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Morphological and biochemical changes during formocresol induced cell death in murine peritoneal macrophages: apoptotic and necrotic features

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Abstract The present study was conducted to investigate the role of Formocresol (FC)-induced apoptosis and necrotic cell death in murine peritoneal macrophages (pMø). Macrophages were cultured with 1:100 FC for 2 to 24 h. The viability (trypan blue assay), cell morphology (scanning electronic microscope), and apoptotic and necrotic indexes (light and fluorescent microscopy) were determined at different scheduled times. Simultaneously, the expressions of proteins related to stress, survival, and cell death were measured by western blotting. FC-exposed macrophages exhibited maximal apoptosis from 2 to 6 h, coincident with Bax overexpression ($P<0.001$). Additionally, Bcl-x_L showed maximal expression between 12 and 24 h suggesting its survival effect in pMø. The lowest pMø viability and the increment of the necrotic rate from 4 to 12 h were observed in accordance to Fas and Hsp60 overexpressions. In summary, all the experimental data suggest that two different pathways emerge in pMø exposed to FC, one leading Bax-dependent apoptosis (2–6 h) and the other one favoring necrosis (4–18 h), related to Fas-receptor and Hsp60 stress signal.

Keywords Macrophages · Formocresol · Apoptosis · Bax · Necrosis · Fas

Introduction

Biological effects of Formocresol (FC) are still under debate by several authors, and concerns about the safety of FC have been appearing in the dental and medical literature for more than 20 years (Lewis and Chestner 1981; Yodaike 1981; Perrera and Petito 1982). Its safety has also been questioned because it is known to have a toxic, mutagenic, and carcinogenic potential (Lewis 1998; Hagiwara et al. 2006). Accumulation of FC has been demonstrated in the pulp, dentin, periodontal ligament, and bone surrounding the apexes of pulpotomized teeth (Fulton and Ranly 1979). In addition, this drug suppresses cellular metabolism, acting as a cytotoxic agent to account for the tissues fixation (Loos and Han 1971). In fact, the International Agency for Research on Cancer of the World Health Organization reclassified formaldehyde as a known human carcinogen. Hereby, European pediatric dentists reject FC clinical use. However, American pediatric dentists use FC full strength (19% or 48.5% formaldehyde) in primary tooth pulpotomy (Casas et al. 2005). Thus, the major motivation of this study was to explore the effects of FC on macrophage cell death.

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Macrophages (Mø) participate in a number of (patho) physiological settings due to high plasticity of their functional responses (Gordon 2003). They are involved in chronic inflammation and the repair of pulpal as well as periapical tissues (Toriya et al. 1997; Kawashima et al. 1996).

It has been reported that in murine peritoneal macrophage (pMø) FC interferes with the adhesion of membrane macromolecules, reducing the phagocytic and adhesion activities (Segura et al. 1998). Moreover, the cytotoxic effects of FC have been studied in vitro in a variety of systems (Hagiwara et al. 2006; Ramos et al. 2008), while in vivo, FC treatment promotes necrosis and inflammatory reaction in the connective tissue (Sant' Anna et al. 2008). Apoptosis and necrosis have traditionally been addressed as two forms of cell death that exhibit very distinctive biochemical and morphological characteristics (Majno and Joris 1995), but these processes are currently viewed as two extremes of a continuum of possible types of cell demise (Denecker et al. 2001). Programmed cell death (apoptosis) is essential for homeostatic inflammation, playing an important regulatory function upon Mø (Jacobson 1997).

Generally, two signaling pathways are known to lead to the apoptotic fate of the cells: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. Ligation of death receptors (such as Fas/CD95 or TNF receptor) with their respective ligands recruits the adapter protein FADD and activates death proteases (Krammer 1999). The mitochondrial apoptosis pathway can be driven by many proteins of the Bcl-2 family (Adams and Cory 1998). The anti-apoptotic molecules Bcl-2 and Bcl-x_L prevent the translocation of cytochrome *c* from the mitochondria, while the induced expression of Bax results in mitochondrial dysfunction, leading to cytochrome *c* release. Activation of both pathways triggers and amplifies a cascade of executioner caspases that, after cleavage of vital death substrates, leads to the final demise of the cells (Marsden and Strassen 2003).

