Human Fetal Retinal Pigment Epithelium-Induced Cell Cycle Arrest, Loss of Mitochondrial Membrane Potential and Apoptosis

Lili Farrokh-Siar,1 Kourosh A. Rezai,1 Ellen M. Palmer,2 Samir C. Patel,1 J. Terry Ernest,1 and Gijs A. van Seventer2

PURPOSE. To investigate the mechanism of action of the soluble immune suppressive product secreted by human fetal retinal pigment epithelial (HFRPE) cells in a model system using the human T-cell line Jurkat (Jkt).

METHODS. Pure HFRPE cells were isolated and cultured. The supernatants of both nonactivated and IFN-γ-activated HFRPE cells were isolated. Cells from the human T-cell line Jkt were incubated either in standard culture medium or in the supernatant isolated from HFRPE cells. In the first assay Jkt cell proliferation was measured by [3H]thymidine incorporation. In the second assay Jkt cell apoptosis was examined for annexin V staining by flow cytometry. In the third assay Jkt cell division was evaluated with carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye. In the last assay the mitochondrial transmembrane potential of Jkt cells was measured with the cationic lipophilic fluorochrome 3,3'-dihexyloxacarbocyanine iodide [DiOC(6)]. In all the assays the effect of supernatants isolated from both nonactivated and IFN-γ-activated HFRPE cells were compared with standard culture medium. The involvement of antiapoptotic human gene bcl-xL was determined by using a Jkt cell line that was stably transfected with bcl-xL.

RESULTS. The supernatant isolated from HFRPE cells significantly suppressed the cell division in Jkt cells and induced apoptosis. These effects were stronger when the supernatant was isolated from IFN-γ-activated HFRPE cells. The apoptosis pathway induced by the secreted product of HFRPE cells involved the early disruption of mitochondrial transmembrane potential. Although the overexpression of bcl-xL gene rescued the Jkt cells from supernatant-induced apoptosis, it could not restore the proliferation of Jkt cells.

CONCLUSIONS. These data suggest that HFRPE cells secrete a product that initiates an early cell cycle arrest in the human T-cell line Jkt, which is followed by the activation of an apoptotic pathway that involves the loss of mitochondrial membrane potential. The latter could be prevented by bcl-xL overexpression. Also these data suggest that the HFRPE-induced T-cell apoptosis may play a significant role in maintaining the immune privilege in the subretinal space. (Invest Ophtalmol Vis Sci. 2000;41:3991–3998)

Immune privilege, the phenomenon whereby grafts placed in certain sites of the body enjoy an unexpectedly prolonged acceptance, was described more than 100 years ago.1–4 The eye is one of the body organs enjoying this privilege. Although the mechanisms involved in maintaining the immune privilege in the anterior chamber of the eye are well studied, relatively little is known about the immune privilege in the subretinal space.5,6 Recently, it was shown that human fetal retinal pigment epithelial (HFRPE) cells transplanted into the subretinal space of human eyes with intact Bruch’s mem-

brane survived for an extended period without a severe immune response.7

We and others showed that HFRPE cells contributed to the immune privilege in the subretinal space by induction of apoptosis in human T cells.8–10 The mechanism(s) involved in the HFRPE-mediated T-cell apoptosis are currently not well established. However, we showed that the pathway involved in HFRPE-induced apoptosis in T cells was Fas ligand independent.8,9 We also demonstrated that PGE2, TGF-β, IL-10, or TNF-α were not involved in HFRPE’s inhibition of T-cell activation and proliferation.9

