IONOMYCIN-INDUCED CA\textsuperscript{2+} CYTOSOLIC INCREASE IS NOT INDUCING MASSIVE APOPTOSIS OF BA/F3 CELLS

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IONOMYCIN-INDUCED Ca\textsuperscript{2+} CYTOSOLIC INCREASE IS NOT INDUCING MASSIVE APOPTOSIS OF Ba/F3 CELLS (Abstract): It was shown that a novel immunotherapy using \textit{ex vivo} activated splenocytes is capable of promoting survival and hematopoietic recovery after syngeneic and semiallogeneic bone marrow transplantation in BALB/c mice subjected to a lethal dose of total body irradiation. We tested the hypothesis that the low number of B cells obtained in the above conditions might be due to the effects of calcium overload, induced by calcium ionophore ionomycin, through the apoptosis of the early B cells. We used flow cytometry, together with calcein and propidium iodide as indicators of mitochondrial permeability transition pore (MPTP) activation and apoptosis. From the normal Ba/F3 cells 69.83±3.90% were associating high calcein fluorescence of mitochondria in the presence of 80 μM cobaltous chloride. In some experiments Ba/F3 cells were treated with 2 μM ionomycin and 2.5 mM Ca\textsuperscript{2+} in culture medium for 24 hours. In this case, 85.68±2.66% of the Ba/F3 cells were associating high calcein fluorescence of mitochondria, thus a large proportion of them remaining living cells, being unaffected by the calcium overloading induced by ionomycin. Moreover, only about 8% of cells are necrotic as shown by propidium iodide. Thus, calcium ionophore ionomycin induced higher mitochondrial calcein fluorescence than in normal cells. For comparison we used as positive control the effects of staurosporine 10 μM, a well known inducer of apoptosis. After 24 h of treatment only 5.98±1.32% of cells were having mitochondria loaded with calcein (live cells), more than 80% being necrotic. To demonstrate the involvement of MPTP activation we administered the inhibitor cyclosporine A (CsA), also administered in parallel in some experiments (1 μM) for 24 hours. CsA dramatically reduced the staurosporine apoptotic effects on Ba/F3 cells, having no significant influence on ionomycin treatment. Thus, we can consider that the calcium ionophore ionomycin is not inducing MPTP opening and apoptosis of Ba/F3 cells, although is increasing \([\text{Ca}^{2+}]_{\text{c}}\) and \([\text{Ca}^{2+}]_{\text{mt}}\). This conclusion is further supported by the propidium iodide staining, since a very low proportion of Ba/F3 cells treated with ionomycin for 24 h were accumulating specific red fluorescence. These results are also in contrast to those obtained by staurosporine treatment, which induced accumulation of specific red fluorescence characteristic to propidium iodide (necrotic and apoptotic) in more than 80% of Ba/F3 cells. We can conclude that the calcium overload induced by ionophore ionomycin is not responsible for the low numbers of B cells obtained through selective selection of splenocytes used for hematopoietic recovery after syngeneic and semiallogeneic bone marrow transplantation in BALB/c mice.

KEY WORDS: PRO-B CELLS, APOPTOSIS, CALCEIN, IONOMYCIN, PROPIDIUM IODIDE, LASER CONFOCAL MICROSCOPY

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INTRODUCTION

It was shown that a novel immunotherapy using ex vivo activated immune cells is capable of promoting survival and hematopoietic recovery after combined chemotherapy and radiotherapy and after syngeneic and semiallogeneic bone marrow transplantation in BALB/c mice subjected to a lethal dose of total body irradiation [1]. Splenocytes were used for cell activation. During the 2 days of culture, some cells survived and expanded upon the stimulation with granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2), and calcium ionophore. Many unresponsiveness cells died. Cell numbers after the cell culture were significantly smaller than those before the cell culture and the cell yields were usually 50%. The therapeutic cells consisted of a mixture of immunologically active cells, including T cells, B cells, monocytes, NK cells and CD14 negative monocytes. Analysis of lineage markers revealed expression of CD3 (T cells) in 66.08%, CD19 (B cells) in 8.13%, CD14 (monocytic cells) in 11.7%, and CD56 (natural killer cells) in 3.06% of the nucleated cells. The major change was found in the subpopulation expressing CD14 marker which was greatly down-regulated by calcium ionophore [2].

The low number of B cells obtained in the above conditions might be also due to the effects of calcium overload, induced by calcium ionophore, on the early B cells stages of development.

