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Letter to the Editor

Long-distance apoptotic killing of cells is mediated by hydrogen peroxide in a mitochondrial ROS-dependent fashion

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Dear Editor,

Reactive oxygen species (ROS) can initiate programmed cell death under various (patho)physiological conditions. Apoptosis is often accompanied by the generation of large amounts of ROS, which are involved in transduction and amplification of intracellular signals.

It has been hypothesized[1] that apoptogenic activity of hydrogen peroxide is used by organisms to form a 'dead' zone around a virus-infected cell (as in a tobacco leaf infected by tobacco mosaic virus), thus limiting expansion of the infection. It was suggested that in a tissue the front of elevated concentration of H_2O_2 produced by an infected cell spreads much faster than the front of viral particles formed in the same cell. This hypothesis was supported by an observation on clustering of dead cells in monolayer culture during spontaneous apoptosis, which was prevented by catalase.[2] However, no direct evidence for H_2O_2-mediated bystander killing or measurements of H_2O_2 levels and identification of the source(s) of ROS have been presented (see also Ref.[3]).

In this study, we observed clustering of apoptotic cells in monolayers of HeLa cells treated with tumor necrosis factor α (TNF) in the presence of the protein synthesis inhibitor emetine. The cluster formation was statistically significant, and their average size (10–20 cells) was larger than possible clones formed during cell growth (not shown in figures).

For further studies of bystander killing, we suggest a new approach. A coverslip covered with apoptogen-treated cells (the inducer) is placed in a Petri dish side by side with another coverslip with nontreated cells (the recipient). When the inducer cells were treated for 3 h with TNF, the following incubation was shown to result in massive apoptosis not only on the inducer but also on the recipient coverslip. Apoptotic cells were identified by chromatin condensation (Figure 1a), which was accompanied by collapse of the mitochondrial membrane potential, BAX translocation from cytosol to mitochondria, cytochrome *c* translocation in the opposite direction, and a burst in ROS formation. It was found that such apoptosis is arrested in HeLa cell line overproducing Bcl-2 (not shown in figures). TNF-specific monoclonal antibodies did not affect recipient cell death, a fact excluding migration of TNF from the inducer to recipient coverslip (Figure 1b). This conclusion was strengthened by experiment with oligomycin, which inhibited TNF-induced apoptosis but did not affect apoptosis induced by H_2O_2 and staurosporin, as was shown by our group.[4] This inhibitor was found to arrest the TNF-induced apoptosis if added not later than in 1.5 h after TNF. If oligomycin was added when the TNF pretreated inducer coverslip was placed near the recipient coverslip (i.e. 3 h after TNF), no inhibition of apoptosis of the recipient cells was observed (Figure 1b).

Consistent with the suggested bystander killing mediated by a water-soluble messenger, it was shown that the percentage of apoptotic cells on the recipient coverslip did not depend on distance from the inducer coverslip (the maximal distance was 0.7 cm) but was strongly decreased when thickness of the layer of the medium above the cells was increased (not shown). Catalase did not affect apoptosis of inducer cells and strongly decreased the number of apoptotic cells on the recipient coverslip (Figure 1b), indicating that extracellular hydrogen peroxide was the signal molecule in the long-distance killing of bystander cells.

To test the possible mitochondrial origin of messenger hydrogen peroxide, we used 10-(6′-ubiquinolyl)decyltriphenylphosphonium (MitoQ), an antioxidant electrophoretically accumulating in the mitochondrial matrix.[5] Inducer cells pretreated with 20 nM MitoQ remained fully sensitive to the TNF-induced apoptosis (in line with previously reported data[6]), but did not induce apoptosis in the recipient cells (Figure 1b). The effect of MitoQ was abolished if it was added together with the protonophorous uncoupler trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP), indicating that accumulation of antioxidant in the matrix was critical for the effect (not shown). At 1–4.5 h after the inducer and recipient coverslips were placed together, measurable increase in $[H_2O_2]$ in the medium was observed (Figure 1c). This increase took place in spite of very short lifetime of the added H_2O_2 in the presence of HeLa cells (not shown). These data indicate the sustained hyperproduction of H_2O_2 in TNF-treated cells. The H_2O_2 level was much lower when the inducer cells were pretreated with MitoQ. When the recipient cells (instead of the inducer) were pretreated with MitoQ, the H_2O_2 level in the medium was not affected (Figure 1d) in spite of the fact that the bystander killing was inhibited (see above, Figure 1b). These data suggest a protective, uncoupler-sensitive action of MitoQ against H_2O_2-induced apoptosis in HeLa (Figure 1e) and some other cell lines.[6] It can be suggested that such an effect was mediated by cessation of 'ROS-induced ROS-release'[7] triggered in mitochondria by exogenous H_2O_2. In

