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ORIGINAL ARTICLE

Salmonella induce autophagy in melanoma by the downregulation of AKT/mTOR pathway

C-H Lee[1,2], S-T Lin[2], J-J Liu[1], W-W Chang[3,4], J-L Hsieh[5] and W-K Wang[6]

Salmonella have been demonstrated to inhibit tumor growth. However, the mechanism of *Salmonella*-induced tumor cell death is less defined. Autophagy is a cellular process that mediates the degradation of long-lived proteins and unwanted organelles in the cytosol. Tumor cells frequently display lower levels of basal autophagic activity than their normal counterparts and fail to increase autophagic activity in response to stresses. Autophagy is involved in the cell defense elimination of bacteria. The signaling pathways leading to activation of *Salmonella*-induced autophagy in tumor cells remain to be elucidated. We used autophagy inhibitor (3-Methyladenine) and apoptosis inhibitor (Z-VAD-FMK) to demonstrate that *Salmonella* may induce cell death via apoptosis and autophagic pathway. Meanwhile, we suggested that *Salmonella* induce autophagy in a dose- and time-dependent manner. The autophagic markers were increased after tumor cell infected with *Salmonella*. In addition, the protein express levels of phosph-protein kinase B (P-AKT), phosph-mammalian targets of rapamycin (P-mTOR), phosph-p70 ribosomal s6 kinase (P-p70s6K) in tumor cells were decreased by western analysis after *Salmonella* infection. In conclusion, our results point out that *Salmonella* induce the autophagic signaling pathway via downregulation of AKT/mTOR pathway. Herein, our findings that *Salmonella* in controlling tumor growth may induce autophagic signal pathway.

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INTRODUCTION

The use of preferentially replicating bacteria as oncolytic agents is one of the innovative approaches for the treatment of tumor. This is based on the observation that some obligate or facultative anaerobic bacteria are capable of multiplying selectively in tumors and inhibiting their growth. *Salmonella* have been employed as an antitumor agent that is capable of preferentially amplifying within tumors and inhibiting their growth.[1] Previous studies demonstrated that the induction of tumor apoptosis was correlated with *Salmonella* accumulation n the tumor sites.[1,2] Bacteria in tumors induced apoptosis by multiple mechanisms including competition for nutrients, stimulation of immune response.[3] Moreover, toxin from bacteria may induce the apoptosis of tumor. As bacterial replication in tumors and subsequent lysis of tumor cells may induce cell-mediated immune responses to tumor cells, higher oncolysis could account, in part, for an increased infiltrate of immune cells in tumors. The cells undergoing bacteria-induced cell death exhibit heterogeneous morphological features.[4,5] It is clear that more than one mechanism is involved in the bacteria-induced killing of cells.[6]

Autophagy is a cellular process that mediates the degradation of long-lived proteins and unwanted organelles in the cytosol. Autophagy pathway interacts with intracellular bacteria in a variety of ways.[7] Autophagy is involved in the cell defense elimination of bacteria. The signaling pathways leading to the activation of bacteria-induced autophagy in tumor cells remain to be elucidated. Autophagy is regulated by a multitude of factors, including nutritional status, hormones and intracellular signaling pathways.[8] Malignant cells frequently display lower levels of basal autophagic activity than their normal counterparts and fail to increase autophagic activity in response to stresses.[9] To date, a possible interaction of *Salmonella* with tumor cells has not been examined. Herein, we propose a role for *Salmonella* in controlling tumor growth by inducing apoptosis and autophagy.

RESULTS

Tumor-targeting potential of *Salmonella* in tumor-bearing mice and inhibition of subcutaneous tumor growth at distance by *Salmonella*

We monitored the kinetics of bacterial distribution in murine melanoma K1735 (Figure 1a) and B16F10 (Figure 1b) mice models after injection with 2×10^6 colony-forming units (c.f.u.) of *Salmonella*. The bacterial amount was much higher in tumors than that in livers and spleens in both tumor models of mice at each examined time. They were approximately three to four orders of magnitude higher than those found in livers or spleens. Antitumor effects of *Salmonella* were evaluated in terms of tumor growth of the mice bearing K1735 or B16F10 tumors. As shown in Figures 1c and d, tumor growth was significantly retarded in mice treated with *Salmonella* in comparison with that in PBS-treated control mice. Supplementary Figures S1a and b demonstrated that the survival of the mice injected with *Salmonella* was significantly prolonged compared with that of the mice injected with PBS.

