoptotic bodies formed by cell disintegration are removed by phagocytes or neighboring cells, resulting in cellular deletion without inflammation. Autophagy is a self-degradative process involved in the basal turnover of cellular components in response to nutrient starvation or organelle damage in a wide range of eukaryotes and is a form of programmed cell death controlled by autophagy-related Atg proteins including beclin 1. During necrosis, the cell membrane is ruptured and the cytoplasmic contents [e.g. nuclear protein high mobility group box 1 (HMGB1)] are released into the extracellular space causing a massive inflammatory response. Apoptotic and autophagic cell death may suppress tumour progression, whereas necrotic cell death is thought to promote tumour growth and angiogenesis either by increasing the probability of proto-oncogenic mutation or by the action of HMGB1 (27-32). Thus, the consequences of the cell death mode are quite different in terms of its influence on the whole organism. In solid tumours, necrosis is commonly found in the core region in response to oxygen and glucose depletion (OGD) due to insufficient vascularization (33). HMGB1 is a highly conserved, DNA-binding protein in nearly all cell types and was originally identified to function as a structural co-factor critical for proper transcriptional regulation in somatic cells (34). It may be passively released into the extracellular milieu by necrotic and damaged somatic cells and extracellular HMGB1 represents a necrotic marker. It is also secreted in the extracellular milieu through active secretion by stimulated macrophages or monocytes in a process that depends on a secretion signal mediated by either extracellular lysophosphatidyl-choline or ATP. Extracellular HMGB1 acts as a cytokine by signaling via the receptor for advanced glycated end-products and members of the Toll-like receptor family. It may cause inflammatory responses, including the production of multiple cytokines, thereby contributing to the pathogenesis of diverse inflammatory and infectious disorders. Furthermore, HMGB1 has been demonstrated to possess tumour-promoting and angiogenenic activities in addition to acting as a pro-inflammatory mediator (34-36). A growing number of studies indicate that HMGB1 is a successful therapeutic target in the experimental models of inflammatory and infectious disorders and cancer. Thus, the therapeutic potential of blocking HMGB1 in the treatment of inflammatory diseases and cancer is highly evaluated (37-40).

Previously, we demonstrated GD-induced necrosis through the production of ROS in A549 lung carcinoma cells and that protein kinase C-dependent ERK 1/2 activation switched GD-induced necrosis to apoptosis through the inhibition of ROS production, possibly by inducing MnSOD expression and the prevention of GD-induced down-regulation of CuZnSOD (41). Cellular damage by ROS is compromised by the levels of defense antioxidant enzymes such as cytosolic CuZn superoxide dismutase (CuZnSOD), mitochondrial MnSOD, glutathione peroxidase (GPx) and catalase (42,43). Cytosolic CuZnSOD and mitochondrial MnSOD efficiently detoxify O_2^- formed on the two sides of mitochondrial inner membranes initially to H_2O_2, which in turn is further converted to H_2O, with the help of GPx and catalase. During metabolic stress-induced necrosis, CuZnSOD has been shown to be released into the extracellular space in an active form upon GD, thereby accelerating ROS (possibly O_2^-) damage and facilitating necrotic cell death (44). In this study, to better understand the anti-inflammatory and tumour preventive properties of sodium salicylate and aspirin, we examined their effects on GD-induced necrotic cell death and HMGB1 release in A549 cells. Herein we show that sodium salicylate and aspirin prevented HMGB1 release by inducing a switch from GD-induced necrosis to autophagy through the prevention of GD-induced CuZnSOD release and ROS production. Thus, sodium salicylate and aspirin appeared to have the potential to suppress HMGB1 release from necrotic cells, thereby contributing to their activity to prevent inflammatory response and tumour progression.

Materials and methods

Cell culture and glucose deprivation. Human lung adenocarcinoma cell line A549 cells were obtained from American Type Culture Collection and grown in RPMI-1640 medium (Gibco BRL), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco BRL) and 1% penicillin-streptomycin (PS, Gibco BRL) in a 37˚C humidified incubator with 5% CO_2. For glucose deprivation, cells were gently rinsed twice with glucose-free RPMI-1640 and incubated in GD medium [glucose-free RPMI-1640 medium (Gibco BRL) containing 10% dialyzed and heat-inactivated FBS and 1% PS].