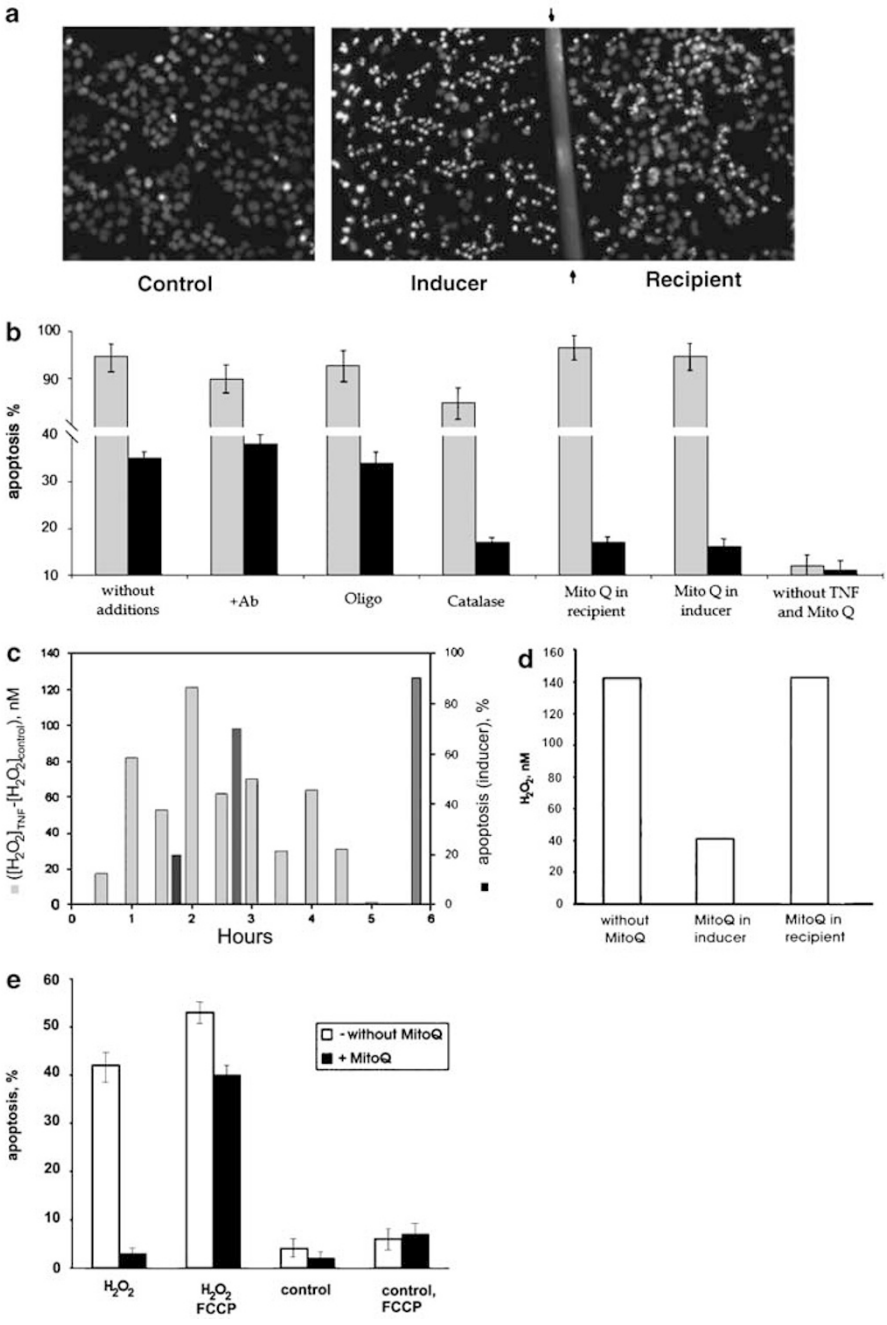

Figure 1 Bystander killing induced by TNF-pretreated cells (**a**–**d**) and apoptosis induced by added H_2O_2 (**e**). (**a**) Typical distribution of apoptotic cells on the inducer and recipient coverslips. Arrows indicate the area where two coverslips are in contact. (**b**) An inducer cover-slip covered with HeLa cells (grown as described in Shchepina *et al.*[4]) was treated with TNF (10 ng/ml) and 1 μM emetine for 3 h, washed, and then placed side by side with the recipient coverslip with nontreated cells for 17 h. The cells were stained with Hoechst 33342, and apoptotic nuclei were counted. Where indicated, monoclonal anti-TNF antibodies (Ab, 700 ng/ml), oligomycin (Oligo 5 μg/ml), or catalase (2500 U/ml) were added when the inducer and the recipient coverslips were placed together. In control experiments (not shown), antibodies and oligomycin completely blocked the TNF-induced apoptosis when added simultaneously with TNF. Where indicated, the inducer or the recipient cells were grown with 20 nM MitoQ for 6 days (two passages) before the two coverslips were placed together. (**c**) Concentration of H_2O_2 in the incubation medium (light columns) and the level of apoptosis on the inducer coverslip (dark columns) at various durations of coincubation of the inducer and the recipient coverslips. The H_2O_2 level in the medium with emetine but without TNF was used as a control. The H_2O_2 concentration was measured fluorimetrically using Amplex Red and horseradish peroxidase. (**d**) Effect of MitoQ on the H_2O_2 concentration in the incubation medium after 24 h coincubation of the inducer and recipient coverslips. The conditions of MitoQ treatment were as in (**b**). (**e**) Uncoupler FCCP abolishes the MitoQ protection of HeLa cells from apoptosis induced by added H_2O_2. The cells were grown with 20 nM MitoQ with or without 1 μM FCCP for 7 days (two passages) and then without MitoQ and FCCP for 24 h. FCCP was added 1 h before MitoQ (where indicated). On the 8th day 50 μM H_2O_2 was added, and on the 9th day apoptosis was measured. In (**b**) and (**e**) the average data from three to five experiments and s.e.are presented. (**c**) and (**d**) show results of typical experiments