[1]Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan; [2]Graduate Institute of Basic Medical Science, School of Medicine, China Medical University, Taichung, Taiwan; [3]Department of Biomedical Sciences, College of Medical Science and Technology, Chung Shan Medical University, Taichung, Taiwan; [4]Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan; [5]Department of Nursing, Chung Hwa University of Medical Technology, Tainan, Taiwan and [6]Department of Environmental Engineering and Science, Feng Chia University, Taichung, Taiwan. Correspondence: Dr C-H Lee, Department of Microbiology, School of Medicine, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan.
E-mail: chlee@mail.cmu.edu.tw
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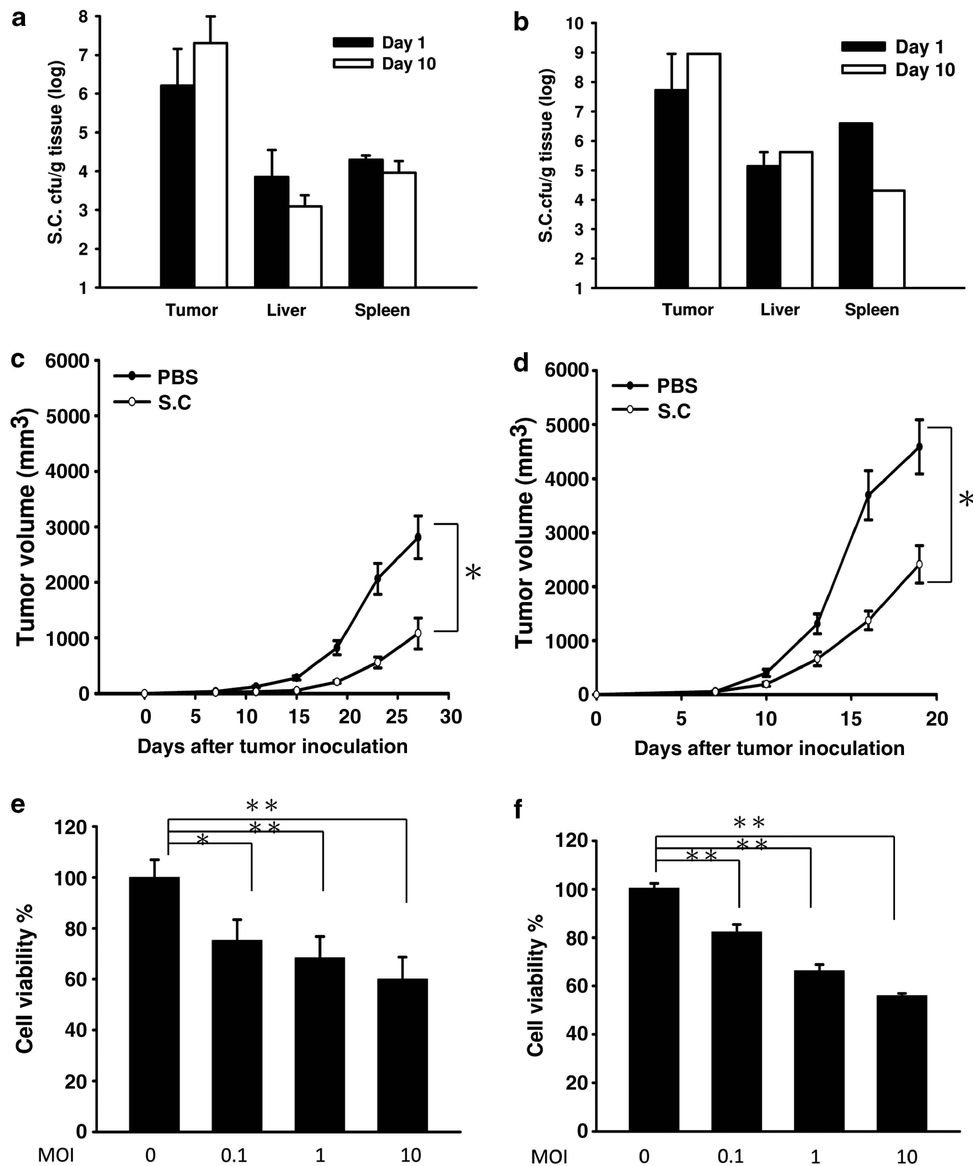

Figure 1. Antitumor effects of *Salmonella* (S.C.) on tumor growth *in vivo* and *in vitro*. The spatial and temporal distribution of S.C. in tumor-bearing mice. The mice bearing (**a**) K1735 or (**b**) B16F10 tumors ranging from 50 to100 mm^3 were injected intravenously (i.v.) with S.C. (2×10^6 c.f.u.) and the amounts of S.C. in the tumor, livers and spleens were determined on 1 and 10 day (mean ± s.d., $n = 3$–4) post infection. Groups of 7 mice that had been inoculated subcutaneously (s.c.) with (**c**) K1735 cells (10^6) or (**d**) B16F10 (10^6) on day 0 were treated i.v. with S.C. (2×10^6 c.f.u.) or PBS on day 7. Tumor volumes among different treatment groups were compared on day 19. The effects of S.C. on cell viability *in vitro*. The (**e**) K1735 (10^6) and (**f**) B16F10 (10^6) tumor cells were infected with various doses of S.C. for 90 min. Cell were harvested and stained with trypan blue. *$P < 0.05$; **$P < 0.01$. Data are expressed as mean ± s.d. of hexaplicate determinations.

As indicated in Supplementary Figures S1c and d, mice treated with S.C. had a 9% lower average body weight compared with mice treated with PBS. The body weight of mice recovered after 1 week. Two tumor models had similar results. To examine the effect of *Salmonella* on cell death in melanoma cells, cells were incubated with different multiplicities of infection of *Salmonella* and then analyzed by cell viability assay. We also observed that *Salmonella* induced melanoma cell death in a dose-dependent manner in K1735 (Figure 1e) and B16F10 (Figure 1f) cells *in vitro*. Taken together, these results indicate that systemic delivery of *Salmonella* can target tumor sites and delay tumor growth.

Salmonella induced apoptosis-related and -independent cell death

The induction of tumor death was correlated with *Salmonella* accumulation in the tumor sites. When the amount of *Salmonella* accumulated in tumor sites, *Salmonella* significantly induced tumor cell death. We next investigated whether autophagy has a role in *Salmonella*-induced cell death in melanoma cells. Either the pan-caspase inhibitor Z-VAD-FMK or 3-MA, an autophagy inhibitor, could partially protect the cells from *Salmonella*-induced death, as assessed by cell viability assay. Determination of cell viability by trypan blue exclusion assay shows that the treatment of melanoma cells with Z-VAD-FMK resulted in the inhibition of *Salmonella*-induced apoptosis (Figure 2a). Treatment of Z-VAD-FMK decreased the expression of cleaved-caspase 3 after *Salmonella* infection in two tumor models (Figures 2a and b). In marked contrast, treatment of 3-MA also decreased the cell death after *Salmonella* infection. 3-MA could partially protect melanoma cell from *Salmonella*-induced cell death (Figures 2b and c), suggesting that 3-MA protected cells from autophagy-related cell death. Immunoblot analysis revealed that treatment with