On the other hand, the intracellular chaperone heat shock protein (Hsp60) is induced from monocytes/macrophages undergoing necrotic or apoptotic cell death after infection, trauma, and neoplasia (Zheng et al. 2004).

The aim of this study was to examine in vitro the primary mechanism involved in FC macrophage injury (necrosis and/or apoptosis) through cellular morphological descriptions and the analysis of pro-apoptotic and survival proteins.

Materials and methods

Experimental design

Peritoneal murine macrophages were taken from four 2-month-old CF-1 Swiss mice (26–28 g) from the Animal Facilities at the National Northeast University, Argentina. All experimental procedures were conducted according to the principles in the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health (NIH), Bethesda, MD, 1996). Mice were killed by ethyl ether inhalation, followed by a peritoneal lavage with 5 ml of phosphate buffer saline pH 7.2 (PBS), after i.p. injection of 1 ml 3% thioglycollate broth (Difco, Detroit, MI) 3 days before the experiment. Cells were seeded in a culture medium (MEM, Alpha Modification Sigma Co.) supplemented with 10% fetal bovine serum (FBS/Genser, Argentina), 2 mM glutamine, 30 mM HEPES, 0.4% sodium bicarbonate, and penicillin–streptomycin (100 IU/ml and 100 μg/ml) and plated onto glass cover slips and tissue culture tubes. After 60 min incubation at 37°C 5% CO_2, unattached cells were removed and adherent cells were left overnight in a 37°C cell culture incubator prior to FC exposure. Mø enrichment was monitored by nonspecific esterase staining and was ≥95%. Viability was monitored by trypan blue exclusion and was routinely ≥95%. Thereafter, the cells were seeded in the absence or presence of FC (1:10, 1:100 and 1: 1,000 in PBS) for 0, 2, 4, 6, 12, 18, and 24 h. The selected concentration of FC 1:100 (Buckley's formulation, Sultan Chemists Inc, Englewood, NJ), was calculated from the maximum nonlethal dose applied in primary human teeth (Segura et al. 1998).

Cell viability

Adherent pMø were directly stained with 0.4% trypan blue dye (Sigma Co) in a 1:1 ratio after exposure to FC (0–24 h). Two hundred cells were counted in triplicate, and the percentage of viable cells was calculated at indicated time points of the protocol.

Morphological analysis using May Grünwald-Giemsa

Untreated or FC-exposed pMø cells were examined with a light microscope for morphological changes, as previously described (Aquino Esperanza et al. 2008). Briefly, adhered pMø cells were directly stained with May Grünwald-Giemsa (MGG), and 500 cells were counted. Results are expressed as apoptotic or necrotic percentage at indicated time points of the protocol.

Morphological analysis using fluorescence microscopy

Acridine orange/ethidium bromide staining (AO/EB) was performed as described previously (Lopes et al. 2001). Briefly, 2×10^6 pMø cells were stained with 200 μl of a combined dye of 100 μg/ml AO and 100 μg/ml EB (Sigma Co). Cells were observed under a fluorescence microscope (Olympus CX-35 microscope equipped with a Y-FL epifluorescence attachment and an Olympus Coolpix Digital Camera). Three hundred cells were counted per sample, and they were classified as follows: viable, apoptotic, and necrotic cells. Results are expressed as apoptotic or necrotic percentages at indicated time points of the study.

Scanning electronic microscopy

Direct observation of pMø morphological features (cellular shape and plasma membrane aspect) in controls and FC-exposed cells was essentially performed as previously described (Juaristi et al. 2007). Cells were dehydrated and critical-point dried (Dento Drier). They were coated with gold-palladium for 3 min. Samples were observed with a scanning electronic microscope (SEM) (Jepl JSM-5800 LV) and images (×1.500–2,500) were obtained in triplicates at indicated time points of the protocol.

