Imaging the Different Mechanisms of Prostate Cancer Cell-killing by Tumor-targeting *Salmonella typhimurium* A1-R

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Abstract. *Background/Aims: We previously developed a genetically-modified bacterial strain of Salmonella typhimurium, auxotrophic for leucine and arginine, which also expresses green fluorescent protein (GFP), termed S. typhimurium A1-R. S. typhimurium A1-R was found to be effective against metastatic human prostate, breast, pancreatic, cervical and ovarian cancer, as well as osteosarcoma, fibrosarcoma and glioma, in clinically relevant nude mouse models. Materials and Methods: To understand the tumor cell-killing mechanism of S. typhimurium A1-R-GFP, we studied the interaction of S. typhimurium A1-R-GFP with three different prostate cancer cell lines in vitro. S. typhimurium-GFP invasion, proliferation, and means of killing in three different human prostate cancer cell lines were visualized by confocal fluorescence microscopy with the Olympus FV1000. Results: We found that S. typhimurium A1-R-induced cancer-cell death had different mechanisms in different prostate cancer cell lines, occurring through apoptosis and necrosis in the PC-3 prostate cancer cell line, and by cell bursting in the LNCaP and DU-145 prostate cancer cell lines. The time required for S. typhimurium A1-R-GFP to kill the majority of cancer cells varied from line to line, ranging from 2 hours to 48 hours. Conclusion: Understanding the various mechanisms of cancer-cell killing by S. typhimurium A1-R will be important for its use as a general therapeutic for cancer.*

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Key Words: Salmonella typhimurium A1-R, tumor targeting, GFP, RFP, prostate cancer, cell line, PC-3, LNCaP, DU-145, apoptosis, necrosis, cell burst.

Cancer patients were treated by William B. Coley at the New York Cancer Hospital, the precursor of the Sloan-Kettering Memorial Cancer Center, with *Streptococcus pyogenes* (*S. pyogenes*) and, in the late 19th and early 20th centuries, often with good results. Coley subsequently used killed *S. pyogenes* along with *Serratia marcescens*, the combination of which became known as Coley's Toxins (1, 2). Hoption Cann *et al.* (1) compared the 10-year survival rates of patients treated with Coley's regimens and modern conventional therapy. The study found that patients receiving current conventional therapies did not fare better than patients receiving Coley's bacterial treatment.

Anaerobic bacteria, for example *Bifidobacterium* (3) and *Clostridium* (4, 5), which replicate only in necrotic areas of tumors, have been used for cancer treatment. Anaerobic bacteria cannot grow in viable tumor tissue, which limits their efficacy.

Salmonella typhimurium (*S. typhimurium*) is a facultative anaerobe, which has important advantages compared to obligate anaerobes, since it can grow in the oxic viable region of tumors as well as necrotic regions (6).

The *S. typhimurium* A1-R strain developed by our laboratory has high tumor colonization efficacy and antitumor efficacy. *S. typhimurium* A1-R is auxotrophic for Leu--Arg, which prevents it from mounting a continuous infection in normal tissues. *S. typhimurium* A1-R as monotherapy was able to inhibit or eradicate primary and metastatic tumors in nude mouse models of prostate (7, 8), breast (9-11), lung (12, 13), pancreatic (14-18), ovarian (19, 20) and cervical cancer (21), as well as sarcoma (22-25) and glioma (26, 27), all of which are highly aggressive tumor models.

Green fluorescent protein (GFP) has been used as a genetic reporter to label *S. typhimurium* A1 such that single bacteria *in vivo* can be visualized (7, 28). In the present study, different prostate cancer cell lines with red fluorescent