It is known that the dysregulation of Ca^{2+} (down- or upregulation) has long been involved to be important in cell injury. A Ca^{2+}-linked process important in necrosis and apoptosis (or necroptosis) is the mitochondrial permeability transition (MPT). In the MPT, large conductance permeability transition (PT) pores open that make the mitochondrial inner membrane abruptly permeable to solutes up to 1500 Da. The importance of Ca^{2+} in MPT induction varies with circumstance. Ca^{2+} overload is sufficient to induce the MPT. By contrast, after ischemia-reperfusion to cardiac myocytes, Ca^{2+} overload is the consequence of bioenergetic failure after the MPT rather than its cause. In other models, such as cytotoxicity from Reye-related agents and storage-reperfusion injury to liver grafts, Ca^{2+} appears to be permissive to MPT onset. Lastly, in oxidative stress, increased mitochondrial Ca^{2+} and oxygen radicals generation act synergistically to produce the MPT and cell death. Thus, the exact role of Ca^{2+} for inducing the MPT and cell death might depend on the particular biologic setting [3].

The goal of our study was represented by the characterization of ionomycin (calcium ionophore)-induced effects on Ba/F3 (pro-B) cells using flow cytometry, together with calcein and propidium iodide as indicators of MPTP activation and apoptosis.

MATERIALS AND METHODS

The IL-3-dependent mouse pro-B cell line Ba/F3 was maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% heat-inactivated fetal bovine serum and 10% WEHI-3-conditioned media as a source of murine IL-3, in an atmosphere with 5% CO2 and at 37°C [4].

Cells were grown at a density of around 5x10^5 per ml before experiments, each one being performed using 1x10^6 cells/ml. For some experiments (in triplicate) Ba/F3 cells were treated with 2 µM ionomycin and 2.5 mM Ca^{2+} in culture medium for 24 hours. For comparison we used as control the effects of staurosporine 10 µM, a well known inducer of apoptosis, also in triplicate. To demonstrate the involvement of MPTP activation we administered the inhibitor cyclosporine A (CsA), also administered in
parallel in some experiments (1 μM) for 24 hours. The control Ba/F3 cells received no treatment for 24 hours. After that, all batches of Ba/F3 cells were incubated in the presence of 1 μM calcein-AM and 80 μM cobaltous chloride as well as propidium iodide 1 μg/ml (Sigma-Aldrich) at 37°C for 30 minutes. For the determination of calcein loading in mitochondria, in the presence of cobaltous chloride, and propidium iodide staining we used a FACS Calibur (Beckton-Dickinson). The final concentration for dimethyl sulfoxide (DMSO) in the medium, used as a drug solvent, did not exceed 0.1%, having no cellular effects at this concentration.

The statistical significance of test results was highlighted using the Variance One-Way ANOVA, All Pairwise Multiple Comparison Procedures (Holm-Sidak method) and the results were expressed as mean ± S.E.M (n = 3). Value of p<0.05 was considered statistically significant always.

RESULTS

From the normal Ba/F3 cells (20,000 events gated) 69.83±3.90% are associating high calcein fluorescence of mitochondria in the presence of 80 μM cobaltous chloride as shown in fig. 1, R1 region (triplicate experiment). As shown, 69.83±3.90% of the cells are associating high calcein fluorescence of mitochondria (R1 region). p<0.001 as compared to control cells with no calcein, data are expressed as mean ± S.E.M. (n = 3).

The calcein uptake is evident if we compare these cells with the control ones (no treatment) (Fig. 2). For the control cells, the vast majority is found in the R2 region (bottom left), that means 96.80±1.41%, data are expressed as mean ± S.E.M. (n=3).

For comparison we used as positive control the effects of staurosporine 10 μM, a well known inducer of apoptosis, also in triplicate. After 24 h of treatment the R1 region contains only 5.98±1.32% of cells having mitochondria loaded with calcein (live cells), more than 80% being necrotic (the R3 region, fig. 3, n=3). p<0.001 as compared to cells with only calcein and to cells with no calcein, data are expressed as mean ± S.E.M.
In some experiments (in triplicate) Ba/F3 cells were treated with 2 μM ionomycin and 2.5 mM Ca^{2+} in culture medium for 24 hours (fig. 4). In this case, 85.68±2.66% of the Ba/F3 cells are associating high calcein fluorescence of mitochondria, thus a large proportion of them remaining living cells, being unaffected by the calcium overloading induced by ionomycin. Moreover, only about 8% of cells are necrotic as shown by propidium iodide (R3 region). p<0.001 as compared to cells with only calcein and to cells with no calcein, data are expressed as mean ± S.E.M (n=3).

