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Mitochondrial apoptosis is amplified through gap junctions

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ABSTRACT

The death of one cell can precipitate the death of nearby cells in a process referred to as the bystander effect. We investigated whether mitochondrial apoptosis generated a bystander effect and, if so, by which pathway. Microinjection with cytochrome *c* mimicked function of the mitochondrial apoptosis-induced channel MAC and caused apoptosis of both target and nearby osteoblasts. This effect was suppressed by inhibiting gap junction intercellular communication. A bystander effect was also observed after exogenous expression of tBid, which facilitates MAC formation and cytochrome *c* release. Interestingly, in connexin-43 deficient osteoblasts, microinjection of cytochrome *c* induced apoptosis only in the target cell. These findings indicate that a death signal was generated downstream of MAC function and was transmitted through gap junctions to amplify apoptosis in neighboring cells. This concept may have implications in development of new therapeutic approaches.

Introduction

Apoptosis is essential to the development of multicellular eukaryotic organisms and tissue homeostasis. Several internal and external signals can instigate apoptosis through different pathways, but they all end with cell death and rapid removal of apoptotic bodies. Extrinsic apoptosis begins when conditions in the extracellular environment, such as recognition of Fas ligand, induce the cell to commit suicide through events that lead to activation of the initiator caspase 8 [1]. In contrast, intrinsic apoptosis is a mitochondria-dependent pathway that begins in response to either extracellular cues or internal cellular damage. Various forms of cellular stress, such as DNA damage, can activate the intrinsic pathway, where formation of a channel called MAC (mitochondrial apoptosis-induced channel) is the commitment step of apoptosis. MAC opening leads to the crucial release of pro-apoptotic factors including cytochrome *c* from mitochondria [2,3] and this release is abolished by agents that block MAC [4]. There is some crosstalk between these pathways, as tBid, which is generated by caspase 8 cleavage, also catalyzes MAC formation and cytochrome *c* release from mitochondria [5–7]. Released cytochrome *c* triggers apoptosome formation, which then activates caspase 9, and ultimately caspase 3.

The bystander effect is an extension of the apoptotic cascades whereby cell death is propagated to nearby cells. Currently, the mechanisms underlying the bystander effect are poorly understood. The bystander effect seems to occur through either GJIC (gap junction intercellular communication) or extracellular pathways. The GJIC pathway refers to a bystander effect propagated by the diffusion of death signals through gap junctions. Alternatively, the extracellular mechanism involves the release of death signals from dying cells to the extracellular fluid. The phenomenon of bystander death has gained attention for it opens up the possibility of therapeutically limiting the wave of secondary injury during stroke or brain trauma [8,9], and of amplifying the potency of radiation and other cancer treatments [10]. For example, bystander effects are key to gene therapy whereby malignant cells are transfected with the herpes simplex virus-thymidine kinase gene, followed by treatment with the prodrug ganciclovir. Phosphorylation generates ganciclovir-triphosphate, which is a cytotoxin that diffuses through gap junctions and induces apoptosis in neighboring cells [10]. Another form of bystander killing involving gap junctions includes transfer of small HIV viral peptides that cause cytotoxic T-cell recognition of adjacent non-infected cells [11]. Alternatively, the extracellular pathway mediates bystander death in response to radiation [12], as well as in breast cancer cells that exogenously express the death domain of the nuclear receptor interacting factor-3 transcriptional coregulator [13]. In those cases,

Abbreviations: βGA, 18-β glycyrrhetinic acid; Bid, BH3 interacting domain death agonist; GJIC, gap junction intercellular communication; MAC, mitochondrial apoptosis-induced channel; tBid, truncated Bid

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bystander killing is presumably through release of DNA fragments or reactive oxygen species. However, in many cases, the death signal has not yet been identified [14–16] and little is known about the bystander effects on other important cancer models, like osteosarcomas. A recent study used the enzyme/prodrug gene approach to induce bystander killing in mouse bone tumors, but the possible role of gap junctions remained unclear [17].