Western blotting analysis

Fas, Bax, Hsp60, and Bcl-x_L western blots were performed as previously described (Aguirre et al. 2005). At each time period after FC treatment, control and treated pMø cells ($2\text{-}4\times10^6$) were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 2.5 mg/ml deoxycholic acid, 1 mM EDTA, 10 μg/ml Nonidet-40, pH 7.4, 2.5 mM supplemented with protease inhibitors: 2.5 μg/ml leupeptin, 0.95 μg/ml aprotinin, and 2.5 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 14,000×*g* for 20 min, and the protein concentration in the supernatant was determined by Bradford method (Bradford 1976).Whole lysate (20 μg) was separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes (Bio-Rad), and probed with primary anti-Fas (sc-7886), anti-Bax (sc-6236), anti-Hsp60 (sc-1722), and anti-Bcl-x_L (sc-7122) (Santa Cruz Biotechnology Inc., Santa Cruz.), followed by incubation with secondary antibodies (horseradish peroxidase-conjugated anti-IgG: CN111-035-045 and CN 705-035-147) (Jackson Immunoresearch Inc, USA). An antibody against β-tubulin (Sigma-Aldrich) was used as an internal loading control. Immunocomplexes were detected by an Opti4CN kit (Bio-Rad) and quantified by densitometric analysis using the NIH-image system.

Statistical analysis

Each experiment was performed in triplicate. Data were expressed as mean ± SEM. They were statistically analyzed using a one-way ANOVA. A significant difference from the control was determined by Tukey's test or Dunnett's *t* test (Graph Pad Software Inc., San Diego, USA). p values <0.05 were considered significant. Correlation analyses were performed using Spearman rank correlations test.

Results

Cytotoxicity

To evaluate FC cytotoxic effects in pMø cells, we monitored the viability with trypan blue exclusion assay at different time points. As shown in Fig. 1, 90% of control cells were viable for 24 h of incubation. In contrast, a significant decrease of viable cells was found in FC-exposed pMø cells after 4 to 12 h ($p<0.001$), suggesting a cytotoxic effect at this particular period.

Morphological changes in pMø cells induced by FC

To evaluate the morphological changes induced by FC in pMø, we examined samples through SEM. Cells in the control group were typically star shaped with filopodium emission accompanied by cytoplasm projections (Fig. 2a). Apoptotic cells, with characteris-

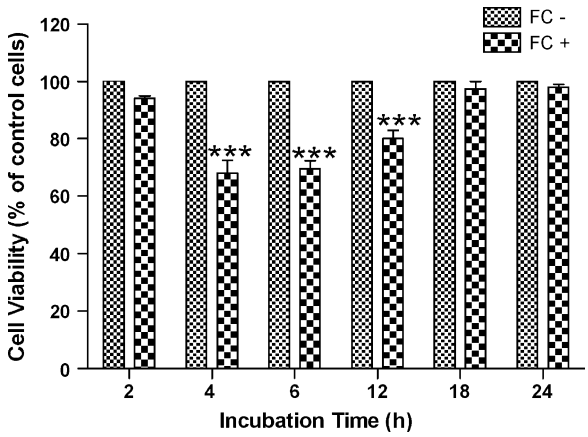

Fig. 1 Macrophage viability upon FC treatment. Cellular viabilities of pMø cells treated and untreated with FC (FC+ or FC−, respectively) for 24 h were determined using a trypan blue dye exclusion assay. Significant percentages of death cells were observed in FC-treated pMø cells from 4 to 12 h of incubation. ***$p<0.001$ indicates significant differences from the control. All results are shown as mean ± SEM from minimum three separate experiments

tic plasma membrane blebbing that resembles apoptotic bodies, are shown at 4 h of incubation (Fig. 2b). A direct loss of the membrane integrity was assumed as necrotic damage. This was the prevalent process following 6 h of incubation (Fig. 2c). Afterwards, 24 h FC-treated pMø cells exhibited stellate morphology, resembling the control group (Fig. 2d, e). Interestingly, pMø exposed to FC revealed both kinds of cell death, necrosis, and apoptosis, between 4 and 6 h post treatment.

