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Communication

Differentiated Paju cells have increased resistance to toxic effects of potassium ionophores★✪

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In this study we have investigated the impact of differentiation of neuronal cells on their sensitivity to microbial toxins. We used the human neural crest-derived tumor cell line Paju, which can be induced to differentiation *in vitro* by treatment with phorbol 12-myristate 13-acetate. Addition of the highly toxic potassium ionophores cereulide (4.5 and 9.0 ng/ml) or valinomycin (20 ng/ml), to cultures of undifferentiated Paju cells caused collapse of the mitochondrial membrane potential — measured with the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrabenzimidazole carbocyanine iodide (JC-1) followed by detachment of the cells and their apoptotic death. After induced differentiation of the Paju cells, their mitochondria retained the membrane potential upon exposure to the toxins and the cells displayed increased resistance to apoptosis as compared with undifferentiated cells. This effect may be

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Abbreviations: $\Delta\Psi_m$, mitochondrial transmembrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrabenzimidazole carbocyanine iodide; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate.

caused by an elevated expression of the anti-apoptotic protein Bcl-2 and of the neuroprotective factor, stanniocalcin, in differentiated cells.

When neural Paju cells are induced to differentiation in culture, the expression of Bcl-2 rises (Zhang *et al.*, 1996). Bcl-2 is known to protect against the mitochondrial permeability transition induced by Ca^{2+} and/or prooxidants (Shimizu *et al.*, 1998; Murphy *et al.*, 2001). Neuronal differentiation, both *in vivo* and *in vitro*, also leads to a strongly upregulated expression of stanniocalcin (Zhang *et al.*, 1998). Stanniocalcin confers a protective effect on neurons during cerebral ischemia (Zhang *et al.*, 2000). Mitochondria have a central role in the signal transduction of apoptosis and necrosis (Gottlieb, 2000; Lemasters *et al.*, 2002). Valinomycin, a potassium ionophore, facilitates the selective transport of K^+ ions across the inner membrane of mitochondria (Duax *et al.*, 1996) and induces apoptosis in ascites hepatoma cells (Inai *et al.*, 1997) by disrupting the mitochondrial membrane potential. Valinomycin has been reported to cause apoptosis in other mammalian cell lines as well (Penning *et al.*, 2000; Li & El-Mallahk, 2000; Krick *et al.*, 2001). Andersson *et al.* (1998a) isolated from an indoor environment *Streptomyces griseus* strains that produce valinomycin. It inhibited human NK cell activity and induced apoptosis as did commercially available valinomycin (Paananen *et al.*, 2000). Cereulide is a toxin, which may cause serious food poisoning in man. We have characterised cereulide isolated from *Bacillus cereus* and found that it acts as a potassium ionophore with valinomycin-like effects on mitochondrial functions (Mikkola *et al.*, 1999). It induced loss of mitochondrial membrane potential ($\Delta\Psi_m$) in human cells (HeLa, Caco-2 and Calu-3) (Jääskeläinen *et al.*, 2003) and initiated apoptosis of human NK cells as a consequence of mitochondrial damage (Paananen *et al.*, 2002).

Here we report that the mitochondria in Paju cells that have been induced to neural differentiation are more resistant to the toxic effects of cereulide and valinomycin than the mitochondria in undifferentiated Paju cells. It is suggested that this could be due to higher expression of Bcl-2 and stanniocalcin in differentiated Paju cells.

MATERIALS AND METHODS

RPMI 1640 medium, EDTA, phorbol 12-myristate 13-acetate (PMA), propidium iodide (PI), L-glutamine, penicillin G, streptomycin sulfate, valinomycin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrabenzimidazole carbocyanine iodide (JC-1) was from Molecular Probes Inc. (Eugene, OR, U.S.A). Other chemicals were of the highest purity available commercially.

The Paju cell line was established in the Department of Pathology, Haartman Institute (Zhang *et al.*, 1996) from the pleural fluid of a girl who had a widespread metastatic neural crest-derived tumour. The cells grow surface-adherent in RPMI 1640 medium, supplemented with 10% FCS (fetal calf serum), penicillin G (6 mg/ml), streptomycin sulfate (10 mg/ml), and 1 mM glutamine. For subculturing, the cells were detached by treatment with 0.5 M EDTA. After detachment the cells were seeded (40 000 cells/chamber) onto 8-well chambered cover glasses (Naperville, U.S.A.), grown to 20% confluence whereafter 10 nM PMA was added for 4 days to induce terminal neural differentiation.