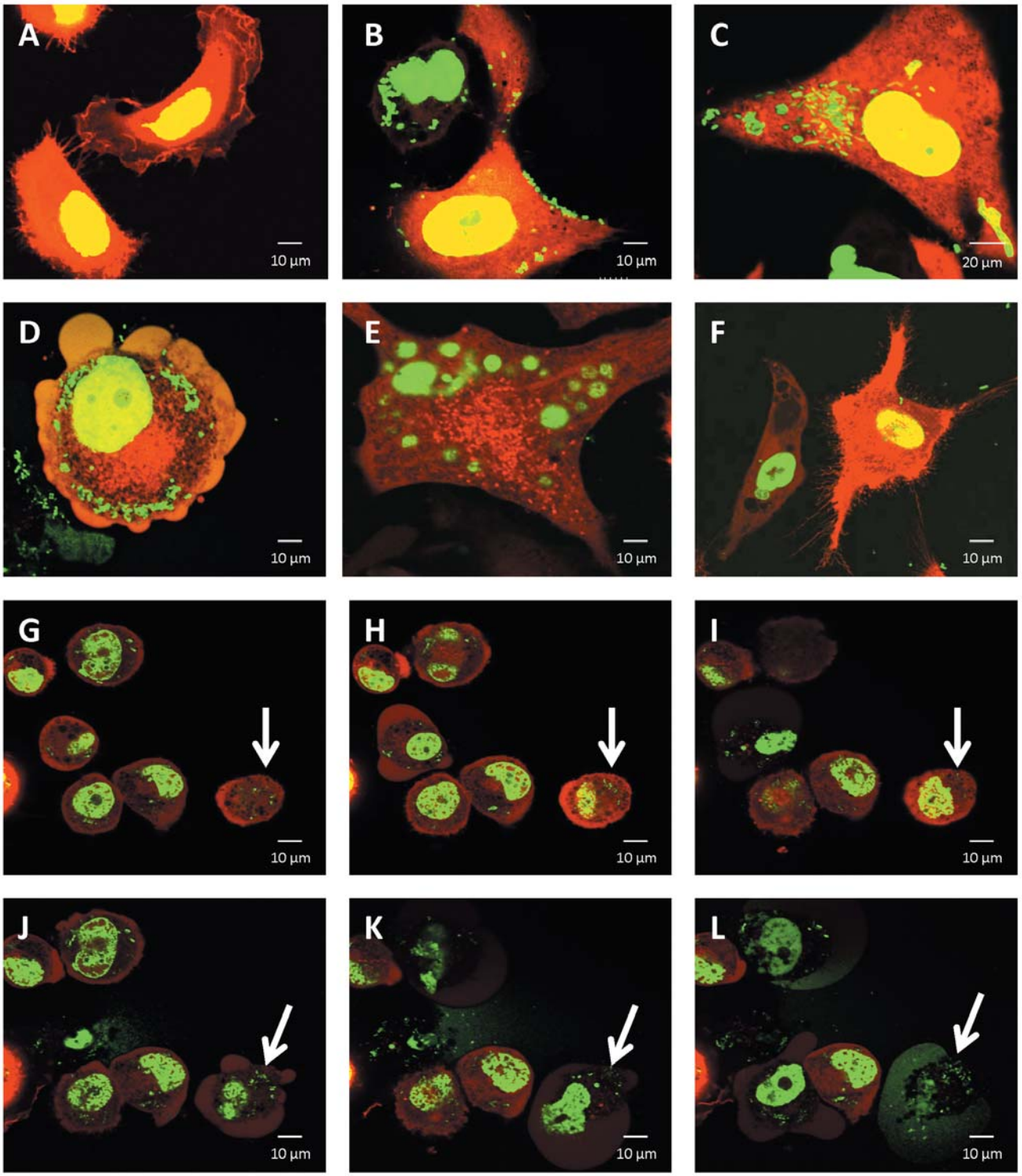

Figure 1. *Dual-color PC-3 human prostate cancer cell death induced by Salmonella typhimurium (S. typhimurium) A1-R-GFP. A: Untreated dual-color PC-3 human prostate cancer cells with green fluorescent protein (GFP) expressed in the nuclei and red fluorescent protein (RFP) expressed in the cytoplasm (0 time point); B: S. typhimurium A1-R-GFP attached to the membrane of PC-3 cells (45-minute time point); C: S. typhimurium A1-R-GFP invasion and proliferation in PC-3 cells (45-minute time point); D: cytoplasmic swelling in PC-3 cells induced by S. typhimurium A1-R-GFP (2-hour time point); E and F: Apoptotic bodies in PC-3 cells induced by S. typhimurium A1-R-GFP invasion (2-hour time time point); G to L: PC-3 necrotic cell death (arrows) induced by S. typhimurium A1-R-GFP invasion (G=45-minute time point; H=55-minute time point; I=65-minute time point; J=75-minute time point; K=85-minute time point; L=95-minute time point).*