Evaluation of apoptosis with MGG and double fluorescence staining (AO/EB)

FC-induced apoptosis was observed by examining the morphology of the cell and its changes after MGG and AO/EB staining.

Morphological features of apoptosis by MGG (Fig. 3a, upper panel) were detected from 2 to 6 h of incubation ($p<0.01$ and $p<0.05$, respectively).

Apoptosis was detected early in pMø-FC- exposed cells with AO/EB staining (Fig. 3a, lower panel). Experimental values were significantly increased from 2 to 4 h of incubation (7.1±0.4 and 6.5±0.5% $p<0.001$, respectively), showing that their maximal levels were four times higher than the control (1.5± 0.25%) and remained elevated until 6 h of incubation ($p<0.05$). Thereafter, no significant increase in the number of apoptotic cells was observed. These results indicated that FC triggers a classic apoptotic process within the first 6 h of incubation.

Evaluation of necrosis with MGG and double fluorescence staining (AO/EB)

Morphological characterization of FC-induced necrosis in pMø cells was examined with MGG and a double fluorescent dye (AO/EB) staining method. A significant increase in necrotic cells stained with MGG (Fig. 4a, upper panel) was observed following a 4 to 18 h FC exposure (17±0.2, $p<0.00$ l and 16.2±1.0%, $p<0.05$, respectively) versus control cells (5±0.35%). Moreover, pMø stained with AO/EB (Fig. 4a, lower panel) showed significant increments of the necrotic indexes from 4 to 24 h upon FC treatment (28±1.5%, $p<0.001$, and 17±1.1% $p<0.05$, respectively). These findings suggest that FC induces necrotic cell death as a late cellular effect.

Bax and Bcl-x_L expressions in pMø cells exposed to FC

Bax expression (Fig. 5a) was strongly induced in pMø during FC exposure showing a maximum at 2 h ($p<0.001$). It remained at a high level throughout 6 h ($p<0.001$) and decreased to control level by 12 h. In contrast, the intracellular levels of the anti-apoptotic Bcl-x_L protein (Fig. 5b) remained unchanged until 12 h ($p<0.05$). It reached a maximum by 18 h and sustained its expression up to 24 h ($p<0.001$). These results suggest that FC-exposed pMø induces the intrinsic apoptotic pathway throughout Bax expression (2 to 6 h). In addition, the overexpression of the anti-apoptotic protein Bcl-x_L from 12 to 24 h seemed to be associated to pMø survival.

Fas and Hsp60 expressions in pMø cells exposed to FC

Stress may stimulate the expression of Fas, which can trigger the extrinsic apoptotic pathway. Immunoblottings were performed in order to examine if necrosis induced in FC-exposed pMø was accompanied by Fas and Hsp60 overexpression.

Fas expression was detected at 4 h of incubation (Fig. 6a), continued to increase up to 18 h ($p<0.01$ and