Bacterial toxin from *S. griseus* was purified by HPLC and shown to be valinomycin as previously described (Andersson *et al.*, 1998a). The toxin is hydrophobic and therefore it was diluted in methanol and applied to cells in 8-well chambers at a concentration of 5 ng/ml (isolated from *S. griseus* strain 10/A1) or 20 ng/ml (from the same strain or commercial from Sigma). In controls, methanol alone was

used as a solvent. Cereulide, the emetic toxin, was isolated from *B. cereus* strain F4810/72 as described (Andersson *et al.*, 1998b) and applied to the cells at a concentration of 4.5 and 9 ng/ml.

Changes in $\Delta\Psi_m$ in intact cells were examined with fluorescence microscope after staining with the fluorescent probe JC-1 (Reers *et al.*, 1995). The plasma membrane integrity was assessed by PI, which enters into dead cells only (Yeh *et al.*, 1981). These dyes were used in combination as a cell viability test. Staining of the slide cultures was done at 3 μM JC-1 and 5 μM PI for 20 min at 37°C in the dark in culture medium under humidified atmosphere containing 5% CO_2. Free dye was removed by washing with RPMI-1640 medium and the slide was analysed immediately with a Leitz photomicroscope with epifluorescence optics and a 100-W mercury lamp. To visualize green and red fluorescence simultaneously, a long-pass filter system LP 520 nm, dichromic mirror RKP 510, and exciter (450–490 nm) were used.

RESULTS

The morphology of Paju cells before (WT) and after induced differentiation is shown in Fig. 1. Treatment with PMA provoked neural sprouting of dendrites carrying growth-cone like structures at their tips (Fig. 1B). The PMA-treated cells also largely exit the cell cycle and accumulate in G2 (not shown).

A collapse of $\Delta\Psi_m$ in connection with the mitochondrial permeability transition often is an early, irreversible event in cell death. Therefore we followed $\Delta\Psi_m$ by staining with the JC-1 fluorescence probe. The integrity of the plasma membrane was probed by staining with PI. Figure 2 shows epifluorescence micrographs of WT (left panels) and differentiated (right panels) Paju cells exposed to the microbial toxins. In the control, the cells were exposed to the solvent (methanol) only. The mitochondria display yellow-orange fluorescence, indicating a high $\Delta\Psi_m$, both in WT (panel A) and differentiated (panel B) cells. WT cells lost their $\Delta\Psi_m$ after exposure to 4.5 ng cereulide/ml for 20 min and only a few mitochondria emitted yellow fluorescence while the cytoplasms emitted green (panel C). Treatment of WT cells with 9 ng cereulide/ml for 20 min caused an almost complete loss of the $\Delta\Psi_m$ (green colour), rounding up and detachment of the cells (panel E). Treatment of differentiated Paju cells with the same concentrations of cereulide induced only small if any changes (panels D and F). In the case of valinomycin, the effects were analogous. Normal cells lost their $\Delta\Psi_m$ after exposure for 20 min to 5 ng/ml valinomycin isolated from *S. griseus*. In contrast, the differentiated cells were unaffected (not shown). Even 20 ng/ml valinomycin isolated from *S. griseus* (panel H)