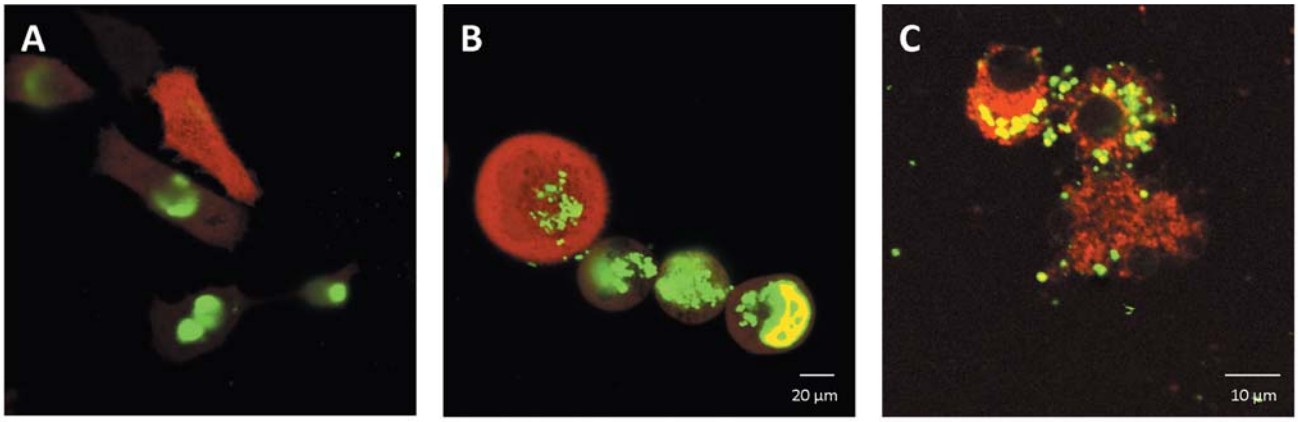

Figure 2. *Dual-color DU-145 human prostate cancer cell death induced by Salmonella typhimurium (S. typhimurium) A1-R-GFP. A: Untreated dual-color DU-145 human prostate cancer cells (0 time point); B: S. typhimurium A1-R-GFP invasion and proliferation in the DU-145 cells (2-hour time point); C: DU-145 cell death caused by bursting after extensive intracellular bacterial proliferation (24-hour time point).*

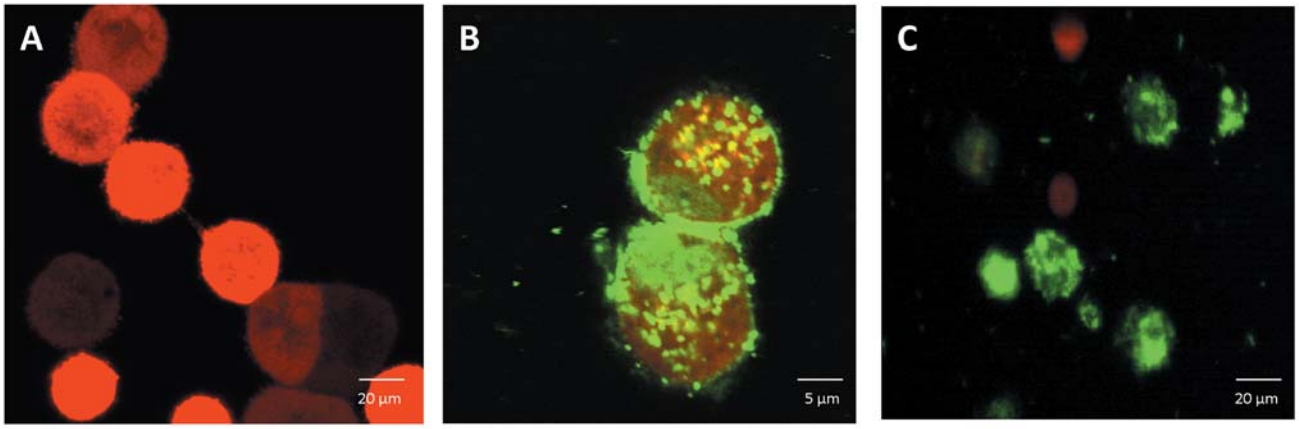

Figure 3. *LNCaP-RFP human prostate cancer cell death induced by Salmonella typhimurium (S. typhimurium) A1-R-GFP. A: Untreated LNCaP-RFP human prostate cancer cells (0 time point); B: S. typhimurium A1-R-GFP invasion and proliferation in the LNCaP-RFP cells (24-hour time point); C: LNCaP cell death caused by bursting after extensive intracellular bacterial proliferation (48-hour time point).*

protein (RFP) in the cytoplasm and GFP in the nucleus were used for simultaneous imaging of intracellularly infecting GFP-expressing bacteria, such that cell death behavior of the infected cancer cells could be observed by confocal microscopy.