![Fig. 2 Representative flow cytometry recordings of 1x10^6 control Ba/F3 cells/ml.](image)

![Fig. 3 Representative effects of 24 h treatment with staurosporine 10 μM on 1x10^6 Ba/F3 cells/ml. The cells were stained with 1 μM calcein-AM and 80 μM cobaltous chloride at 37°C for 30 minutes.](image)
To demonstrate the involvement of MPTP activation we administered the inhibitor cyclosporine A (CsA), also administered in parallel in some experiments (1 μM) for 24 hours. CsA dramatically reduced the staurosporine apoptotic effects on Ba/F3 cells, having no significant influence on ionomycin treatment (personal observation, data not shown).

**DISCUSSIONS**

Inhibition of mitochondrial oxidative phosphorylation progresses to uncoupling when opening of cyclosporin A-sensitive permeability transition pores increases permeability of the mitochondrial inner membrane to small solutes. Involvement of the mitochondrial permeability transition (MPT) in necrotic and apoptotic cell death is implicated by demonstrations of protection by cyclosporin A against oxidative stress, ischemia/reperfusion, tumor necrosis factor-alpha exposure, Fas ligation, calcium overload, and a variety of toxic chemicals. Confocal microscopy directly visualizes the MPT in single mitochondria within living cells from the translocation of impermeant fluorophores, such as calcein, across the inner membrane. Simultaneously, mitochondria release potential-indicating fluorophores. Subsequently, mitochondria swell, causing outer membrane rupture and release of cytochrome c and other proapoptotic proteins from the intermembrane space. In situ a sequence of decreased NAD(P)H, increased free calcium, and increased reactive oxygen species formation within mitochondria promotes the MPT and subsequent cell death. Necrotic and apoptotic cell death after the MPT depends, in part, on ATP levels. If ATP levels fall profoundly, glycine-sensitive plasma membrane permeabilization and rupture ensue. If ATP levels are partially maintained, apoptosis follows the MPT. The MPT also signals mitochondrial autophagy, a process that may be important in removing damaged mitochondria.

![Fig. 4 Representative effects of 24 h treatment with 2 μM ionomycin and 2.5 mM Ca²⁺ in culture medium on 1x10⁶ Ba/F3 cells/ml. The cells were stained with 1 μM calcein-AM and 80 μM cobaltous chloride as well as with propidium iodide 1 μg/ml at 37°C for 30 minutes.](image)
Cellular features of necrosis, apoptosis, and autophagy frequently occur together after death signals and toxic stresses. A new term, necrapoptosis, describes such death processes that begin with a common stress or death signal, progress by shared pathways, but culminate in either cell lysis (necrosis) or programmed cellular resorption (apoptosis), depending on modifying factors such as ATP [5].

Combining direct assays for the mitochondrial permeability transition pore (MPTP) and changes in mitochondrial membrane potential provides a unique window on early events in apoptosis. The MPTP is activated in response to environmental conditions in the mitochondria such as depleted ATP levels, increased reactive oxygen species production and, in particular, mitochondrial calcium \([\text{Ca}^{2+}]_{\text{mt}}\) overload. Activation of the pore has been associated with release of apoptogenic molecules from mitochondria such as cytochrome c, resulting in apoptosome formation, activation of caspases, and execution of apoptosis [6,7].

Downstream events of pore activation range from normal pore flicker to irreversible opening of the pore leading to programmed cell death, both associated with changes in mitochondrial membrane potential (\(\Psi_{\text{mt}}\)). Pore activation is observed by changes in mitochondrial calcine fluorescence, when cytoplasmic fluorescence is quenched by cobalt. Using the cobalt quenched assay (CoQC) by flow cytometry, there was possible to demonstrate cyclosporine A (CsA)-sensitive ionomycin-induced pore opening in Jurkat cells, MH1C1 hepatocytes, BPAE cells, HeLa cells. While it is likely that collapse of \(\Psi_{\text{mt}}\) indicates irreversible pore opening, MPTP measurement by CoQC detects more subtle changes with respect to pore opening. A combination of the CoQC method with potentiometric probes should facilitate the elucidation of the subtle phenomena associated with mitochondrial permeability transition leading to programmed cell death [8].

The role of calcium elevations in apoptotic and necrotic cell death has been a focus of research in recent years. Evidence has been presented that calcium oscillations can effectively trigger apoptosis under certain conditions and that dysregulation of calcium signalling is a common cause of cell death. These effects are regularly mediated through calcium signal propagation to the mitochondria and the ensuing mitochondrial membrane permeabilization and release of pro-apoptotic factors from mitochondria to the cytoplasm [9].