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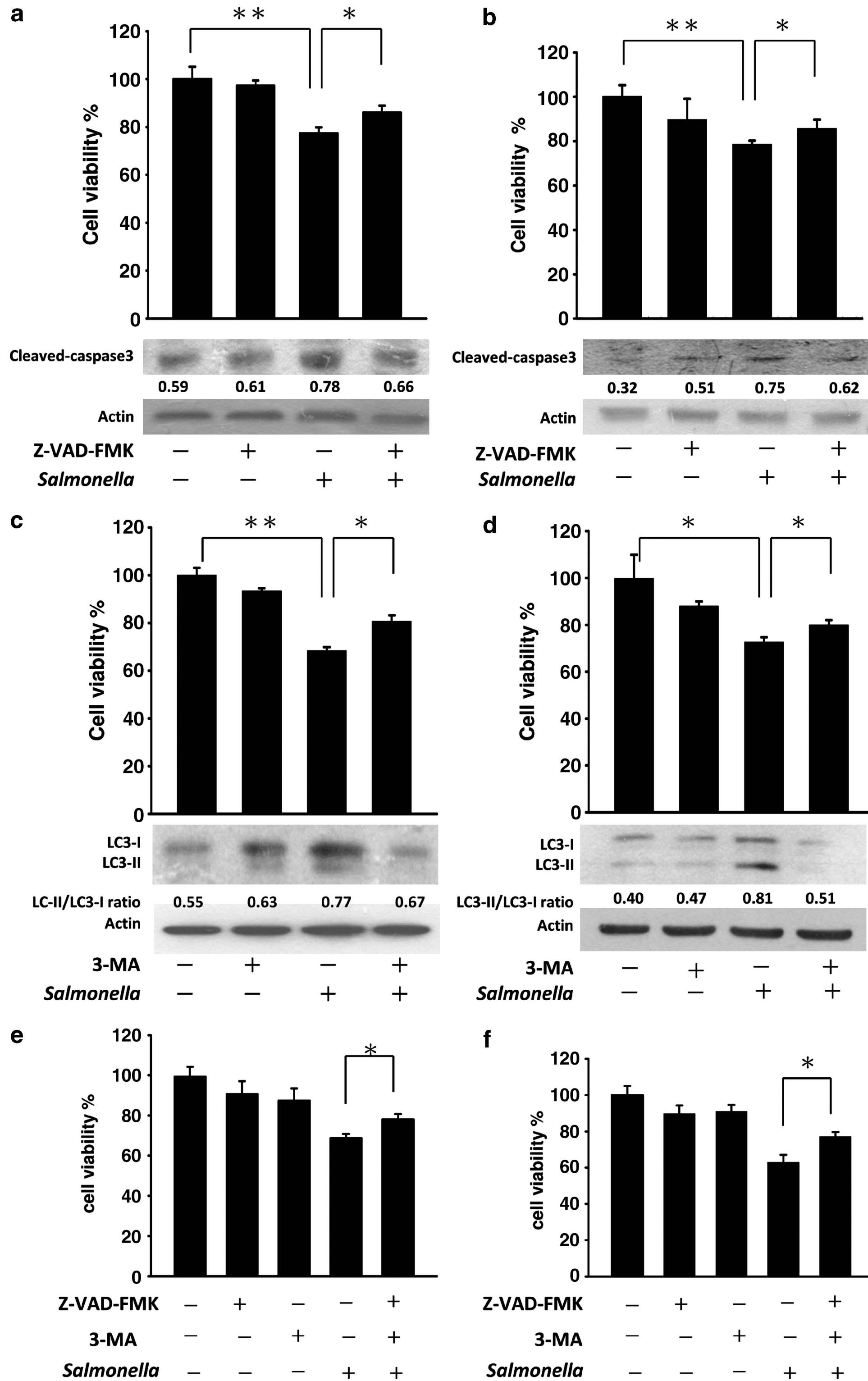

Figure 2. *Salmonella* induced apoptotic and nonapoptotic cell death. The K1735 (**a, c, e**) and B16F10 cells (**b, d, f**) were treated with Z-VAD-FMK (20 μM) or 3-MA (5 mM) for 4 h and then infected with *Salmonella* (multiplicity of infection = 1) for 90 min. Cell were harvested and stained with trypan blue. The expression of cleaved-caspase 3 levels in (**a**) K1735 and (**b**) B16F10 cells were determined by immunoblot analysis. The expression of LC3 levels in (**c**) K1735 and (**d**) B16F10 cells were determined by immunoblot analysis. Inserted values indicated relative proteins expression in comparison with β-actin. $^{*}P<0.05$; $^{**}P<0.01$. Data are expressed as mean ± s.d. of hexaplicate determinations.

Salmonella in melanoma cells enhanced the conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II (Figures 2b and c). Z-VAD-FMK and 3-MA partially protected cells from *Salmonella*-induced death (Figures 2e and f). The results point out that *Salmonella* induced apoptotic and autophagic cell death in melanoma cells.