There are reports suggesting the involvement of both cell–cell contact dependent and independent pathways in the induction of apoptosis by HFRPE cells.8,10 These contradictory findings suggest the possibility that HFRPE cells may induce apoptosis through multiple mechanisms. We previously described studies that focused largely on the characteristics of HFRPE cell-induced T-cell apoptosis in which cell–cell contact was possible.8,9 In light of these findings we performed a more detailed study to evaluate the effect of HFRPE supernatant on human Jurkat (Jkt) T-cell line in a HFRPE-free system.
We showed previously that HFRPE cells can induce apoptosis in Jkt T cells. In many aspects Jkt T cells represent the phenotype of an activated human primary T cell. One of the aims of this study was to further characterize the mechanisms involved in the HFRPE cell-mediated apoptosis of Jkt cells. We analyzed therefore the effect of supernatant isolated from HFRPE cells on the cell division of Jkt cells. We also examined the supernatant-induced apoptosis to see if it was mediated by an alteration in the mitochondrial membrane of Jkt cells. Major changes in the function of mitochondrial membrane are critically important in the early stages of apoptosis. A breakdown in the mitochondrial transmembrane potential (ΔΨm) mediated by opening of the so-called mitochondrial permeability transition pores or megachannels marks the point-of-no-return in the apoptotic process.

It is known that various members of the bcl-2 family are actively involved in enhancing or preventing apoptosis, in part by regulating the physical properties of mitochondria. Specifically, the antiapoptotic bcl-2 family member bcl-xL has been shown to help maintain the integrity of mitochondrial membrane. We showed previously that bcl-xL overexpression can rescue the HFRPE-mediated apoptosis in Jkt T cells in an assay in which cell–cell contact was possible. In this study we investigated whether the overexpression of bcl-xL gene could rescue the supernatant-induced loss of mitochondrial membrane potential and subsequent apoptosis in Jkt cells.

**MATERIALS AND METHODS**

**Isolation of HFRPE Cells**

HFRPE cells were obtained from human fetal eyes at 18 to 22 weeks of gestational age (Anatomic Gift Foundation, Woodbine, GA) as described previously. Microdissection was performed under sterile conditions using a dissecting microscope. The eyes were opened by a circumferential incision just above the ora serrata near the limbus, and the anterior segment and lens were separated. The posterior segment of the eye was cut into four quadrants and placed in a Petri dish containing Dulbecco’s minimum essential medium (DMEM; Sigma, St. Louis, MO). The neural retina and any remaining vitreous were removed. Sheets of RPE cells were separated from the choroid using fine forceps and immediately placed into a Petri dish containing Dulbecco’s modified essential medium (DMEM, Sigma, St. Louis, MO). The neural retina and any remaining vitreous were removed. Sheets of RPE cells were separated from the choroid using fine forceps and immediately placed into a Petri dish containing Dulbecco’s minimum essential medium (DMEM, Sigma, St. Louis, MO). The neural retina and any remaining vitreous were removed. Sheets of RPE cells were separated from the choroid using fine forceps and immediately placed into a Petri dish containing Dulbecco’s modified essential medium (DMEM, Sigma, St. Louis, MO).

**Preparation of HFRPE Supernatant**

HFRPE cell supernatants were isolated from either activated or nonactivated HFRPE cells. HFRPE cells were cultured until they reached confluency. Activated HFRPE cells were obtained by their incubation with 1000 U/ml of IFN-γ (Pharmingen, San Diego, CA). Nonactivated HFRPE cells were incubated with culture medium without IFN-γ. After 72 hours the culture medium was discarded, and cultures were washed twice with PBS without Ca2+/Mg2+ (BioWhittaker) and then reincubated with freshly prepared culture medium (without IFN-γ) for an additional 48 hours. The supernatants from both nonactivated and activated HFRPE cells were collected and centrifuged for 5 minutes at 2000 rpm to separate the remaining cell debris.