Western blot analysis and antioxidant enzyme activity assay. Western blotting with antibodies to PARP (Santa Cruz), active caspases-3 and -9 (Cell Signaling), CuZnSOD, MnSOD, catalase (Santa Cruz), ERK2 (Cell Signaling) and HMGB1 (BD Pharmingen) was performed as previously described (41). The SOD activity was monitored with nitroblue tetrazolium negative staining after native gel electrophoresis on 7% polyacrylamide gels as previously described (41). The lower band was preconfirmed as CuZnSOD and the upper one as MnSOD in the gel by which incubation with 5 mM sodium cyanide showed only the upper band. The catalase activity in non-denaturing 7% polyacrylamide gels was monitored as previously described (41).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. For the cell viability assay, A549 cells (10^4 cells/well) were seeded in a 96-well tissue culture plate and incubated for 24 h. After treatment of the GD medium for the indicated times, MTT solution (0.5 mg/ml) was added to each well. After incubation for 4 h at 37˚C, formazan crystals in viable cells were solubilized with 150 μl of DMSO. The solubilized formazan product was spectro-photometrically quantified using an ELISA reader at 595 nm.

Measurement of intracellular ROS. To determine the production of intracellular H_2O_2 and O_2^-, cells were plated in 48-well plates (at $2x10^4$ cells/well) or on cover-glass for a fluorocount measurement or fluorescence microscopic detection, respectively. After the GD treatment, cells were incubated in a 37˚C, CO_2 incubator loaded with 2,7-dichloro-fluorescin diacetate (DCFH-DA, Molecular Probes, 50 μM) or dihydroethidium (HE, Molecular Probes, 10 μM) for the last 30 min of the indicated incubation times. Intracellular

ROS was also determined using a fluorescence microscope (DM5000, Leica, Germany), an L5 filter cube (excitation at BP 440-520 nm and emission at BP 497-557 nm) and a TX2 filter cube (excitation at BP 520-600 nm and emission at BP 570-720 nm) for DCFH-DA and HE, respectively.

Hoechst 33342 (HO)/propidium iodide (PI). Cells were incubated either with 1 μg/ml HO or 5 μg/ml PI at 37˚C, 5% CO_2 for 15 min in the dark. Floating and attached cells were collected by centrifugation and trypsinization of the medium. The pooled cell pellets were immediately fixed in 3.7% formaldehyde, washed with phosphate-buffered saline (PBS), which was resuspended with a fraction of the suspension centrifuged in a cytospinner (Thermo Shandon, Shandon Inc.). The slides were then washed in PBS to remove excess dye, air-dried, mounted in FluroGard antifade and examined with fluorescence microscopy (340/425 nm) (HO) and 580/ 630 nm (PI) (Carl Zeiss, Axioskop 2 plus).

HMGB1 release assay. Cell culture medium was collected at the indicated time points whereas the cells and debris were removed by centrifugation at 2400 g for 20 min at 4˚C. The supernatant was first filtered through Centricon YM-100 (Millipore) to clear the samples from cell debris and macromolecular complexes formed during clotting. Samples were then concentrated to 15-fold with Centricon YM-30 and analyzed by Western blotting with antibody to polyclonal anti-HMGB1 antibodies (BD Pharmingen). HMGB1 was identified as a 29 kDa protein.

Lactate dehydrogenase (LDH) assay. Cells were plated at a concentration of $1x10^4$/well in 96-well plates 1 day before heat-shock treatment. After 2 h 30 min of incubation at 41˚C for heat-shock or 37˚C for control, the medium was replaced by GD medium and incubated for another 12 or 18 h. The released LDH was determined using the cytotoxicity assay kit II (BioVision, CA, USA) as per manufacturer's protocol. Briefly, the plates were centrifuged at 600 g for 10 min, and the cell-free supernatant was transferred to a 96-well plate, mixed with LDH reaction mix, incubated for 30 min and the absorbance was measured at 450 nm. The percentage of specific LDH release was calculated by the following formula: % cytotoxicity = [(experimental LDH release)-(spontaneous LDH release by effector and target)/(maximum LDH release) - (spontaneous LDH release)] x 100. The spontaneous release of LDH activity from the control cells was <2% of the maximal release of LDH activity, which was determined from the complete lysis by adding lysis buffer. All assays were performed in triplicate.

Transmission electron microscopy. For transmission electron microscopy, the collected cells were fixed in 2.5% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.2) for 2 h at 4˚C and washed twice with cold PBS, post-fixed in OsO_4, dehydrated in graded ethanol and embedded in epon mixture. Sections were prepared with ultra-microtome (MT-7000), mounted in copper grids and counterstained with uranyl acetate and lead citrate. Photographs were taken using an electron microscope (Hitachi H-7600).