Here, the ability of mitochondrial apoptosis to induce a bystander effect was determined using two different means of mimicking MAC function in osteosarcoma-derived osteoblasts, i.e., cytochrome *c* microinjection and exogenous expression of tBid. Cytochrome *c* microinjection imitates MAC's functional release of pro-apoptotic proteins from mitochondria while exogenous expression of tBid is just upstream as this protein induces MAC formation [5–7]. Development of a bystander effect was assessed through observation of dead cells around the targeted cells usually with time-lapse video microscopy. A bystander effect occurred in osteoblasts with functional GJIC and was suppressed by GJIC inhibitors. Onset of apoptosis markers in target cells preceded that of bystanders and indicates most cells died by apoptosis. Calcium's role as a death signal in these events was explored. The results indicate mitochondrial apoptosis generates a death signal downstream of MAC formation that overcomes the enhanced pro-survival machinery of osteoblasts and propagates to neighboring cells through a GJIC pathway.

Materials and methods

Cell culture. MG63 osteoblasts (osteosarcoma-derived cell line, ATCC) and UMR106-01 osteoblasts (osteosarcoma-derived cell line) were grown in MEM (minimum essential media with Earl's salts) containing 10% FBS (fetal bovine serum), 1% L-glutamine, 1% penicillin, 1% streptomycin, and 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid). Cells were grown at 37 °C in 5% CO_2 on glass coverslips for 24 h prior to experiments.

Microscopy. Time-lapse images were captured every 5–10 min using a SPOT RT, Nikon DS-2MBW, or a Roper Coolsnap HQ2 monochrome camera on a NIKON Eclipse TE 300 or Nikon Eclipse TE2000-E microscope equipped with epifluorescence and Uniblitz shutters. Fluorescent images were merged with their respective differential interference contrast (DIC) or phase contrast images using NIS-element AR 3.0 imaging program. Images were analyzed for the occurrence of apoptosis in transfected or microinjected (target cells) and neighboring (bystander) cells. Cells were scored apoptotic by morphological changes such as rounding up, blebbing and nuclear condensation/fragmentation. The % dead bystander cells was calculated as $(a-b)/(c-b)*100$, where a is the number of dead cells, b is the number of target microinjected cells, and c is the number of cells in the cluster. Routinely, cells were stained with FITC-Annexin-V and Hoechst 33342 (Invitrogen) to detect the apoptotic markers of phosphatidylserine exposure and nuclear condensation/fragmentation, respectively.

Microinjections. Cultured cells were grown on 25 mm glass coverslips, which were mounted in open Rose chambers. Micropipettes were fabricated on a horizontal puller model P-87 (Sutter Instruments). MG63 cells were microinjected typically with cytochrome *c* (3 mg/mL inside the pipette) or calcium (0–80 mM) and monitored for 24–48 h with time-lapse video microscopy. Routinely, 1 mg/mL rhodamine B isothiocyanate–dextran (10 kDa, Sigma) was added to the cytochrome *c* mixture to confirm microinjection. Intercellular communication was assessed by microinjecting cells with 10 mM Lucifer yellow CH (457 Da, Invitrogen) and determining the spread of dye to adjacent cells ~10 min later. Intercellular communication and propagation of Lucifer yellow was blocked by incubating cells with 75 μM 18-β glycyrrhetinic acid (βGA) for 24 h prior to microinjection. The final concentration of calcium microinjected was estimated assuming 5% cell volume was microinjected and reported as 5% of concentration in the micropipette as previously described [18].

Plasmids and transfections. YFP control, YFP-Bid and YFP-tBid plasmids were generated as previously described [19]. For transfections, MG63 cells at ~20% confluence were permeabilized using a mixture of 50 μg polyethylenimine (Sigma) and 8 μg plasmid for 2 h [20]. After transfection, cells were monitored for 24 h by DIC and fluorescence time-lapse microscopy.

Results

Does mitochondrial apoptosis induce a bystander effect and, if so, which pathway is involved in its propagation? A bystander effect was observed after inducing mitochondrial apoptosis in target osteoblasts by microinjection of cytochrome *c* into single cells. These bystander effects were seen in normal cells and suppressed by inhibitors of GJIC or genetic GJIC incompetence. A second means of inducing MAC function via exogenous expression of tBid verified the notion that mitochondrial apoptosis can induce a bystander effect. These results indicate mitochondrial apoptosis generates a death signal downstream of MAC formation that can propagate to neighboring cells through an intercellular pathway reliant on GJIC to trigger apoptosis in neighboring cells.