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Salmonella induced autophagy in melanoma

As our results revealed that *Salmonella* induced nonapoptotic cell death as well as caspase-dependent apoptotic cell death in melanoma cells, we sought to examine whether *Salmonella* induced autophagic cell death in melanoma cells. During the autophagic process, LC3 is concentrated in autophagosome membranes, and the punctate fluorescence produced by green fluorescent protein (GFP)-fused LC3 (GFP-LC3) can be used as a good indicator of autophagy.[10] We transfected the GFP-LC3 expression plasmid into melanoma to observe autophagy. As shown in Figure 3a, control cells showed diffuse cytoplasmic distribution of green fluorescence, whereas punctate fluorescence of GFP-LC3 was significantly observed in *Salmonella*-treated cells. The percentage of GFP-LC3 punctuated dots in cells were significantly increased in the cells infected with *Salmonella* compared with PBS group (Figure 3b). We also used transmission electron microscopy to observe the ultrastructure of autophagy in *Salmonella*-treated cells. Whereas PBS-treated cells exhibited few autophagic features, numerous autophagic vacuoles were observed in melanoma cells treated with *Salmonella*. The presence of double-membrane containing cellular organelles was observed in *Salmonella*-treated cell at higher magnification (Figure 3c). Autophagy formation is associated with various signaling pathway. The above findings prompted us to further explore the detailed mechanism underlying the autophagic effects of *Salmonella* in melanoma. The AKT/mTOR/p70S6K signaling pathway negatively regulates autophagy.[11] We next examined the AKT/mTOR/p70S6K signaling pathway in *Salmonella*-induced autophagy. In a dose- (Figure 4) and time-dependent (Supplementary Figure S2) manner, treatment of *Salmonella* decreased the phosphorylation of AKT, mTOR and p70S6K, indicating downregulation of the AKT/mTOR/p70S6K pathway by *Salmonella* in K1735 cells (Figure 4a). Furthermore, very similar results were observed when *Salmonella* was treated with B16F10 cells (Figure 4b). Modulation of beclin 1 expression can affect the induction of autophagy.[9] Figure 4 also showed that treatment of *Salmonella* in melanoma cells dramatically increased the expression of beclin1 and enhanced of the conversion of LC3-1 to LC3-II, which is indicative of autophagic induction.[11] p62 binds directly to LC3 proteins via a specific sequence motif. The protein is itself degraded by autophagy.[12] As shown in Figure 4 and Supplementary Figure S2, treatment of *Salmonella* in melanoma cells dramatically decreased the expression of p62. These results suggested that *Salmonella* induced melanoma autophagy. Taken together, these results indicated that induction of autopagy by *Salmonella* in melanoma cells was associated with the downregulation of AKT/mTOR/p70S6K pathway.

Salmonella induced autophagy via the downregulation of AKT signaling pathway

We found that *Salmonella* induced autophagy by reducing AKT phosphorylation. The AKT/mTOR/p70S6K signaling pathway was

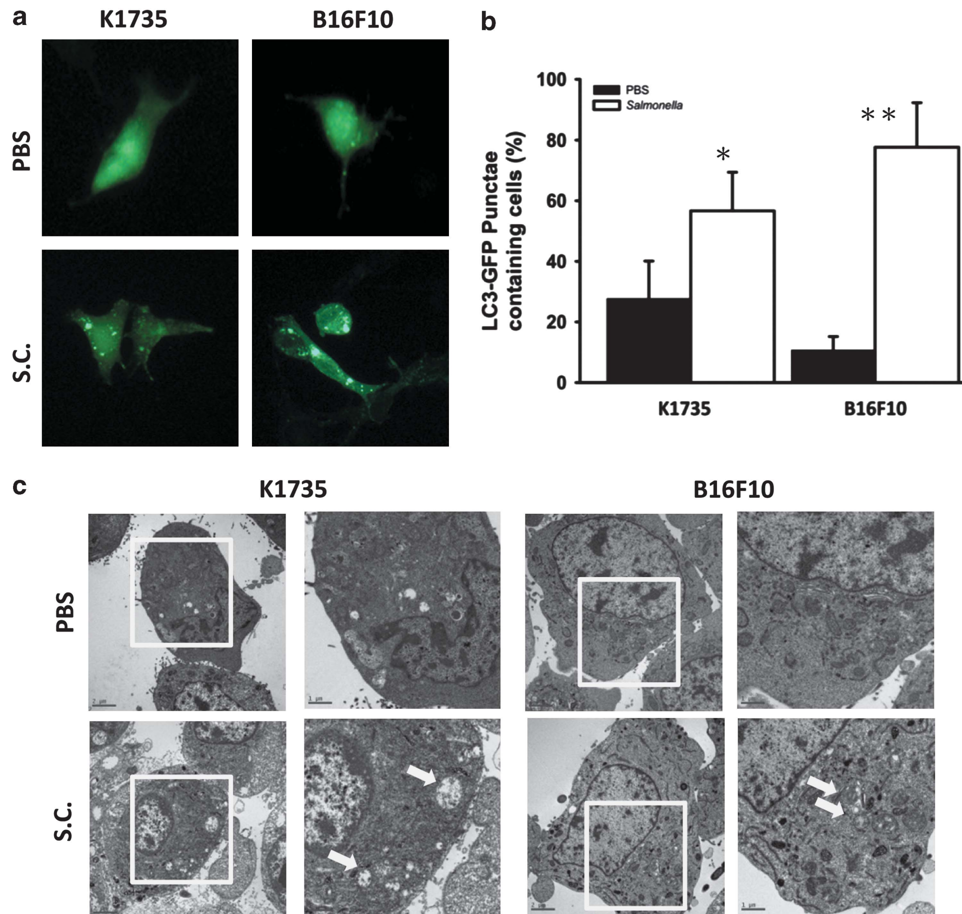

Figure 3. *Salmonella* induced autophagy in melanoma cells. (**a**) K1735 and B16F10 cells were transfected with the plasmid encoding GFP-LC3 followed by infection with *Salmonella* (S.C.) for 90 min. The GFP-LC3 punctate containing cells were visualized by fluorescence microscopy. (**b**) Quantitation of percentage of cells with autophagsomes. *$P<0.05$; **$P<0.01$. Data are expressed as mean ± s.d. of hexaplicate determinations. (**c**) Ultrastructural analysis of *Salmonella*-induced autophagy by transmission electron microscopy in melanoma cells (× 5000 magnification). The right panel showed the magnified image (× 10 000 magnification) of the area indicated by the box in the left panel. The arrow indicates an autophagosome. Scale bar, 1 μm.