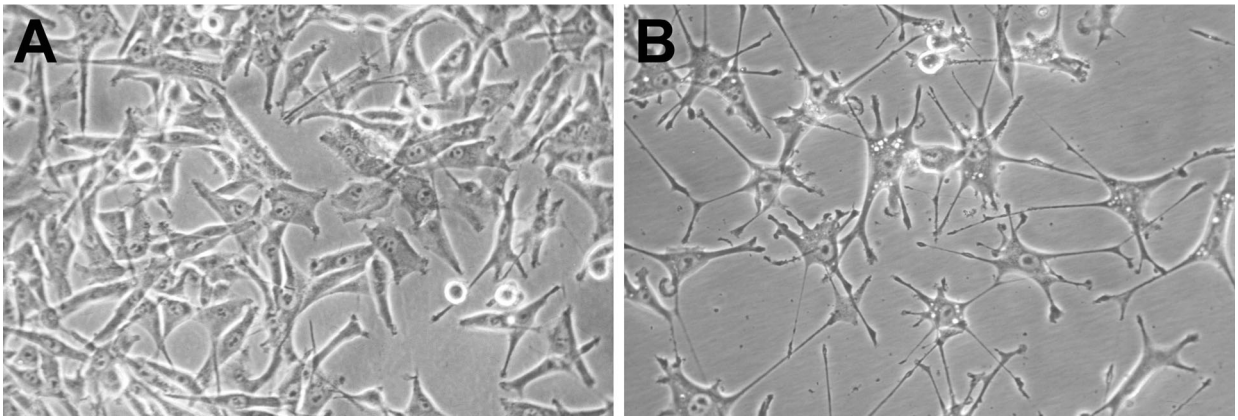

Figure 1. The morphology of Paju cells before (WT) (panel A) and after induced differentiation (panel B).

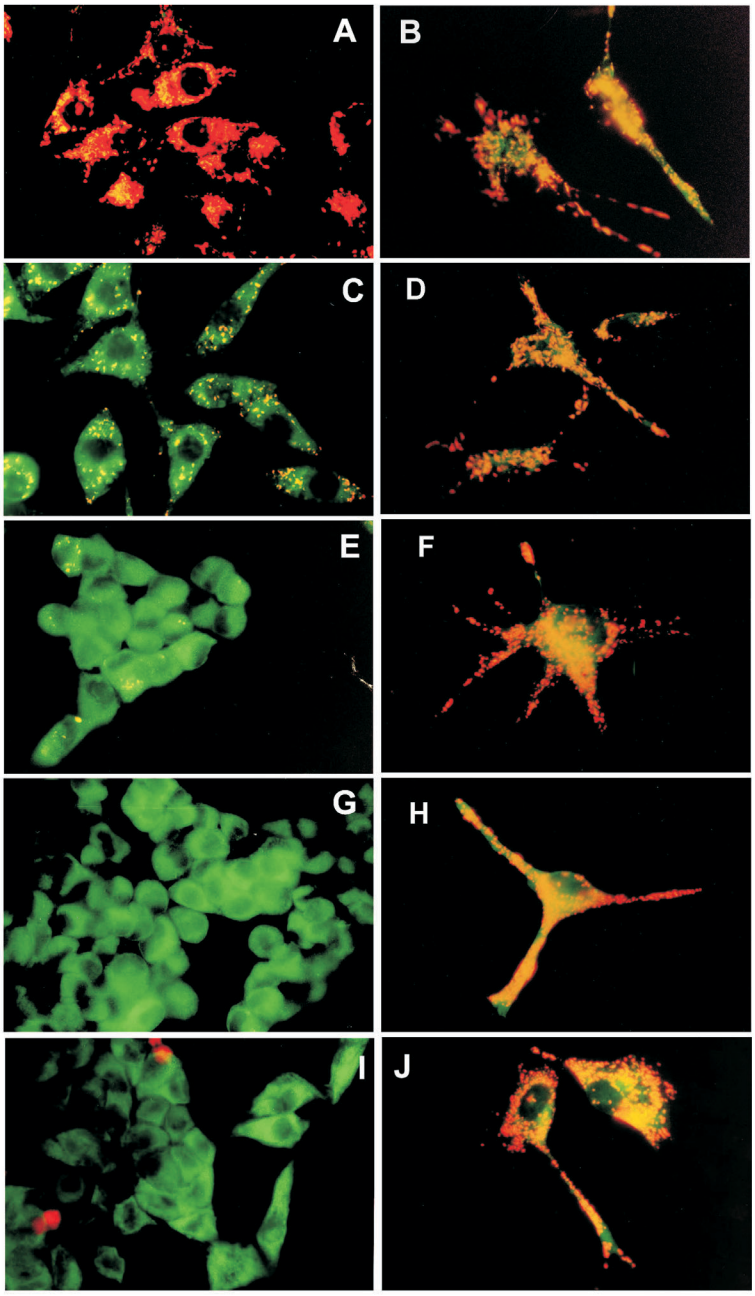

Figure 2. JC-1 and PI-stained human neural undifferentiated (WT) (left panel) and differentiated (right panel) Paju cells exposed to the microbial toxins.