The effect of GJIC on the bystander effect induced by mitochondrial apoptosis

Many cell types including cardiomyocytes, epithelial cells, and osteoblasts rely upon GJIC for tissue homoeostasis and normal cell function. As shown in Fig. 1, MG63 osteoblasts are GJIC competent and transferred Lucifer yellow between cells. Microinjection of cytochrome *c*, which mimics MAC function, induced cell death in target osteoblasts with a dose (3 mg/mL inside the pipette) similar to that reported by others to kill several cell types [18,21,22]. Importantly, this action also induced a bystander effect, since several nearby cells along with the target cell died (Fig. 1, top panels). Typically, no death was observed in target cells or bystanders upon microinjection with Lucifer yellow, rhodamine–dextran, or BSA (not shown). It could be hypothesized that a death signal is generated during apoptosis in a microinjected cell that crosses gap junctions to induce bystander death. If this hypothesis is correct, then blockade of gap junctions should modify the bystander effect. Fig. 1 shows cytochrome *c* microinjection killed target cells, but bystanders were only observed with GJIC competent cells. The bystander effect was suppressed after treatment with the GJIC blocker 18-β glycyrrhetinic (βGA). The action of βGA (75 μM) was confirmed as this agent prevented the spread of Lucifer yellow stain to neighboring cells after microinjection of a single cell (Fig. 1). The strength of the bystander effect was quantified as the % dead cells in each cluster excluding the microinjected cell. Microinjection of single MG63 cells in a cluster with cytochrome *c* induced apoptosis in 31 ± 6% of its neighbors ($n = 17$ clusters, 104 cells). However, only 9 ± 4% ($n = 14$ clusters, 61 cells) of the neighboring cells died when pretreated with βGA (Fig. 1B). The effect of closing GJIC via βGA was significant as the p value was < 0.001, which indicates that GJIC was requisite to this bystander effect.

The bystander effect induced by cytochrome *c* microinjection was also assessed in UMR cells to further explore the role of gap junctions. UMR and MG63 are similar as both are osteoblast-like cells derived from osteosarcomas but UMR cells have reduced GJIC and connexin-43 expression [23,24]. Communication was evaluated by the spread of Lucifer yellow from microinjected to neighboring cells (Fig. 1). Like βGA-treated MG63, only the microinjected UMR cells died; no bystander effect was observed