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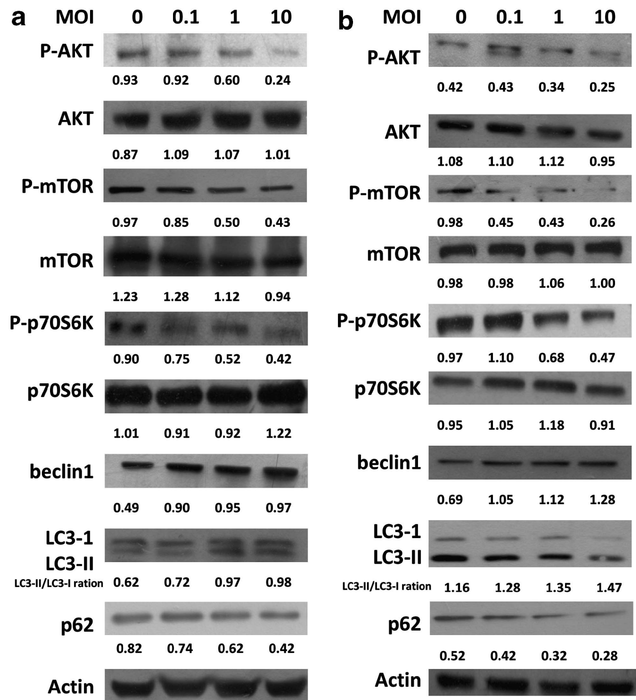

Figure 4. *Salmonella* induced autophagic signaling pathway. The (**a**) K1735 and (**b**) B16F10 cells were infected with various multiplicities of infection of *Salmonella* for 90 min. The expression of AKT/mTOR proteins and autophagic marker in cells were determined by immunoblot analysis. Inserted values indicated relative proteins expression in comparison with β-actin.

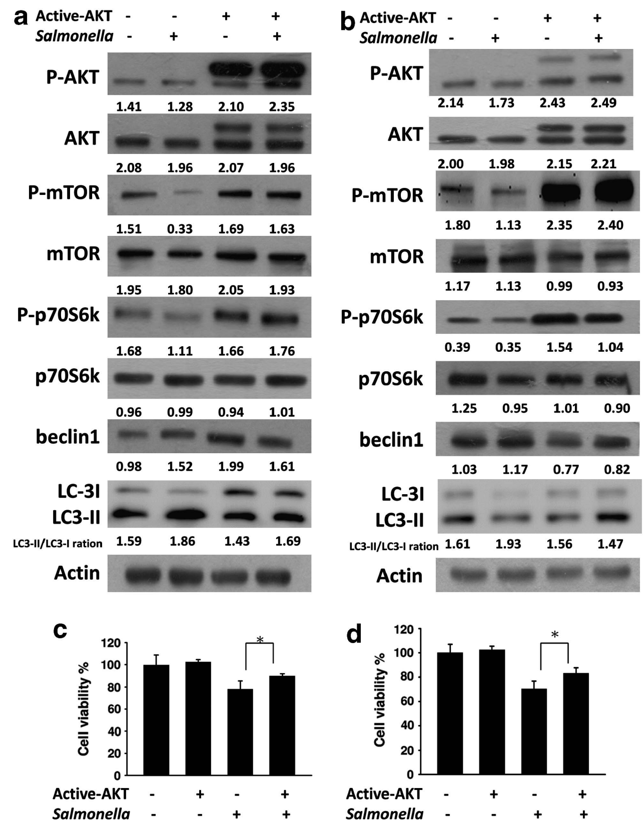

Figure 5. Effect of *Salmonella* on AKT phosphorylation and autophagic pathway. The (**a**) K1735 and (**b**) B16F10 cells transfected control or constitutively active AKT plasmids were treated with *Salmonella*. The expression of AKT/mTOR proteins and autophagic marker in cells were determined by immunoblot analysis. Inserted values indicate relative protein expression in comparison with β-actin. The K1735 (**c**) and B16F10 (**d**) cells transfected control or constitutively active AKT plasmids were treated with *Salmonella*. Cells were harvested and stained with trypan blue. $^{*}P < 0.05$. Data are expressed as mean ± s.d. of hexaplicate determinations.

reversed by transfecting constitutively active AKT plasmid. Suppressive effect of *Salmonella* on the AKT/mTOR/p70S6K signaling pathway was relieved by transfecting constitutively active AKT in K1735 (Figure 5a) and B16F10 (Figure 5b) cells. Transfection of constitutively active AKT plasmid reduced the expression of becline 1 and the conversion of LC3-I to LC3-II by *Salmonella* treatment in comparison with vector only control transfection. The *Salmonella*-induced cell death was also reduced after *Salmonella* treatment by transfecting constitutively active AKT plasmid (Figures 5c and d). Our results suggest that downregulation of AKT is required for *Salmonella*-induced autophagy in melanoma cells.

Salmonella induced apoptosis and autophagy *in vivo*

Although *Salmonella* was effective in inducing autophagy *in vitro*, autophagic marker was not observed *in vivo*. To investigate the apoptosis and autophagy *in vivo* after *Salmonella* treatment, mice bearing melanoma were injected with *Salmonella*, and the levels of apoptotic cells and autophagic marker in the tumors were determined by terminal dUTP nick-end labeling (TUNEL) and immunohistochemistry (Figure 6). TUNEL assay shows an increase in the amount of cells undergoing apoptosis in the *Salmonella*-treated tumors compared with PBS-treated tumors (Figure 6a). There was 3–10-fold increase in the number of apoptotic cells induced by *Salmonella* compared with that induced by PBS (Figure 6b). Meanwhile, the expression of becline 1and LC3 were significantly upregulated after *Salmonella* treatment compared with the groups treated with PBS (Figure 6c). Induction of autophagy was confirmed by western blotting for LC3 in B16F10 model (Figure 6d). LC3-II was increased by *Salmonella* treatment. The effects of Z-VAD-FMK and 3-MA were further confirmed in *Salmonella*-treated B16F10 tumor model. Tumor growth was not suppressed more significantly with combination treatment than with inhibitor treatment only (Figure 6d). Taken together, these results indicate that *Salmonella* therapy resulted in retarding tumor growth and increasing apoptosis and autophagy in the tumors.