Results and Discussion

Sodium salicylate prevents GD-induced necrosis and HMGB1 release. Previously, we showed that GD induced necrosis and released the inflammatory and tumour-promoting cytokine HMGB1 in A549 lung adenocarcinoma cells (41,44). We examined the effect of sodium salicylate on GD-induced necrosis. Cell death mode, necrosis or apoptosis, were determined by observing the Ho33342/PI staining pattern and nuclear shape. Ho33342 is cell-permeable, and has adenine-thymine-specific dyes that bind to the minor groove of DNA and stain all cellular nuclei to blue, while PI only penetrates cells with damaged membranes and leads to nuclear red fluorescence. As demonstrated previously, GD induced a marked PI staining, but no nuclear condensation or fragmentation in Ho33342 staining (Fig. 1A), confirming our previous results that GD induces necrosis. The addition of sodium salicylate during GD significantly suppressed the population of PI-stained cells, but unlike the treatment with catalase, the cells showed no condensed or fragmented nuclei stained by Ho33342, thus indicating that sodium salicylate prevents GD-induced necrosis without switching the cell death mode to apoptosis. Similar results were obtained with aspirin, but not with other non-steroidal anti-inflammatory drugs such as indomethacin, NS398 and sulindac (data not shown). The inhibitory effect of sodium salicylate on GD-induced necrosis was further confirmed by inhibition of the HMGB1 release (Fig. 1B). The inhibitory effect of sodium salicylate on GD-induced necrosis was also confirmed by a decrease in lactate dehydrogenase (LDH) activity in the medium (Fig. 1C).

Sodium salicylate switches GD-induced necrosis to autophagy. Since Ho33342/PI double staining or LDH assay showed neither necrotic nor apoptotic features in the cells that were treated with sodium salicylate in the GD condition, we assessed cell viability to see if sodium salicylate prevented GD-induced cytotoxicity. The cells were cultured in normal or GD condition in the presence or absence of 2 mM sodium salicylate and MTT assay was carried out of up to 30 h. Comparable cytotoxic effects were observed in the cells that were treated with sodium salicylate in the GD condition, similar to those treated with GD only in A549 cells (Fig. 1C), despite the significant difference in the LDH release pattern (Fig. 2A).

The absence of necrotic or apoptotic features in sodium salicylate-treated GD cells led us to examine the effects of treatment on the cellular ultrastructure using electron microscopy. Upon treatment with GD alone, the cells exhibited the characteristic signs of necrotic changes, swelling of cytoplasm and mitochondria, disintegrated membrane, loose cytoplasm and cell debris as a result of necrosis (Fig. 2B). However, the addition of sodium salicylate or aspirin to GD changed the cells which had dense cytoplasm, intact membrane and typical autophagic vesicles containing cytoplasmic constituent and organelles such as endoplasmic reticulum or mitochondria, which are typical features of autophagic death (Fig. 2B). In addition, we did not detect the activation of caspase 3, which is known to play a key role in apoptosis by metabolic stress (Fig. 2C). GD-treated cells in the presence of sodium salicylate