JC-1 fluoresces yellow-orange in cell areas with high $\Delta\Psi_m$ and green in areas of lower potential. PI fluoresces red in cells with damaged cell membrane, whereas there is no fluorescence in intact cells. Paju cells were exposed (20 min, 37°C) to the solvent only (panels A and B), to 4.5 ng/ml (C and D), or to 9 ng /ml of cereulide (panels E and F), to 20 ng/ml of valinomycin isolated from *S. griseus* (panels G and H), and to 20 ng/ml of valinomycin from Sigma (panels I and J).

or commercial valinomycin (panel J) caused no visible damage in differentiated cells. Normal (WT Paju) cells treated under identical conditions showed green fluorescence with JC-1, corresponding to a decreased $\Delta\Psi_m$. The cells were, however, still not permeable to PI (panels G and I), indicating an intact plasma membrane.

Taken together, these results show that, under conditions where all three tested micro-

bial toxins caused loss of $\Delta\Psi_m$ in WT Paju cells, the mitochondria in differentiated Paju cells displayed resistance to the toxins.

DISCUSSION

In this study Paju cells were used to monitor for neurotoxicity of cereulide from *B. cereus* (Mikkola *et al.*, 1999) and of valinomycin, both commercial and isolated from *S. griseus* (Andersson *et al.*, 1998a). We examined the effect of neuronal cell differentiation on the sensitivity to these microbial toxins. Profound effects of the toxins (all potassium ionophores) on undifferentiated Paju cells were observed (Fig. 2). JC-1 staining showed a loss of $\Delta\Psi_m$ in normal cells within 20 min of exposure to the toxins, the cells rounded and detached from the monolayer. The cells lost viability without obvious damage to the plasma membrane. In contrast, in differentiated cells, mitochondria were largely intact after similar exposure to the same toxins, indicating an increased resistance.

Mitochondria may loose their $\Delta\Psi_m$ by three main mechanisms: 1) $\Delta\Psi_m$-driven uptake of K^+ as a positively charged ionophore complex; 2) Opening of the permeability transition pore; 3) Increased transmembrane transport of protons, i.e. protonophorous uncoupling. Since we deal here with K^+ ionophores, mechanism 1) is mainly responsible for the depolarisation. Given that K^+ is the main cation in the cytosol, the uptake of K^+ could be considerable, resulting in extensive swelling of mitochondria. We might in addition have stimulation of the mitochondrial permeability transition. This and swelling will cause some secondary increase in H^+ flux, i.e. uncoupling with decreased synthesis of ATP, and hydrolysis of ATP by the F_oF_1-ATPase. Mitochondrial volume is regulated by the combined activities of the ATP-sensitive K^+ channel and the K^+/H^+ antiporter (Garlid & Paucek, 2003). In the presence of potassium ionophores it is to be expected, that the stimulated influx of K^+ and swelling results in activation of the antiporter, which in turn causes K^+ efflux and influx of H^+. This cycling mechanism may contribute to an uncoupling.

The toxicity of the studied potassium ionophores is mainly due to their effects on mitochondria, i.e. loss of $\Delta\Psi_m$ and swelling. This generally occurs in the mitochondrial permeability transition, however, valinomycin was found also to cause release of cytochrome *c* without inducing permeability transition (Shinohara *et al.*, 2002). Swelling itself would rupture the outer membrane and release proapoptotic factors like cytochrome *c* (Cai *et al.*, 1998). The associated decrease in ATP would be expected to result in cell necrosis (Lemasters *et al.*, 2002), but the cell death seen in this study rather corresponds to apoptosis.

The molecular mechanism(s), conferring higher resistance to the toxins in differentiated Paju cells, is not clear. The expression of several proteins is increased during induced neural differentiation, among which Bcl-2 is known to suppress the mitochondrial permeability transition (Shimizu *et al.*, 1998; Murphy *et al.*, 2001). This property of Bcl-2 is not of interest since valinomycin was found to release cytochrome *c* and induce apoptosis without permeability transition (Shinohara *et al.*, 2002). Stanniocalcin has a protective effect on neurons during cerebral ischemia (Zhang *et al.*, 2000). Moreover, binding of stanniocalcin to the inner membrane of mitochondria was recently reported and stanniocalcin was found to stimulate electron transport in submitochondrial particles *in vitro* (McCudden *et al.*, 2002). These observations suggest that stanniocalcin may contribute to the maintenance of the energy state even under stressful conditions. Studies are in progress to elucidate whether stanniocalcin or/and some other factors are instrumental for the increased resistance to bacterial toxins in differentiated neuronal cells.

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