**Preparation of Jkt Cells**

Two different Jkt cell lines were used in this study: bcl-xL/Jkt or Neo/Jkt cells. Both cell lines were generously provided by Craig B. Thompson and Mathew G. Vander Heiden (Department of Medicine, University of Chicago, Chicago, IL). The bcl-xL/Jkt cell line was generated by the stable transfection of Jkt cells with both the neomycin-resistant gene and the bcl-xL antiapoptosis human gene (bcl-xL/Jkt). The Neo/Jkt cell line, which served as control, was only transfected with the neomycin-resistant gene (Neo/Jkt). Both Jkt cell lines are type II cells, as defined by their ability to prevent Fas-mediated apoptosis by overexpression of the antiapoptotic bcl-2 family member bcl-xL. The Jkt cells were cultured in standard culture medium containing RPMI 1640 (BioWhittaker) supplemented with 20 mM glutamine (BioWhittaker), 10% heat inactivated fetal calf serum (GIBCO, Life Technologies, Grand Island, NY), 100 IU/ml penicillin, 100 μg/ml streptomycin (BioWhittaker), and 20 mM HEPES (BioWhittaker). The cultures were incubated at 37°C in a humidified mixture of 95% air and 5% CO2. For selection purposes 0.5 mg/ml of G418 (GIBCO, Life Technologies) was added to the cultures.

**Jkt Cell Proliferation Assay.** In this assay the rescue effect of bcl-xL overexpression on supernatant-induced (supernatant isolated from either activated or nonactivated HFRPE cells) apoptosis was analyzed by measuring the proliferation of Jkt cells. Supernatants were isolated from both nonactivated or IFN-γ-activated HFRPE cells (see above). The bcl-xL/Jkt or Neo/Jkt cells (serving as control) were suspended either in the isolated supernatants or in standard culture medium (see above) in flat-bottomed 96-well-plate tissue culture clusters (Costar, Cambridge, MA). After an incubation period of 48 hours, the cultures were pulsed with a [3H]thymidine solution (5 mCi/ml, 2 mCi/mmol specific activity; New England Nuclear, Boston, MA) for 4 hours before harvesting on glass fiber filters. Incorporation of the radioactive label was measured by liquid scintillation counting and was expressed as the arithmetic mean of counts per minute (cpm) of triplicate cultures. In Figure 1 the mean and SD counts per minute of for the two cell lines in standard media was 415,000 ± 33,889 cpm. The 100% on the y-axis in Figure 1 indicates the proliferation of either bcl-xL/Jkt or Neo/Jkt cells in standard culture medium.

**Apoptosis Assay: Annexin V Staining.** The rate of supernatant-induced apoptosis in bcl-xL/Jkt or Neo/Jkt cells was evaluated in this assay. The bcl-xL/Jkt or Neo/Jkt cells were incubated in the supernatants isolated from either nonactivated or IFN-γ HFRPE cells (as mentioned above). After 24, 48, 72, or 96 hours of incubation the percentage of cells stained...
with annexin V-phycocerythrin (PE; R&D, Minneapolis, MN) was determined by flow cytometry (FACScan). Annexin V binds to phosphatidyl serine (exposed on the cell membrane), which is one of the earliest indicators of cellular apoptosis. Staining procedures were performed according to the manufacturer’s instructions (R&D).

**Cell Division Rate Assay: Carboxyfluorescein Succinimidyl Ester (CFSE) Staining.** The effect of HFRPE cell supernatant on the cell division of Jkt cells was evaluated with the CFSE fluorescent dye. Carboxy-fluorescein diacetate succinimidyl ester (CFDASE), the diacate form of CFSE is a nontoxic chemical that readily diffuses into cells. After binding to the plasma membrane, its acetyl groups are cleaved by cellular esterases to generate CFSE, which is an active fluorescent dye. After binding to the plasma membrane, its acetyl groups are cleaved by cellular esterases to generate CFSE, which is an active fluorescent dye. After binding to the plasma membrane, its acetyl groups are cleaved by cellular esterases to generate CFSE, which is an active fluorescent dye. After binding to the plasma membrane, its acetyl groups are cleaved by cellular esterases to generate CFSE, which is an active fluorescent dye.

Upon cell division CFSE segregates equally between daughter cells resulting in the sequential dilution of fluorescence intensity in successive generations. This dilution of fluorescence is visualized with flow cytometry, tracking down the number of cell divisions in proliferating cell populations.