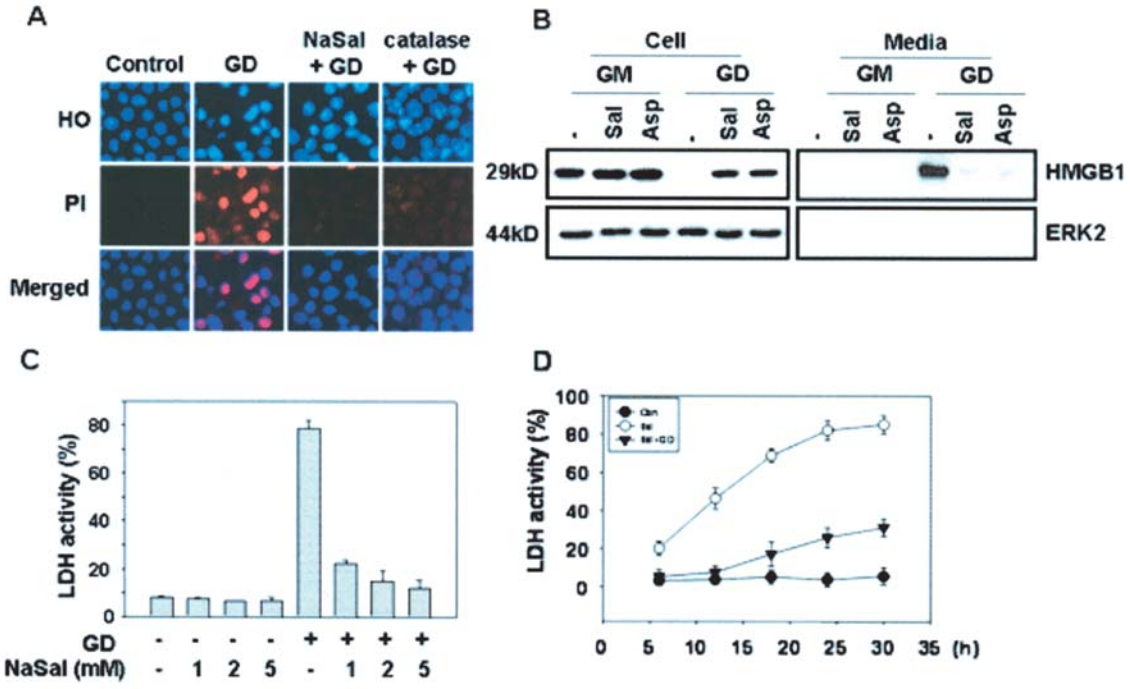

Figure 1. Sodium salicylate prevents GD-induced necrosis and HMGB1 release. (A) A549 cells were exposed to the GD medium for 18 h in the absence or presence of 2 mM sodium salicylate or catalase (1,000 U/ml). The cells were stained with HO/PI and observed under a fluorescence microscope. (B) A549 cells were cultured in normal growth or GD medium for 18 h in the presence of 2 mM sodium salicylate or 2 mM aspirin and then the cells and medium were prepared and analyzed by SDS-PAGE and Western blotting using antibodies to HMGB1 and ERK2. (C-D) A549 cells were incubated in normal growth or GD medium for 18 h in the presence of 1, 2 or 5 mM sodium salicylate (C) or incubated in GD medium for up to 30 h in the presence or absence of 2 mM sodium salicylate (D). The media were subjected to LDH assay. Data are the percent of the maximal release of LDH activity and are expressed as the mean ± SEM from three independent experiments.