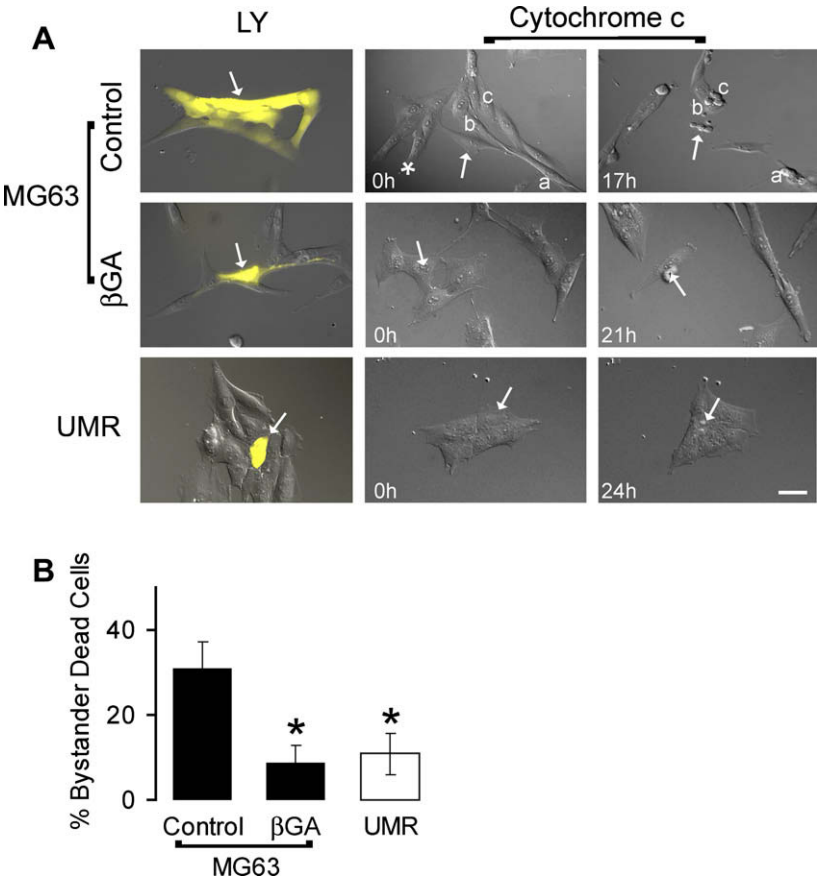

Fig. 1. Gap junction intercellular communication is needed for propagation of apoptosis to adjacent cells. (A) Images show MG63 and UMR cells after microinjection of single cells (arrow) with Lucifer yellow (LY) or cytochrome *c*. Left panels show merged DIC and fluorescence images taken 10 min after microinjection with Lucifer yellow (0.4 kDa) of single UMR or MG63 cells that were or were not treated with the gap junction channel blocker βGA (75 μM) for 24 h as indicated. Microinjection with cytochrome *c* (middle and right panels) induced apoptosis in all target cells. Dividing cells were present (asterisk) attesting to health of the culture. Bystanders (a–c) were observed in untreated MG63 cells (top right panel). A videoclip showing a bystander effect is included as supplemental data. Scale bars are 20 μm for all images. (B) Histogram shows % bystander dead cells. Experiments were repeated 5–17 times. Asterisk represents *p* value < 0.0001.

(Fig. 1). That is, 11 ± 5% of the neighboring cells died (n = 5 clusters, 69 cells) which is statistically different from the MG63 cells (p value < 0.001) and similar to βGA-treated MG63 cells (p value = 0.42). These findings support a key role for GJIC in this bystander effect.

Target and bystander cells died by apoptosis

Microinjection of cytochrome *c* induced onset of apoptosis markers in target and bystander MG63 cells as shown in selected frames of a time-lapse sequence in Fig. 2. Three single osteoblasts (arrows) were microinjected with a lethal dose of cytochrome *c* and the indicator rhodamine–dextran. Each microinjected cell entered apoptosis within an hour. Subsequently, several neighboring cells (a–d) also underwent apoptosis, as they shrank and their membranes began blebbing within 3 h. Annexin-V staining revealed that the dying cells exposed phosphatidylserine to the media, consistent with onset of apoptosis, regardless of whether they were microinjected or merely adjacent to those target cells. Hoechst staining revealed that target cells underwent nuclear condensation prior to adjacent cells (Fig. 2D). Importantly, cells that were not connected with the microinjected cluster (asterisks in Fig. 2) displayed no onset of apoptosis markers.

Bystander effects generated by mitochondrial apoptosis induced via tBid expression

A second means of activating MAC function was used to verify that mitochondrial apoptosis induces a bystander effect in osteoblasts. The BH3-only protein tBid is formed by cleavage of Bid by caspase 8, and induces apoptosis by facilitating formation of MAC [6]. Fig. 3 shows DIC and fluorescent images of MG63 cells 24 h post-transfection with plasmids encoding YFP-Bid or YFP-tBid. Target cells (arrow) were fluorescent because of YFP expression and died if YFP-tBid, but not YFP-Bid, was expressed. Furthermore, cells neighboring those expressing YFP-tBid that were not fluorescent also died (asterisk in Fig. 3). While expression of YFP-tBid routinely killed cells, YFP-Bid and YFP did not induce cell death of transfected or neighboring cells. Although an effect of a low level of expression of YFP-tBid on cells scored as bystanders could not be excluded, these results are consistent with those found with microinjection of cytochrome *c*. Hence, these findings reinforce the notion that mitochondrial apoptosis initiated in the target cells was propagated to bystanders.