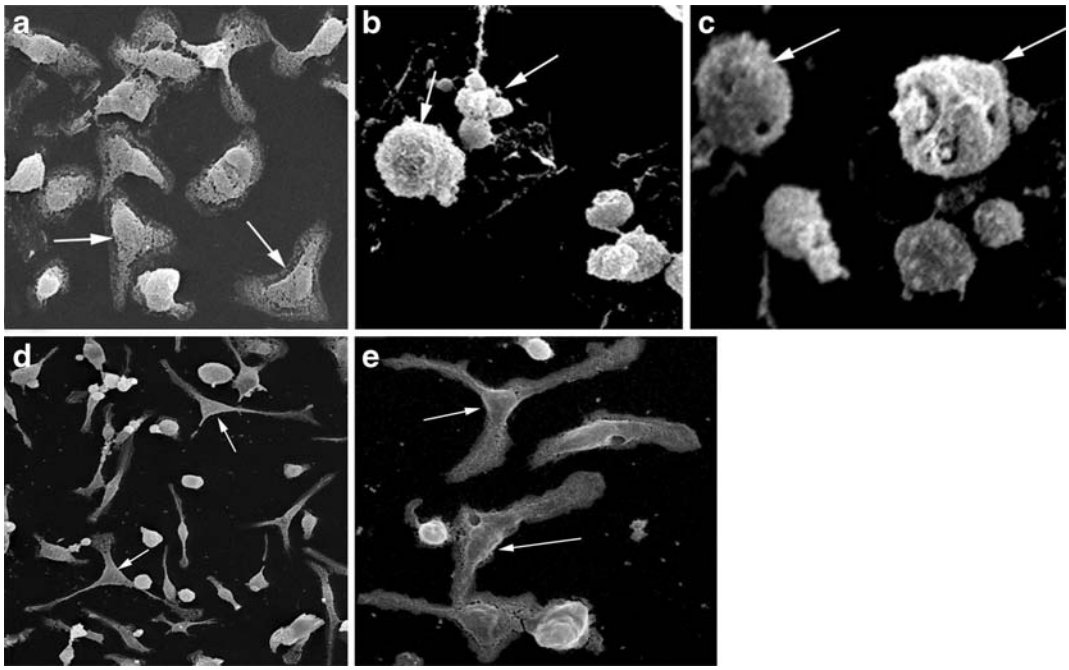

Fig. 2 Morphological features of pMø cells exposed to FC. Representative scanning electronic microscopic images (×2,500) from pMø samples are shown: **a** control cells; **b**, **c** cells exposed to FC at 4 and 6 h, respectively, and for 24 h in absence (**d**) or presence of FC (**e**). A typical star shape with filopodia emission accompanied with cytoplasm projections can be seen in control cells. Apoptotic cells with characteristic plasma membrane blebbing are shown at 4 h (*arrows*). Necrotic cells with typical plasma membrane disruption were identified at 6 h (*arrows*). Thereafter, pMø cells exposed 24 h to FC seem to recover the ability to generate filopodia in a similar way to control samples. *Scale bar* 20 μm

$p<0.001$, respectively), and decreased at 24 h ($p<0.05$). Interestingly, Fas overexpression was in agreement with the maximal necrotic values previously shown.

In addition, the Hsp60 expression was induced at 6 h of incubation ($p<0.05$), peaked at 12–18 h ($p<0.001$), and remained elevated at 24 h ($p<0.01$). This induced protein expression was delayed compared to Fas but showed a similar pattern linked to necrotic values as shown in Fig. 6b. These findings suggest that FC induces necrosis in pMø through a process related to Fas overexpression and Hsp60 (stress signal).

Discussion

FC has been used as acceptable antimicrobial and fixative properties that preserve periapical integrity for many years (Smith et al. 2000; Hill 2007). However, components of FC formulation, especially formaldehyde, have controversial properties, being implicated in the occurrence of periapical changes, alteration of the healing process, systemic distribution in animal organs, and mutagenic effects (Block et al. 1983; Soffritti et al. 2002; Ramos et al. 2008).

Nevertheless, studies using lymphoma cell line and cultured human fibroblast exposed to different dilutions of FC similar to clinical doses did not produce detectable DNA damage and genotoxicity (Ribeiro et al. 2005, 2006; Da Silva et al. 2007).

Macrophages are crucial for host defenses, but they are also able to damage surrounding tissues. Due to their central role in the pathogenesis of the inflammatory response, these cells are relevant in their use for testing the toxicological responses to different biomaterials (Schuster et al. 1996).