DISCUSSION

Some anaerobic and facultative anaerobic bacteria represent novel therapeutic agents that have been recently applied in cancer therapy. Systemic administration of *Salmonella* in tumor-bearing mice leads to its preferential accumulation in tumor sites and thus retards tumor growth. *Salmonella* can effectively eradicate primary and metastatic tumors including bone, prostate, breast, pancreas and sarcoma.[13–15] Many studies suggest the clinical potential of bacterial treatment for critical metastatic tumor targets.[16–18] In this regard, we investigated the antitumor activity of *Salmonella* in the murine melanoma model. Autophagy is an evolutionarily conserved process. Autophagy is a mutifaceted process and alterations in autophagic signaling pathways are frequently found in tumor cells. The AKT/mTOR/p70S6K pathway is known to be a negative regulator of autophagy. We observed that the levels of phosphorylated AKT, mTOR and p70S6K were significantly decreased in *Salmonella*-infected melanoma cells compared with control groups. These results indicated that *Salmonella* can induce autophagic activities in addition to caspase-dependent cell death in melanoma cells *in vitro* and *in vivo*.

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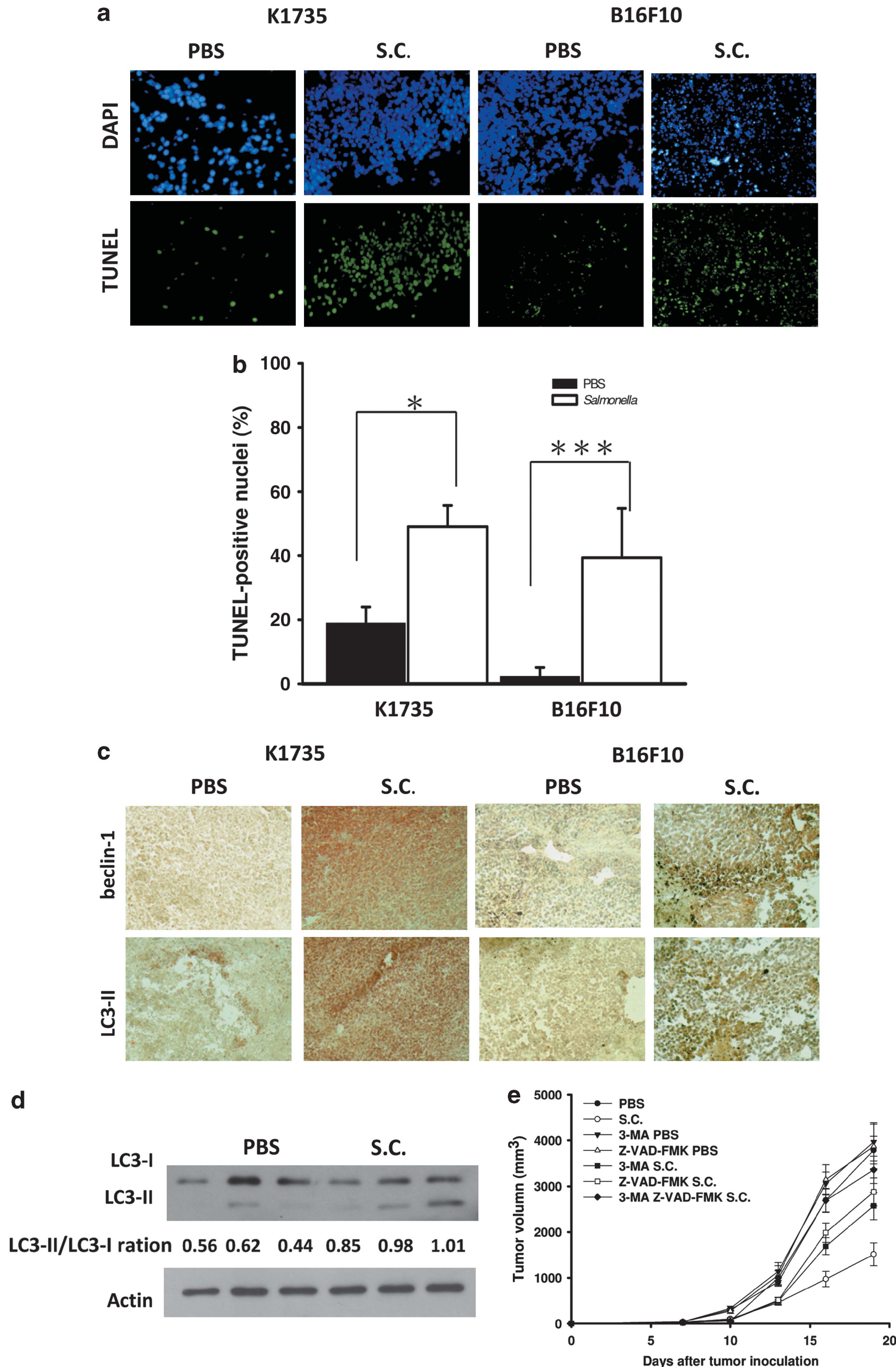