A 5 mM stock solution of CFDASE (Molecular Probes Inc., Eugene, OR) was prepared in DMSO (Sigma). The bcl-xL/Jkt or Neo/Jkt cells were incubated in the supernatants isolated from either nonactivated, IFN-γ-activated HFRPE cells or standard culture medium (see above, serving as control). The cells were subsequently washed in PBS (without Ca\(^{2+}/Mg^{2+}\)) and were resuspended in PBS at a concentration of 5 \(\times\) 10^6 cells/ml. CFDASE stock solution was diluted at a concentration of 1:1500 in PBS. The cells were gently mixed with an equal volume of the diluted CFDASE solution for 10 minutes at room temperature. The labeling process was quenched by adding an equal volume of fetal calf serum (GIBCO, Life Technologies) to the cell suspension solution followed by a 1-minute incubation. The cells were subsequently washed with standard culture medium. The intensity of fluorescence indicating the number of cell divisions, was visualized by flow cytometric analysis on a FACScan. The number of cell divisions was analyzed with the ModFit LT 2.0 software program (Verity Software, Topsham, ME).

**Mitochondrial Membrane Staining.** The cationic lipophilic fluorochrome 3,3-dihexyloxacarbocyanine iodide [DiOC(6)] was used to evaluate whether the supernatant-induced apoptosis was associated with the disruption of transmembrane potential in mitochondria. Neo/Jkt or bcl-xL/Jkt cells were incubated with supernatants isolated from nonactivated or IFN-γ-activated HFRPE cells (as described above) for 12 or 36 hours. The supernatants were then removed, and the cells were incubated with standard culture medium containing 40 \(\mu\)M DiOC(6) for 30 minutes. Subsequently, cells were washed with standard culture medium and immediately analyzed by flow cytometric analysis with a FACScan.

**Statistical Analysis**

For Figure 1 the mean cpm of triplicates and for Figures 2 through 4 the absolute number of cells were used for statistical analysis using a two-factor-with-replication analysis of variance (ANOVA) with \(\alpha = 0.05\). P values \(\leq 0.01\) were accepted as significant.

**RESULTS**

**HFRPE Supernatant Blocked the Proliferation of Jkt Cells**

It was shown that the coculture of IFN-γ-activated HFRPE cells and Jkt cells leads to apoptosis of Jkt cells. The HFRPE-mediated apoptosis was cell–cell contact independent, because the separation of the two cell types by a transwell insert did not prevent the induction of apoptosis. In the assay presented here the effect of supernatant isolated from both nonactivated or IFN-γ-activated HFRPE cells on the proliferation of Neo/Jkt or bcl-xL/Jkt cells was evaluated in a HFRPE-free system. The two Jkt cell lines differed only in their expression levels of bcl-xL. The supernatant isolated from nonactivated HFRPE cells suppressed the proliferation of both Neo/Jkt and bcl-xL/Jkt cells similarly \((P \leq 0.01; \text{Fig. } 1)\). This inhibitory effect was stronger in the supernatants isolated from IFN-γ-activated HFRPE cells. It should be noted that the supernatant isolated from IFN-γ-activated HFRPE cells did not contain IFN-γ as measured by ELISA (data not shown).

The filtration of supernatant through a Centriprep concentrator 100 filter (Amicon, Grace Company, Beverly, MA), which allows the passage of molecules with a molecular weight of less than 100,000 kDa resulted in a similar inhibitory effect (results not shown). This preliminary characterization of the HFRPE-secreted soluble factor indicated that its molecular weight is likely to be less than 100,000 kDa.