The cell culture systems, commonly used as cytotoxicity tests, had cell death as end point, although they did not differentiate between apoptosis and necrosis mechanisms (Schweikl and Schmalz 1996). We performed the present study on murine macrophages using 1:100 FC, which is similar to that found in periapical tissue (Segura et al. 1998); this particular dose was used for the other experiments in

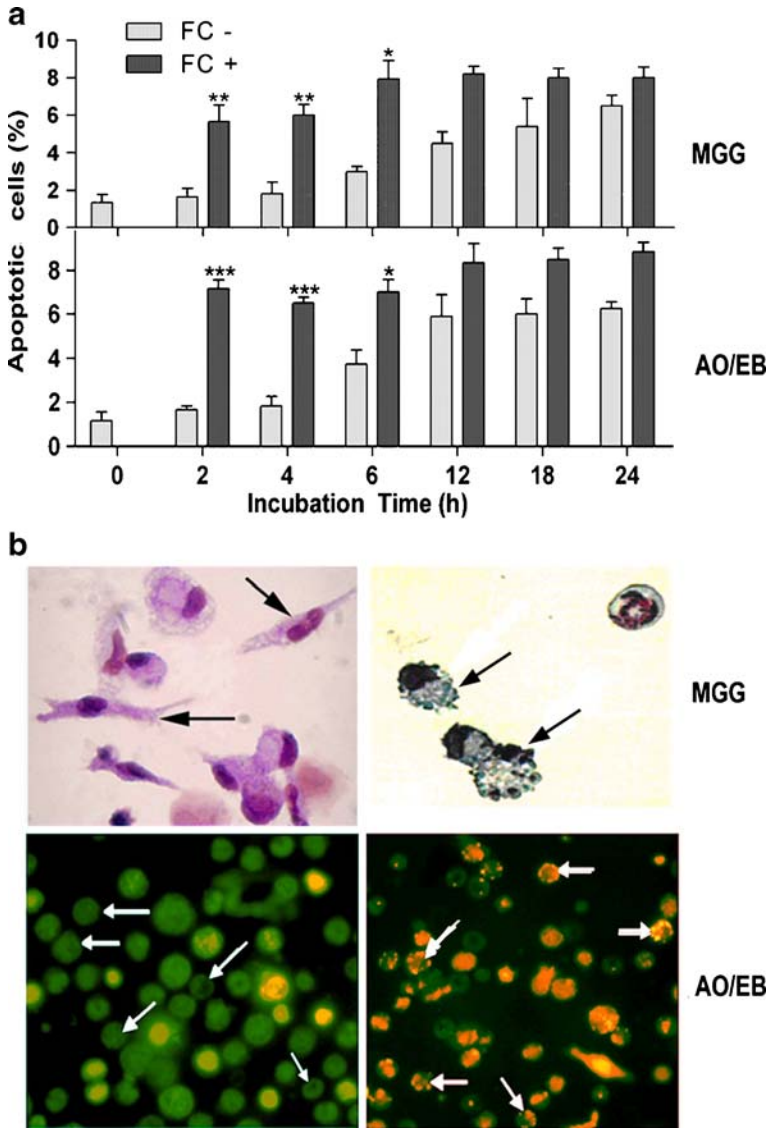

Fig. 3 Macrophage apoptosis upon FC treatment. **a** Changes in apoptotic percentages of pMø cells in the presence or absence of FC (FC+ or FC−, respectively). *Bars* represent percentages of apoptotic cells stained with MGG (*upper chart*) and AO/EB (*lower chart*) at each time point. All results are shown as mean ± SEM from three separate experiments. *Asterisk* depicts FC treatment significantly different from the control; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **b** Representative pictures (×400) of untreated (*left panel*) and 2 h FC-exposed pMø cells (*right panel*). *Arrowheads* in MGG images (*upper panel*) of control pMø cells show a characteristic star shape, with typical long filopodia. However, FC-treated cells display plasma membrane blebbing with chromatin condensation (*arrows*). AO/EB pictures (*lower panel*) show control pMø cells with uniform bright green nuclei and a well-organized cell structure (*arrows*). FC-treated pMø cells show morphological apoptotic features: bright orange condensed chromatin and nuclear fragmentation (*arrows*)

this study. In fact, we made all the experiments using more than 60–90% viable cells.