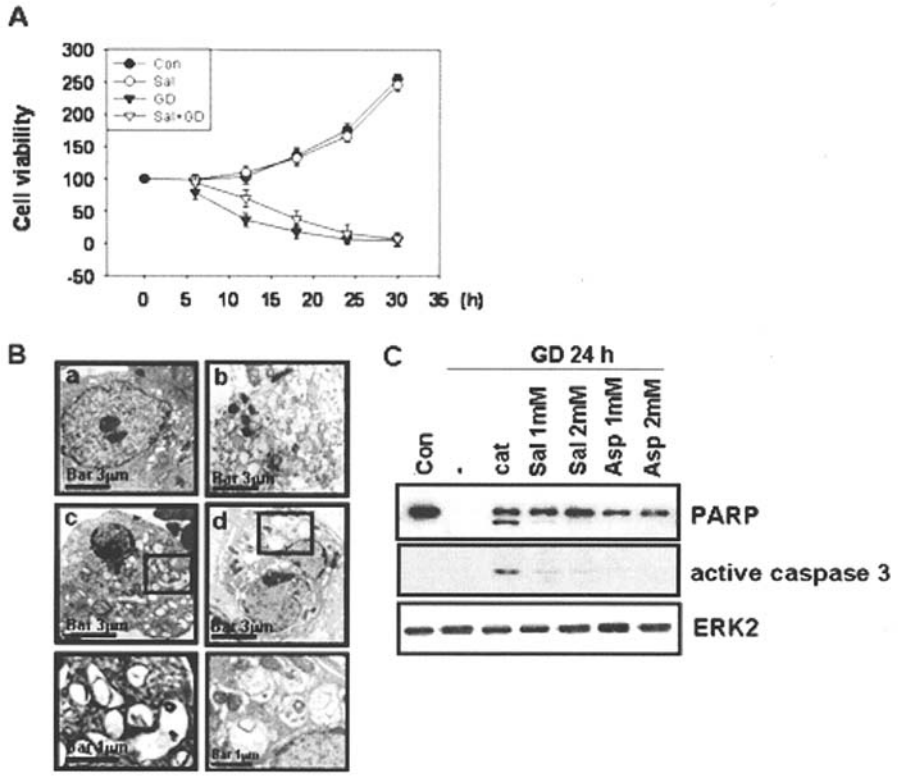

Figure 2. Sodium salicylate switches GD-induced necrosis to autophagic death. (A) The addition of sodium salicylate to GD showed a compatible cytotoxic effect to GD treatment alone. A549 cells were exposed to the GD medium for the indicated times in the absence or presence of 2 mM sodium salicylate and the viability was measured by MTT assay. Data are the percent of the control activity and are expressed as the mean ± SEM from three independent experiments. (B) A549 cells incubated in normal growth and GD medium for 18 h in the absence (b) or presence of 2 mM sodium salicylate (c) or 2 mM aspirin (d) were examined under electron microscopic observation a, control. (C) A549 cells were cultured in normal growth or GD medium in the presence or absence of 1-2 mM sodium salicylate or aspirin for 24 h. The cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to PARP, active caspase-3 and ERK2.

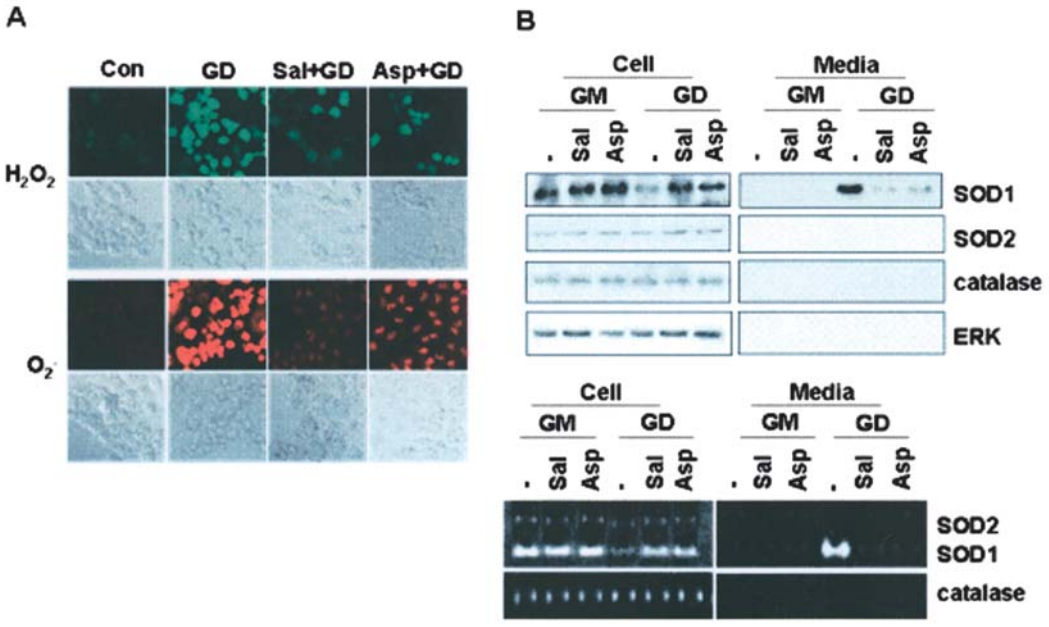

Figure 3. Sodium salicylate and aspirin suppress GD-induced ROS production and CuZnSOD release. (A) A549 cells were exposed to GD medium alone or in the presence of 2 mM sodium salicylate or 2 mM aspirin for 12 h. The cells were treated with DCFH-DA and HE for 20 min and intracellular H_2O_2 and O_2^- were determined under a confocal microscope. (B) Sodium salicylate-induced cell death mode switch is related to inhibition of the GD-induced CuZnSOD release. A549 cells were incubated in normal growth or GD medium for 18 h in the presence or absence of 2 mM sodium salicylate or aspirin. The cells and medium were prepared and analyzed by SDS-PAGE and Western blotting with antibodies to CuZnSOD, MnSOD, catalase and total ERK2 (upper panel). Activity assay for CuZnSOD, MnSOD and catalase was carried out (lower panel).