support of this hypothesis, we observed an increase in intracellular level of H_2O_2 (measured with fluorescent probe 2′,7′-dichlorofluorescein diacetate) 1 h after addition of exogenous H_2O_2 (when this H_2O_2 was already completely consumed). The endogenous [H_2O_2] increase was inhibited by Mito-Q (data not shown). In any case, our data indicate that ROS production in mitochondria is critical both for production of long-distance apoptotic signal by the inducer cells and for reception of this signal by the recipient cell.

H_2O_2 was also employed as an apoptogen for the inducer cells. The inducer cells were pretreated with H_2O_2 for 1 h, carefully washed, and placed to the recipient cells. The bystander killing in this case was smaller since low concentrations of H_2O_2 resulted in small number of apoptotic cells on the inducer coverslip, whereas at high H_2O_2 concentrations necrotic cells appeared. The effect was enhanced when the inhibitor of catalase aminotriazole was included in the incubation medium. Pretreatment of recipient cells with MitoQ protected them from apoptosis (not shown).

Our data supported the hypothesis[1] that H_2O_2 can be used for long-distance transmission of a programmed death signal from apoptotic to healthy cells. Apparently, long-distance phenomena are involved in the H_2O_2-mediated hypersensitive response to infection in plants.[8] H_2O_2 could mediate spreading of apoptosis during self-elimination of organs in ontogenesis such as the disappearance of the tadpole tail. This process was shown to be accompanied by massive H_2O_2-induced apoptosis of the tail cells.[9] Stimulation of the bystander cell killing might be important for chemo-, photodynamic- and radio-therapy of tumours,[10–12] especially for those forms which are insensitive to p53- and Rb-mediated apoptoses but retain sensitivity to the H_2O_2-induced apoptosis (e.g., as HeLa cells). On the other hand, H_2O_2-mediated bystander cell killing could contribute to pathogenesis of myocardial infarct, stroke, septic shock, and aging (for discussion, see[13,14]). In these cases, use of MitoQ and other mitochondria-targeted antioxidants seems promising.

In conclusion, we have presented here strong evidence for H_2O_2-mediated transmission of intercellular apoptotic signal. It was shown that (i) apoptotic cells produce H_2O_2, (ii) catalase inhibits the bystander killing, and (iii) mitochondrial ROS are required for both generation of the death signal by the apoptotic cells and its processing in the healthy cells, since pretreatment of either apoptotic or healthy cells with a mitochondria-targeted antioxidant strongly inhibits the bystander killing.

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