Figure 6. Increase in tumor cells undergoing apoptosis and autophagy in tumor-bearing mice treated with *Salmonella* (S.C.). Groups of four mice that had been inoculated s.c. with K1735 (10^6) or B16F10 cells (10^6) on day 0 were treated i.v. with *Salmonella* (2×10^6 c.f.u.) on day 7. Vehicle control mice received PBS. (**a**) Tumors were excised on day 16 and TUNEL assay was used to detect apoptotic cells ($\times$ 400). (**b**) TUNEL-positive cells were counted from three fields of highest density of positive-stained cells in each section to determine the percentage of apoptotic cells (mean $\pm$ s.e.m., $n = 4$). *$P < 0.05$; ***$P < 0.001$. (**c**) Tumors were excised on day 16 and immunohistochemistry was used to detect beclin-1 and LC3-II expression ($\times$ 200). (**d**) Tumors were excised on day 16 and the expression of LC3 in tumor cells was determined by immunoblot analysis *in vivo*. Inserted values indicated relative proteins expression in comparison with β-actin. (**e**) Groups of eight mice that had been inoculated s.c. with B16F10 cells (10^6) on day 0 were treated i.v. with *Salmonella* (2×10^6 c.f.u.) on day 7. Vehicle control mice received PBS. The mice were injected with 3-MA (24 mg kg^{-1}) and/or Z-VADFMK (10 mg kg^{-1}) i.p. every 3 days for a total of 5 times (days 3, 6, 9, 12 and 15). Tumor volumes among different treatment groups were compared on day 19 ($P < 0.01$ for S.C. versus PBS and S.C. versus Z-VADFMK 3-MA S.C.; $P < 0.05$ for 3-MA PBS versus 3-MA S.C. and Z-VAD-MFK PBS versus Z-VAD-FMK S.C.).

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Autophagy has a very important role in keeping cellular homeostasis. The removal of invading bacteria is crucial for bacterial infection. The ability of autophagy eliminates invasive bacteria or provides a niche for bacterial replication. Previous study demonstrated that autophagy inhibited *Salmonella* replication in cells.[19] Autophagy defends the mammalian cytosol against bacterial invasion. Recent studies discovered that the autophagy receptor CALCOCO2/NDP52, which detects cytosol-invading *Salmonella*, preferentially binds LC3.[20] Meantime, autophagy is activated following bacterial invasion of epithelial cells through a process requiring epithelial cell-intrinsic signaling via the innate immune adaptor protein. Thus, autophagy is an important epithelial cell-autonomous mechanism of antibacterial defense that protects against dissemination of *Salmonella*.[21] When the amount of *Salmonella* accumulates in tumor sites, tumor cells want to clean *Salmonella* and induce strong autophagic response, resulting in cell death. Autophagy is considered to have oppositive roles, promotion and suppression, in tumor cells.[22] Autophagy can provide energy for tumor cells under such stress conditions and thereby have a tumor-promoting role.[23] On the other hand, autophagy has a tumor-suppressing role. Beclin 1 has antitumor activity. Although beclin 1 has multiple functions, these observations suggest a tumor-suppressing role of autophagy. Herein, *Salmonella* dramatically increased the expression of beclin 1 in melanoma cells.

Salmonella-induced cell death of melanoma cells was associated with the induction and processing of the autophagy marker LC3 (Figure 2). *Salmonella*-induced LC3-II conversion was blocked by 3-MA. However, when autophagy was inhibited by 3-MA, *Salmonella* still induced tumor cell death through the activation of caspase 3. Although autophagy and apoptosis constitute distinct processes, their signaling pathways are interconnected through various mechanisms of crosstalk. Thus, either 3-MA or the caspase inhibitor Z-VAD-FMK alone influenced the effect of *Salmonella* in melanoma cells, and in combination they did not significantly rescue *Salmonella*-induced cell death. Autophagy may co-occur with apoptosis in tumor cells exposed to *Salmonella*. Furthermore, at later stages of infection, autophagy may partially participate in the execution of tumor cell death by enhancing apoptosis. When apoptosis is blocked infected tumor cells undergo increased autophagy. These data suggest that *Salmonella* treatment efficiently induces both autophagy and apoptosis, which partner to induce cell death cooperatively by modifying beclin-1 and caspase expression. Apoptosis and autophagy exist crosstalk between two pathways.[23] In this study we found that *Salmonella* induced both apoptosis and autophagy. Both apoptosis and autophagy cooperate to lead to tumor cell death after *Salmonella* infection. In fact, the simultaneous activation of both pathways has been found in preclinical and clinical studies. The treatment of adenovirus carrying XIAP-associated factor 1 induced apoptosis and autophagy in gastric cancer cells.[24] Imatinib activated apoptosis and autophagy pathway in Kaposi's sarcoma.[25] The role of *Salmonella* in apoptosis and autophagy may be important for oncolytic *Salmonella*.

Previously, we showed that *Salmonella* significantly upregulated IFN-γ, which may be responsible for recruiting peripheral immune cells to the tumor in wild-type mice, but not in T-cell-deficient mice. We suggested the T cell is involved in the regulation of *Salmonella*-induced host antitumor immunity in tumor-bearing mice. Thus, our studies may provide a cellular basis for understanding the recruitment of effector immune cells and the synergism between the oncolytic effect of *Salmonella* and adaptive antitumor immune mechanisms.[26]

This study may not only evaluate therapeutic efficacy of *Salmonella* for the treatment of cancer but also elucidate the mechanisms underlying antitumor activities mediated by *Salmonella*, which involve cellular mechanisms.