were stained with MDC (data not shown). Autophagy is a self-degradative process involved in the basal turnover of cellular components and in response to nutrient starvation or organelle damage in a wide range of eukaryotes and is a form of programmed cell death that is controlled by autophagy-related Atg proteins including beclin 1 and other regulatory proteins such as DRAM (damage-regulated autophagy modulator) and Ambra 1 (activating molecule in Beclin 1-regulated autophagy) (25,28,45,46). During autophagy, portions of the cytoplasm are sequestered by double-membraned vesicles called autophagosomes, and are degraded after fusion with lysosomes for subsequent recycling. In vertebrates, this process acts as a pro-survival or pro-death mechanism in different physiological and pathological conditions, such as cancer. ER stress is known to induce autophagy through inducing Atg8 (47,48). Depending on the context, autophagy counterbalances ER stress-induced ER expansion, enhances cell survival or commits the cell to non-apoptotic death (49). Thus, sodium salicylate and aspirin may prevent necrosis and switch the cell death mode to autophagy, by not affecting the autophagic program activating ER stress under GD.

Sodium salicylate prevents GD-induced ROS production and CuZnSOD release. Previously, we demonstrated that GD-induced necrosis occurs through the production of ROS in A549 lung carcinoma cells (41,50) and that CuZnSOD is released into the extracellular space in an active form upon GD, thereby accelerating GD-induced necrotic cell death. We further investigated whether the effects of sodium salicylate and aspirin are linked to the regulation of GD-induced ROS production (44). As shown in Fig. 3, the pretreatment of sodium salicylate and aspirin prevented the production of intracellular H_2O_2 and O_2^- as well as the mitochondrial ROS in response to GD. We showed that the pre-treatment of sodium salicylate and aspirin significantly suppressed GD-induced CuZnSOD release into extracellular space without affecting the protein levels and activities of MnSOD and catalase (Fig. 3B). Thus, the sodium salicylate/aspirin-induced cell death mode switch and inhibition of HMGB1 release are thought to be mediated, in part, by their ability to prevent GD-induced CuZnSOD release. In addition, the antioxidant activity of sodium salicylate/aspirin may contribute to their abilities to prevent necrosis and to switch the cell death mode to autophagy.

The necrosis to autophagy switch by sodium salicylate and aspirin is important in cancer biology. Adult cancers are frequently preceded by a long period of inflammation and necrotic cell death. Tumour cells are prone to die by necrosis when cells are metabolically stressed by hypoxia and GD due to insufficient vascularization that is found in hyperplastic neoplasia and carcinoma *in situ* and, necrosis is commonly found in the core region of solid tumours (33). In clinical studies, the presence of necrosis is almost always deemed a poor prognostic finding and can adversely impact on certain forms of treatment. Thus, metabolic stress-induced necrosis is apparently important for tumour progression. Necrotic cell death is thought to promote tumour growth and angiogenesis either by increasing the probability of proto-oncogenic mutation or by the action of HMGB1 (31,32). In contrast, an autophagic and apoptotic program may suppress tumour progression. Based on our results, we suggest that sodium salicylate may exert tumour suppressive activities by inducing

a necrosis-to-apoptosis switch and preventing the release of the tumour-promoting cytokine HMGB1. HMGB1 has been demonstrated to possess tumour-promoting and angiogenic activities in addition to acting as a pro-inflammatory mediator. The increased expression of HMGB1, as well as its receptor RAGE (receptor for advanced glycation end-products), has been observed in a number of tumours including hepatomas and prostate cancer, which correlates with invasiveness and poor outcome when RAGE is expressed in conjunction with its ligand (37-40). In this study, we used 1-2 mM sodium salicylate and aspirin. The 0.5-2 mM aspirin concentrations approximate to systemic pharmacological concentrations. Although 0.5 mM aspirin is equivalent to a low therapeutic plasma concentration, 2 mM aspirin corresponds to a high therapeutic plasma concentration. Such a concentration (2 mM) is too high to be achieved systemically in the intact organism, but can be locally achieved upon the administration of aspirin during anti-inflammatory therapy, since aspirin concentrations have been suggested to increase in the mildly acidic environments prevailing at inflammatory sites (51) and the tumour micro-environment that is usually acidic (33). Aspirin and other non-steroidal anti-inflammatory drugs such as ibuprofen and indomethacin recently failed to influence the endotoxin-induced active release of HMGB1 in macrophages even at superpharmacological concentrations (up to 10-25 μM) (52). However, we showed that sodium salicylate and aspirin prevented necrotic HMGB1 release. These facts indicate that aspirin and sodium salicylate may exert an inhibitory effect on necrosis-linked HMGB1 release, thereby preventing an inflammatory response. Collectively, our results demonstrate that the cell death mode switch mechanisms may provide a new strategy to control and treat tumour development.

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