Studies on the effect of FC in pMø showed that cell viability decreased between 4 and 12 h, and the percentages of viable cells were similar to control values after 18 h of incubation upon FC. This particular response can be attributed to a viable pool of pMø population that remained available to express the anti-apoptotic protein Bcl-x_L, related to survival and viability of the cells.

The most direct and dramatic consequence in pMø following FC exposure is an altered bioactivity pattern that affects the phagocytic process (Segura et al. 1998). In accordance with these studies, pMø SEM images clearly showed the disruption of cell shape, suggesting an apoptotic or necrotic cell pattern. This process was maximal and concomitant between 4 and 6 h of incubation, and as a consequence, we found the most abrupt shape alterations in pMø cells. Thereafter, pMø cells FC-exposed for 24 h exhibit stellate morphology resembling those observed in control.

Cell death occurs in two ways (apoptosis and necrosis). Apoptosis is a major form of cell death, and it is an important process in a variety of different biological systems, embryonic development, and in

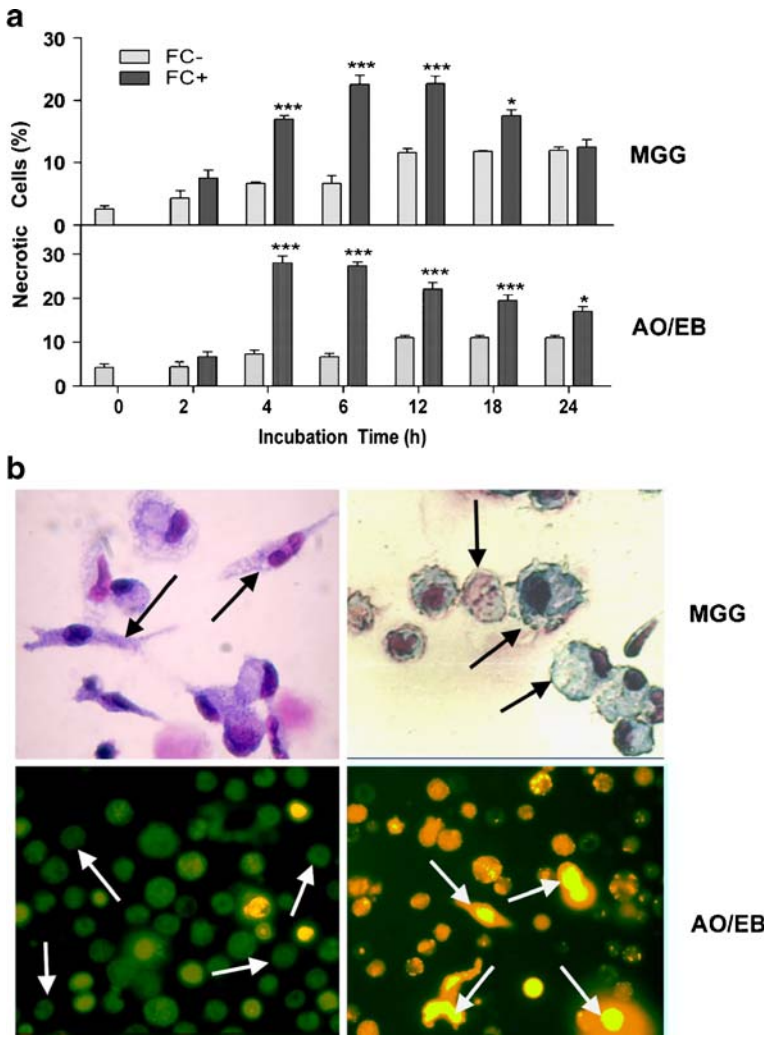

Fig. 4 Macrophage necrosis upon FC treatment. **a** Changes in necrotic percentages of pMø cells in the presence or absence of FC (FC+ or FC–, respectively). *Bars* represent percentage of necrotic cells stained with MGG (*upper chart*) and AO/EB (*lower chart*) at each time point. All results are shown as mean ± SEM from minimum three separate experiments. *Asterisk* depicts FC treatment significantly different from