On the role of Ca^{2+} in the bystander effect

What is the death signal that is propagated through gap junctions in osteoblasts? Several studies suggested calcium was a likely candidate [15,25–27], which precipitated an examination of the possible role of calcium in this bystander effect. Time-lapse video microscopy was used to monitor cell morphology after microinjection of various concentrations of calcium. Microinjection of calcium induced cell death with an LD_{50} of 1.6 mM (Fig. 4A). Surprisingly, transient millimolar levels of calcium must be below some threshold because these levels did not necessarily cause death. Unlike BSA but like cytochrome *c*, we found that about half

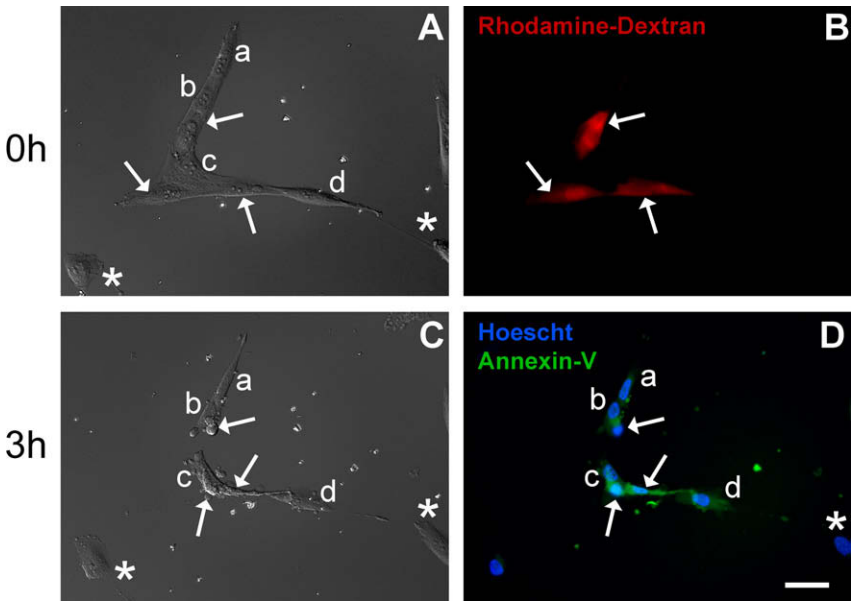

Fig. 2. Cytochrome *c* microinjection induces a bystander effect. DIC (A,C) and fluorescence (B,D) microscopy images show MG63 osteoblasts after microinjection of single cells (arrows) with cytochrome *c* (3 mg/mL) and rhodamine–dextran (10 kDa; 1 mg/mL) inside the pipette. (B) Red fluorescence image shows rhodamine–dextran was limited to microinjected cells. (C,D) Images taken after 3 h reveal all the cells in the cluster, including those not microinjected (a–d), underwent blebbing and shrinkage. (D) Merged blue and green fluorescence image shows those cells also stained positive with FITC-Annexin-V (green), a classical apoptosis marker for phosphatidylserine exposure. Cytochrome *c* injected cells showed more advanced apoptosis and displayed nuclear condensation (Hoechst staining, blue). Cells that were not connected with the microinjected cluster (asterisks) displayed no apoptosis markers. Scale bar is 20 μm for all images.

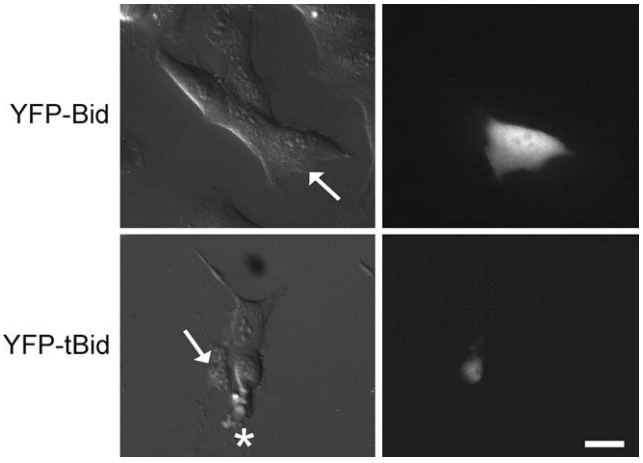

Fig. 3. Apoptosis induced by tBid causes a bystander effect. DIC (left) and fluorescence (right) microscopy images of MG63 cells 24 h after transfection with YFP-Bid and YFP-tBid. Transfected cells are fluorescent and indicated by arrows. Bystander dead cell is indicated with an asterisk. Scale bar is 20 μm for all images.