**bcl-xL Overexpression Rescued Supernatant-Mediated Jkt Cell Apoptosis**

The role and rate of apoptosis in supernatant-mediated inhibition of Jkt cell proliferation was assessed with annexin V-PE staining. bcl-xL/Jkt and Neo/Jkt cells were incubated with the supernatant isolated from nonactivated or IFN-γ-activated HFRPE cells for 24, 48, 72, and 96 hours. Standard culture medium served as control. The supernatant isolated from...
HFRPE cells induced an increased binding of annexin V to Neo/Jkt cells when compared with the cells incubated with standard medium (Fig. 2A). This binding was further increased when Neo/Jkt cells were incubated over a period of 96 hours with the supernatant isolated from activated HFRPE cells ($P < 0.01$; Fig. 2A). In contrast, there was only a slight increase in the percentage cells binding annexin V in the bcl-xL/Jkt cells when they were treated with the supernatant isolated from either nonactivated or activated HFRPE cells. The percentage of annexin V staining in bcl-xL/Jkt cells after treatment with supernatant from either nonactivated or IFN-γ–activated HFRPE cells was always significantly lower than in Neo/Jkt cells. Annexin V–PE is analyzed in FL2-H. The $y$-axis (counts) shows the cell number. The results depicted are a representative of three independent experiments.

**Figure 2.** Overexpression of bcl-xL rescued the supernatant-mediated apoptosis. The rate of supernatant-mediated apoptosis in Jkt T cells was evaluated with annexin V–PE staining. (A) In Neo/Jkt cells the apoptosis rate was significantly increased after incubation with supernatant isolated from nonactivated (normal supernatant) and IFN-γ–activated (IFN-γ supernatant) HFRPE cells. (B) Supernatant-induced apoptosis in bcl-xL/Jkt cells was significantly less than in Neo/Jkt cells.
of annexin V staining of Neo/Jkt cells after the same treatment ($P \leq 0.01$; Fig. 2B).

These results indicate that the supernatant-induced apoptosis was rescued by bcl-xL overexpression. Unexpectedly, these findings did not correlate with the above-mentioned decrease of [$^3$H]thymidine incorporation in the bcl-xL/Jkt cells that were treated with the HFRPE supernatant (Fig. 1).

**HFRPE Supernatant Induced a Cell Cycle Block in Jkt Cells**

In Neo/Jkt cells the supernatant isolated from HFRPE cells increased the rate of apoptosis and suppressed their proliferation. These results were inconsistent with the findings in bcl-xL/Jkt cells. Although the apoptosis rate was not significantly increased in bcl-xL/Jkt cells (annexin V assay) their proliferation was significantly inhibited by the HFRPE supernatant. This indicated that although bcl-xL overexpression rescued apoptosis, it did not restore the Jkt cell proliferation. In this assay we examined whether the supernatant-mediated inhibition of bcl-xL/Jkt cell proliferation was due to a cell cycle arrest rather than apoptosis.

Jkt cells were treated with CFSE before their incubation with HFRPE supernatants. CFSE labeling identifies the progeny of the cells and analyzes the division history of individual cells that have undergone multiple rounds of cell division. Upon cell division CFSE segregates equally between the daughter cells, making the visualization of Jkt cell division possible. The viability of cells was assessed with PI staining.

The supernatants isolated from both nonactivated and activated HFRPE cells induced cell death in Neo/Jkt cells over the period of 96 hours (Fig. 3A). In contrast, the bcl-xL/Jkt cells showed a significant reduction in cell death rate ($P \leq 0.01$) and a significantly higher viability after incubation with HFRPE-isolated supernatant ($P \leq 0.01$; Fig. 3B). The CFSE staining, however, indicated a halt in the cell cycle progression similar to the Neo/Jkt cells (Table 1).

The detailed cell cycle analysis is shown in Tables 1A and 1B. The Neo/Jkt and bcl-xL/Jkt cells that were treated with the nonactivated supernatant were one generation behind the control group (treated with standard medium; Tables 1A, 1B). This decrease of cell cycle progression was even more significant in the cells treated with activated supernatant. In this group both cell lines were two generations behind the control group (Tables 1A, 1B). These results demonstrated that although the supernatant-induced cell death was rescued by the overexpression of bcl-xL gene, the progression of the cell cycle could not be restored.

These findings may explain the supernatant-mediated inhibition of bcl-xL/Jkt cell proliferation in the absence of apoptosis. Additionally, it demonstrated that the supernatant isolated from IFN-γ-activated HFRPE cells was more potent in suppressing the cell cycle progression in Jkt cells.