MATERIALS AND METHODS

Bacteria, cell lines, reagents, plasmid and mice

The *Salmonella enterica* serovar *choleraesuis* (*S.* Choleraesuis; S.C.) (ATCC 15480) vaccine strain was obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan). This rough variant of S.C., which is designated vaccine 51, was obtained by spreading an 18-hour broth culture of the virulent strain 188 of the *S.* Choleraesuis serovar Dublin over the surface of a dried nutrient agar plate, adding a drop of a suspension of salmonella anti-o phage No. 1 and selecting for a phage-resistant colony after incubation at 37 °C for 24 h.[27,28] Murine K1735,[29] B16F10[30,31] melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 μg ml^{-1} gentamicin, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum at 37 °C in 5% CO_2. Murine k1735 cells were kindly provided by Dr MC Hung (The University of Texas MD Anderson Cancer Center). Caspase family inhibitor (Z-Val-Ala-DL-Asp-FMK; Z-VAD-FMK) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Autophagy inhibitor (3-methladenine; 3-MA) were purchased from Merk (Darmstadt, Germany). Constitutively active AKT plasmid was kindly provided by Dr Chiau-Yuang Tsai (Department of Molecular Immunology, Osaka University).[32] Six-to-eight-week-old female C3H/HeN and C57BL/6 mice were obtained from the National Laboratory Animal Center of Taiwan. The animals were maintained in a specialized pathogen-free animal care facility in isothermal conditions with regular photoperiods. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the China Medical University (permit number: 99-20-N).

Animal studies

Groups of mice were subcutaneously (s.c.) inoculated with 10^6 tumor cells. When the tumors had grown to diameters between 50 and 100 mm^3, the mice were intravenously (i.v.) injected with 2×10^6 c.f.u. of S.C. These groups of mice were killed at various time points post infection and the numbers of *Salmonella* in the tumors, livers and spleens were determined on LB agar plates; these data were expressed as c.f.u. per gram of tissue. In a separate experiment, palpable tumors were measured every 3 days in two perpendicular axes using a tissue caliper and the tumor volumes were calculated as follows: (length of tumor) $\times$ (width of tumor)$^2 \times 0.45$.

Infection of tumor cells with *Salmonella*. The body weight and survival of mice were monitored daily. To inhibit autophagy and/or apoptosis, the mice were injected with 3-MA (24 mg kg^{-1}) and/or Z-VAD-FMK (10 mg kg^{-1}) intraperitoneally (i.p.) every 3 days for a total of 5 times (days 3, 6, 9, 12 and 15). Groups of mice were s.c. inoculated with 10^6 tumor cells. When the tumors had grown to diameters between 50 and 100 mm^3, the mice were i.v. injected with 2×10^6 c.f.u. of S.C. at day 7. The palpable tumors were measured every 3 days in two perpendicular axes using a tissue caliper.

Cell viability assay

Cells were pretreated with various inhibitors for 4 h, then *Salmonella* (multiplicity of infection = 0.1, 1 10) was added to cells for 24 h. In a parallel experiment, the adherent cells were measured for cell survival. Cell survival was assessed using the trypan blue exclusion assay.

Immunoblot analysis

The protein content in each sample was determined by bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). Proteins were fractionated on SDS-PAGE, transferred onto Hybond-enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK) and probed with antibodies against LC3 (Novus Biologicals, Littleton, CO, USA), becline 1(Novus Biologicals), p62 (Novus Biologicals), the mammalian target of rapamycin (mTOR) (Cell Signaling, Danvers, MA, USA), phosphor-mTOR (Cell Signaling), AKT (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), phosphor-AKT (Santa Cruz Biotechnology, Inc.), p70 S6 kinase (p70S6K) (Cell Signaling), phosphor-p70S6K (Cell Signaling) or monoclonal antibodies against β-actin (AC-15, Sigma Aldrich). Horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson, West Grove, PA, USA) was used as the secondary antibody and protein–antibody complexes were visualized by enhanced chemiluminescence system (Amersham). The signals were quantified with ImageJ software (rsbweb.nih.gov/ij).[33]

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Transmission electron microscopy

The melanoma cells infected with *Salmonella* for 90 min were fixed for 10 min in 50% Karnovsky fixative. Cells were collected and centrifuged at 1500 *g* for 5 min. The pellet was washed and stored in 70% Karnovsky fixative at 4 °C until embedding and then analyzed by transmission electron microscopy. The sections were observed with a JEOL JEM-1400 electron microscope (Tokyo, Japan).

Analysis of intracellular autophagic vacuoles

The GFP-LC3 was used to detect autophagy as described previously.[10] The melanoma cells were transfected with 5 μg of the GFP-LC3 expression plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The tranfected cells were infected with *Salmonella* for 90 min and the fluorescence of GFP-LC3 was visualized by fluorescence microscopy. Cell number was counted to normalize the measurement and the percentage in cells was calculated.

Immunohistochemical staining

To analyze autophagic marker in the tumors, groups of mice that had been inoculated s.c. with 10^6 melanoma cells at day 0 were injected i.v. with 2×10^6 c.f.u. of *Salmonella* at day 10, and the control mice received PBS. The tumors were excised and snap-frozen on day 20. Cryostat sections (5 μm) were prepared, fixed and incubated with rabbit LC3 (Abgent, San Diego, CA, USA) or rabbit anti-beclin-1 (Novus Biologicals) antibodies. After sequential incubation with the appropriate peroxidase-labeled secondary antibody and aminoethyl carbazole as the substrate chromogen, the slides were counterstained with hematoxylin. TUNEL assay was used to detect cell apoptosis in the tumor area and was performed according to the manufacturer's protocol (Promega, Madison, WI, USA). TUNEL-positive cells were counted under the microscope. The apoptosis index was defined by the percentage of TUNEL-positive among the total cells of each sample.[34]

Statistical analysis

The unpaired, two-tailed Student's *t*-test was used to determine differences between groups for the comparison of control group. A survival analysis was performed using the Kaplan–Meier survival curve and log-rank test. A *P*-value <0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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