the cells in the cluster died if a single cell was microinjected with calcium to bring the cytosol to ~2.5 mM (Fig. 4B and C). It is interesting to note that the cytosol attains these high calcium levels during the death cascades upon loss of plasma membrane integrity. While these findings are consistent with a role for calcium, additional studies would be needed to prove that calcium is the same death signal propagated to bystanders after cytochrome *c* microinjection or tBid transfection.

Discussion

The Bcl-2 family of proteins is a crucial regulator of intrinsic apoptosis. tBid, a pro-apoptotic member of this family, activates another member, Bax and/or Bak, to form MAC. Once formed, MAC releases death factors like cytochrome *c* into the cytosol. In this work, cytochrome *c* microinjection and exogenous expression of tBid were used to mimic MAC function in a human osteosarcoma cell line (MG63). The occurrence of a bystander effect was assessed through observation of dead cells nearby target cells that were microinjected or transfected. To further understand the process of death signal propagation, the bystander effect was analyzed in presence of GJIC inhibitors and in cells in which GJIC had been modified.

Induction of mitochondrial apoptosis in single MG63 cells led to a wave of cell death within the same cluster, but not in unconnected clusters. While variations have been reported, two main pathways could account for propagation of this death wave. Target cells could generate death factors that are then secreted to the extracellular space (extracellular pathway) or be spread via the GJIC pathway through gap junctions to neighboring cells. The GJIC pathway is implicated in this bystander effect, since inhibition of GJIC suppressed both Lucifer yellow transmission and the bystander effect (Fig. 1). Furthermore, a similar osteoblast line (UMR) deficient in GJIC also failed to show a bystander effect. This condition was previously reported in some cancer models, like squamous cell carcinoma of the head and neck, and may account for their inability to propagate cell death [21]. These findings are in contrast to those of Cusato et al. who report that GJIC was essential for a bystander effect in coupled oocytes and developing retina [15,26]. Nevertheless, future studies may reveal some contribution of the extracellular pathway.

To confirm that apoptosis was being propagated from the target to the neighboring cells, the onset of apoptosis markers was monitored. Apoptosis in target cells typically preceded that of bystanders. However, with time, both groups displayed the same classical markers including rounding up, blebbing, phosphatidylserine exposure and nuclear condensation/fragmentation (Fig. 2). Recent studies using patch clamp and ELISA on isolated mitochondria showed that tBid triggers MAC assembly and cytochrome *c* release [6]. In this work, tBid transfection proved sufficient to induce a by-