Materials and Methods

RFP and GFP-histone H2B transduction of human prostate cancer cell lines. PC-3, DU-145 and LNCaP human prostate cancer cell lines were transfected with the pLNCX2 retroviral vector containing the DsRed2 express RFP gene. For establishing RFP and GFP dual-color cells, the PC-3-RFP and DU-145-RFP cell lines were initially established as above, then transfected with a GFP-histone H2B retroviral vector. The resulting cells express GFP in the nucleus and RFP in the cytoplasm (29-31).

Preparation of S. typhimurium A1-R-GFP. The A1-R bacteria (AntiCancer Inc., San Diego, CA, USA) were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS, and then diluted in PBS. Bacteria were then injected into the tail vein of nude mice [5 $\times 10^7$ colony-forming units (cfu) per 100 μl PBS].

Interaction of S. typhimurium A1-R-GFP with prostate cancer cells in vitro. Prostate cancer cells were grown in 24-well tissue culture plates in RPMI-1640 medium to a density of approximately 10^4 cells/well. *S. typhimurium* A1-R-GFP was grown to late log in LB broth, diluted in cell culture medium, and added to the cancer cells (1×10^6/ml) and then placed in an incubator at 37˚C. After 40 minutes, the cells were rinsed and cultured in medium containing gentamycin sulfate (20 μg/ml) to kill external but not internal bacteria. The interaction of *S. typhimurium* A1-R-GFP with cancer cells *in vitro* was observed by confocal fluorescence microscopy (Fluoview FV1000; Olympus, Tokyo, Japan) (32).

Cancer cell killing by S. typhimurium A1-R-GFP in vitro. Human prostate cancer cells were grown in 24-well tissue culture plates to a density of 10^4 cells/well. *S. typhimurium* A1-R-GFP (1×10^6 cfu/well) was added to the cancer cells and placed in an incubator at 37˚C. Cells were cultured in medium containing gentamicin sulfate (20 μg/ml) to kill external but not internal bacteria (7).

Results and Discussion

Dual-color PC-3 human prostate cancer apoptotic and necrotic cell death induced by S. typhimurium A1-R GFP. Untreated dual-color PC-3 human prostate cancer cells expressed GFP in the nucleus and RFP in the cytoplasm (Figure 1A). *S. typhimurium* A1-R-GFP attached to the membrane of PC-3 dual-color cells invaded and proliferated (Figure 1B,C). Cytoplasmic swelling was observed in PC-3 dual-color cells induced by *S. typhimurium* A1-R-GFP invasion. Apoptotic bodies were observed after *S. typhimurium* A1-R-GFP invasion (Figure 1D-F). Cell death was also visualized whereby the cell membrane of the PC-3 cells was disrupted after *S. typhimurium* A1-R-GFP invasion, indicating necrosis (Figure 1J-L). Cell death of PC-3 cells was extensive by two hours.

Dual-color DU-145 human prostate cancer cell death by bursting induced by S. typhimurium A1-R-GFP. Untreated dual-color DU-145 human prostate cancer cells expressed GFP in the nucleus and RFP in the cytoplasm. *S. typhimurium* A1-R-GFP invaded and proliferated extensively in the DU-145 dual-color cells. DU-145 dual-color cell death was caused by bursting after extensive intracellular bacterial proliferation (Figure 2). DU-145 cell death began by two hours and continued through 24 hours.

LNCaP-RFP human prostate cancer cell death by bursting induced by S. typhimurium A1-R. Untreated LNCaP-RFP human prostate cancer cells brightly expressed RFP. *S. typhimurium*-A1-R-GFP invaded and extensively proliferated in the LNCaP cells. LNCaP cell death was caused by bursting after extensive intracellular bacterial proliferation (Figure 3). LNCaP cell death began by 24 hours and continued through 48 hours.

The PC-3 cells appeared to die faster (cell death beginning at 45 minutes continuing through 2 hours) than DU-145 (cell death beginning at 2 hours continuing through 24 hours) and LNCaP cells (cell death from 24 hours continuing through 48 hours). Thus, apoptotic and necrotic cell death may be a more efficient mechanism of cell death due to *S. typhimurium* A1-R-GFP than death induced by cell bursting seen in DU-145 and LNCaP after *S. typhimurium* A1-R-GFP infection. PC-3 is very sensitive to *S. typhimurium* A1-R-GFP *in vivo*. Future experiments will determine the sensitivity of DU-145 and LNCaP *in vivo*. Such experiments will determine if *in vitro S. typhimurium* A1-R-induced cancer cell death predicts *in vivo* sensitivity. This information will be useful to apply *S. typhimurium* A1-R treatment to patients with cancer.

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Received June 26, 2015
Revised July 18, 2015
Accepted July 24, 2015