**HFRPE Supernatant Disrupted the Mitochondrial Transmembrane Potential**

The disruption of the mitochondrial transmembrane potential is one of the early features of apoptosis. In this assay we evaluated whether the supernatant-mediated apoptosis was associated with this disruption. DiOC(6), a cationic lipophilic fluorochrome, is commonly used for quantifying the mitochondrial transmembrane potential ($\Delta \psi_m$). Jkt cells were pre-treated with this dye and were analyzed by flow cytometry. A decrease in fluorescein intensity seen as a bimodal distribution in the flow cytometric analysis correlates with a decrease in $\Delta \psi_m$. Neo/Jkt cells, which were incubated with the supernatant isolated from IFN-γ-activated HFRPE cells, showed a significant decrease in their $\Delta \psi_m$ at an early time point (12 hours), as indicated by the percentage of cells with DiOC(6) staining (Fig. 4). This was further decreased at 36 hours of incubation. In contrast, the bcl-xL/Jkt cells completely overcame the supernatant-induced decrease in $\Delta \psi_m$ ($P \leq 0.01$; Fig. 4). These findings indicated that the supernatant-induced apoptosis was associated with a very early decrease in mitochondrial transmembrane potential, which could be rescued by bcl-xL gene overexpression. These findings further confirm that at least one of the antiapoptotic effects of bcl-xL is mediated through the stabilization of transmembrane potential in mitochondria.

**DISCUSSION**

We previously showed that HFRPE cells were capable of suppressing the activation of human T cells by induction of apoptosis in a cell–cell contact-independent pathway. In this study we further confirmed these findings by showing that the supernatant isolated from HFRPE cells can induce apoptosis in Jkt cells in a HFRPE-free system. The supernatant-induced apoptosis was not secondary to medium starvation because the supernatant isolated from HFRPE cells promoted the proliferation of human umbilical vein endothelial cells (serving as control, data not shown), demonstrating that the supernatant-mediated apoptosis was a T-cell–specific process. This also indicated that HFRPE-induced apoptosis was mediated by soluble factors. These results are in contrast to the previous findings by Jorgensen et al., who suggested that HFRPE cell–mediated apoptosis was cell–cell contact dependent. This discrepancy may be explained by the fact that in their experimental system the supernatant isolated from HFRPE cells was diluted, which we have found to rapidly weaken its effect (data not shown).

The recent discovery that apoptosis can be induced in anucleated cells implies that cytoplasmic structures must control the apoptotic process. It has been shown that nuclear apoptosis is preceded by the disruption of the mitochondrial transmembrane potential. This disruption is mediated by the opening of permeability transition pores in the mitochondrial membrane. In this study, we showed that at early stages of apoptosis the HFRPE cell supernatant altered the mitochondrial membrane potential of Jkt cells. Further, we showed that the overexpression of bcl-xL gene prevented the disruption of mitochondrial transmembrane potential, leading to the rescue of Jkt cells from apoptosis. The function of bcl-xL as an ion channel in the outer mitochondrial membrane, which prevents its osmotic swelling, may explain the significant resistance of bcl-xL to cellular apoptosis.

Although the apoptotic process was rescued in bcl-xL/Jkt cells, their proliferation was not restored in the presence of HFRPE cell supernatant. For a better understanding of this block of proliferation we performed a cell cycle analysis, which provided us with detailed information regarding the different rounds of cellular division. Our results indicated that the supernatant isolated from HFRPE cells delayed the
FIGURE 3. HFRPE supernatant reduced the cell cycle progression in Jkt cells. (A) Neo/Jkt and (B) bcl-xL/Jkt cells were cultured for 96 hours with supernatant isolated from nonactivated (normal supernatant) and IFN-γ-activated HFRPE (IFN-γ supernatant). Two-color FACS analysis was performed on each day of culture to access the number of cell divisions and viability of Jkt cells. CSFE staining was used to assess the progression of the cell cycle (x-axis or FL1-H), and PI (y-axis or FL3-H) was used to assess the viability of Jkt cells. The percentage of dead cells (π-positive, top half) and viable cells (π-negative, bottom half) are indicated in the center of each panel. The results are a representative of three independent experiments.
cell cycle progression of Jkt cells by one generation. The supernatant isolated from IFN-γ-activated HFRPE cells induced an even longer delay (two generations) in proliferation of Jkt cells. This delay in the cell cycle progression explains the block of proliferation of Jkt cells after 48 hours in the absence of apoptosis when the antiapoptotic gene bcl-xL is overexpressed.