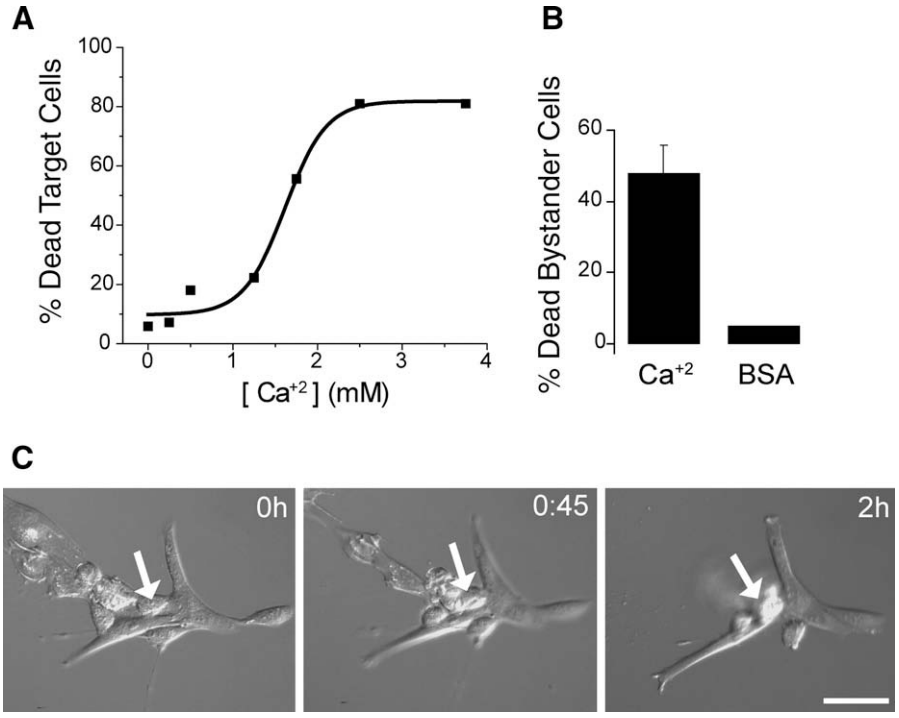

Fig. 4. Calcium microinjection induces a bystander effect. MG63 cells were monitored using time-lapse video microscopy after being microinjected to raise the cytosolic calcium concentration. Cells that underwent shrinkage, blebbing, and nuclear condensation were scored dead. (A) The plot shows the dose dependence of the % target cells dead within 24 h after microinjection to bring the cytosolic calcium concentration to the level indicated (see Materials and methods). There were 9-33 cells microinjected/point. The LD_{50} was 1.6 mM. (B) Histogram shows the % dead bystander cells in a cluster after microinjection with 10 mg/mL BSA or calcium (2.5 mM final concentration). (C) DIC images are shown of a cluster of cells at the indicated times after one cell (arrow) was microinjected to raise the cytosolic calcium level to ~2.5 mM. Scale bar is 50 μm for all images.

stander effect similar to cytochrome *c* microinjection (Fig. 3). Thus, considering that cytochrome *c* microinjection and tBid transfection mimic MAC function, these results suggest MAC may play a key role in generation of a bystander effect.

Induction of mitochondrial apoptosis through MAC formation generated a death factor in the cytosol of single MG63 cells and this factor was propagated through gap junctions to the neighboring cells. However, this death factor has not yet been identified. Cytochrome *c* (~12 kDa) is an unlikely candidate since gap junctions do not allow diffusion of molecules larger than ~1 kDa. Note that microinjected rhodamine–dextran (10 kDa) was never detected in neighboring cells (Fig. 2). Reactive oxygen species are also improbable candidates as blocking GJIC should not prevent propagation of these generally membrane-permeable factors to nearby cells. However, GJIC regulate the intercellular passage of several signaling molecules including ATP, cAMP, IP3, and calcium [14]. Furthermore, calcium transients were linked to cytochrome *c* release from mitochondria during staurosporine treatment, and calcium and IP3 were identified as likely death signals in GJIC-coupled oocytes after cytochrome *c* microinjection [15]. One could speculate that, upon MAC formation and cytochrome *c* release, calcium or some other signaling molecules may increase in the cytosol and propagate to neighboring cells. In fact, microinjection of calcium induces cell death with an LD_{50} of 1–2 mM (Fig. 4), which are levels typically attained after loss of plasma membrane integrity. Like cytochrome *c*, calcium microinjection caused a bystander effect, since 48% of adjacent cells in a cluster died. Hence, calcium might play a role in propagation of mitochondrial apoptosis among adjacent cells. However, these findings need to be extended before the role of calcium in mitochondrial bystander effects is understood.

The bystander effect is an emerging concept that has special implications for development of new therapeutic approaches. A major limitation of gene therapy for cancer is the inability to transduce all the cancer cells in vivo [28]. Bystander killing is thus critical for the eradication of tumors. Here, we showed that mimicking the function of the cytochrome *c* release channel MAC overcame the enhanced pro-survival machinery of two cancer cell lines and ultimately killed them. When GJIC was present, a bystander effect was observed that could be prevented by inhibition of GJIC. Our findings may help in understanding the regulatory mechanisms underlying apoptosis resistance in cancer cells and indicate that mitochondria from different cells can talk through gap junctions and synergistically coordinate cell death.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.09.054.

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