In HepG2 cells and primary hippocampal neurones in culture ionomycin, a well known neutral calcium ionophore, promoted the equilibration of Ca\(^{2+}\) gradients between cellular compartments, including endoplasmic reticulum, mitochondria and cytosol. Thus, cytosolic \([\text{Ca}^{2+}]_{c}\) and mitochondrial \([\text{Ca}^{2+}]_{\text{mt}}\) calcium increased together and then recovered in parallel on removal of the ionophore [10].

At the same time, ionomycin is inducing apoptosis through cytosolic and parallel mitochondrial Ca\(^{2+}\) overload in several cell types as HL-60 human promyelocytic leukaemia cells [10], thymocytes [11], in cultured cortical neurons from embryonic rats [12], but not in peripheral blood lymphocytes [13] or thymic lymphomas [14].

Mitochondrial depolarisation induced by ionomycin in HepG2 cells and primary hippocampal neurones in culture was often modest, independent of cyclosporin A (CsA), was suppressed in the absence of extracellular Ca\(^{2+}\) and was enhanced by pre-incubation of cells with the inhibitor of the mitochondrial Ca\(^{2+}/2\text{Na}^{+}\)-exchanger,
CGP37157, suggesting that the change in potential reflects the prior state of mitochondrial calcium loading [9]. Ionomycin caused some mitochondrial depolarization also in our previous experiments on Ba/F3 cells, although this was low in degree [15]. In addition, since CsA did not significantly influence the ionomycin effects on mitochondrial potential in Ba/F3 cells and mitochondrial calcein loading in the presence of ionomycin (personal observation, data not shown), it might be evident that these effects of ionomycin are not related to MPTP functioning. This is in contrast with the obtained effects of staurosporine on Ba/F3 cells, regarding the MPTP functioning and mitochondrial membrane potential modulation.

Moreover, in our experiments, calcium ionophore ionomycin induced higher mitochondrial calcein fluorescence than in normal cells. Loss or reduction of calcein fluorescence in various circumstances (at least after 24 h treatment) properly reflect the MPTP opening and induction of apoptosis [8]. Thus, we can consider that the calcium ionophore ionomycin is not inducing MPTP opening and apoptosis of Ba/F3 cells, although is increasing $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{\text{int}}$. This conclusion is also supported by the propidium iodide staining, since a very low proportion of Ba/F3 cells treated with ionomycin for 24 h were accumulating specific red fluorescence. These results are also in contrast to those obtained by staurosporine treatment, which induced accumulation of specific red fluorescence characteristic to propidium iodide (necrotic and apoptotic) in more than 80% of Ba/F3 cells.

These results are in accordance with the previous obtained data which showed that the immortalized IL-3-dependent progenitor cell line, Ba/F3, undergoes programmed cell death (apoptosis) when deprived of IL-3. In the absence of IL-3, DNA fragmentation and cell death could be prevented by the calcium ionophores A23187 (1 $\mu$M) and ionomycin (0.5 $\mu$M). This addition of calcium ionophores maintains cell viability while reversibly arresting the cell cycle [16].

CONCLUSIONS

It was shown that a novel immunotherapy using ex vivo activated splenocytes is capable of promoting survival and hematopoietic recovery after syngeneic and semiallogeneic bone marrow transplantation in BALB/c mice subjected to a lethal dose of total body irradiation. Some cells survived and expanded upon the stimulation with granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2), and calcium ionophore. We tested the hypothesis that the low number of B cells obtained in the above conditions might be due to the effects of calcium overload, induced by calcium ionophore ionomycin, through the apoptosis of the early B cells. We used flow cytometry, together with calcein and propidium iodide as indicators of mitochondrial permeability transition pore (MPTP) activation and apoptosis.

In our experiments, calcium ionophore ionomycin induced higher mitochondrial calcein fluorescence than in normal cells. Thus, we can consider that the calcium ionophore ionomycin is not inducing MPTP opening and apoptosis of Ba/F3 cells, although is increasing $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{\text{int}}$. This conclusion is also supported by the propidium iodide staining, since a very low proportion of Ba/F3 cells treated with ionomycin for 24 h were accumulating specific red fluorescence. These results are also in contrast to those obtained by staurosporine treatment, which induced accumulation of specific red fluorescence characteristic to propidium iodide (necrotic and apoptotic) in more than 80% of Ba/F3 cells.
We can conclude that the calcium overload induced by ionophore ionomycin is not responsible for the low numbers of B cells obtained through selective selection of splenocytes used for hematopoietic recovery after syngeneic and semiallogeneic bone marrow transplantation in BALB/c mice.

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