The supernatant isolated from IFN-γ-activated HFRPE cells possessed a stronger immune-suppressive effect than the supernatant isolated from nonactivated HFRPE cells. This may have significant clinical implications. In vivo, during an inflammatory reaction, the activated T cells would produce IFN-γ to further activate the surrounding immune cells. The presence of IFN-γ upregulates the immune suppressive behavior of the RPE cells, which in turn downregulates the inflammatory process, limiting the spread of destruction and maintaining the immune privilege state.

In summary, we showed that the supernatant isolated from HFRPE cells suppressed the proliferation of Jkt cells by induction of apoptosis. This is further evidence that HFRPE-induced apoptosis is mediated by soluble factors. The supernatant-induced apoptosis involved the disruption of the mitochondrial transmembrane potential. Although the overexpression of bcl-xL gene rescued the supernatant-mediated apoptosis, it was not capable of restoring the cellular proliferation. This suggests that the HFRPE supernatant-induced cell cycle arrest may precede the induction of apoptosis. RPE cells may therefore play an active role in maintaining the immune privilege in the subretinal space by secreting a factor that leads to cell cycle arrest and apoptosis in T cells.

### TABLE 1. Analysis of the Jkt Cell Cycle Progression

<table>
<thead>
<tr>
<th></th>
<th>Control* (%)</th>
<th>Supernatant Isolated from Nonactivated HFRPE Cell (%)</th>
<th>Supernatant Isolated from IFN-γ-Activated HFRPE Cell (%)</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>72 h</td>
</tr>
<tr>
<td>A. Neo/Jkt cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent</td>
<td>99.7</td>
<td>79.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Gen 2</td>
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<td>20.3</td>
<td>21.1</td>
</tr>
<tr>
<td>Gen 3</td>
<td>0.2</td>
<td>0</td>
<td>77.8</td>
</tr>
<tr>
<td>Gen 4</td>
<td>0.1</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>B. Bcl-xL/Jkt cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent</td>
<td>99.6</td>
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<tr>
<td>Gen 4</td>
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<td>0.3</td>
<td>0</td>
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* Standard culture medium.

The analysis of cell cycle progression with the ModFit program of the CSFE staining data collected in Figure 3 indicated a decrease in cell cycle progression of both Neo/Jkt and bcl-xL/Jkt cells. The generation of the majority of cells from Neo/Jkt or bcl-xL/Jkt cells after incubation for 96 hours with either standard medium or supernatant isolated from nonactivated or IFN-γ-activated HFRPE cells is shown in bold numbers. The data are a representative of three independent experiments. h, hours.

### FIGURE 4. Apoptosis of Jkt cells by HFRPE cell-derived supernatant is associated with a loss in mitochondrial transmembrane potential. Mitochondrial transmembrane potential as reflected by DiOC(6) staining of Jkt cells was determined after coculture with supernatant isolated from nonactivated (normal supernatant) and IFN-γ-activated HFRPE (IFN-γ supernatant). Mitochondrial transmembrane potentials of Neo/Jkt cells were significantly decreased over the 36-hour incubation time in the presence of the supernatants isolated from IFN-γ-activated HFRPE cells (P < 0.01). DiOC(6) staining was analyzed on FL1-H and the percentage of cells with decreased staining is indicated in the center of each panel. The y-axis (counts) shows the cell number. The results depicted are a representative of three independent experiments.
References