the control; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **b** Representative pictures (×400) of untreated (*left panel*) and 6 h FC-exposed pMø cells (*right panel*), stained with MGG and AO/EB. *Arrowheads* in MGG images (*upper panel*) of control pMø cells show a characteristic star shape, with typical long filopodia. FC-treated cells display cellular swelling with cytoplasmic or nuclear membrane disruption/lysis (*arrows*). AO/EB pictures (*lower panel*) show control pMø cells with uniform bright green nuclei and a well-organized cell structure. FC-treated pMø cells show morphological necrotic features: uniformly orange to red nuclei and a well-organized cell structure (*arrows*)

chemically induced cell death (Cohen 1997). In contrast to apoptosis, cell death by necrosis is typically associated with inflammation, and this difference is related to the activation or maturation of phagocytic cells, like Mø. Although the interaction between apoptotic and phagocytic cells induces an anti-inflammatory response (Fadok et al. 2000), necrosis appears to be critical for the initiation of the immune response (Holler et al. 2000; Sauter et al. 2000).

Significant increases in the number of apoptotic cells were observed in FC-treated pMø cells, from 2 to 6 h. These results were coincident using two different staining methods; the AO/EB tinction was more sensitive than MGG, as it has been previously reported (Leite et al. 1999; Gasiorowski et al. 2001).

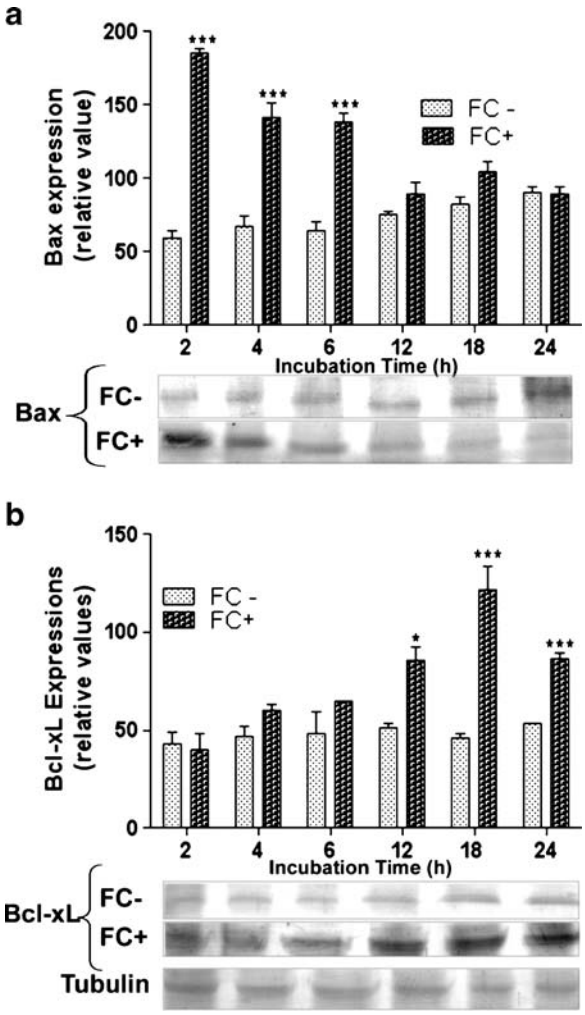

Fig. 5 Bax and Bcl-x_L expressions in pMø cells cultured with FC. Peritoneal macrophages were obtained at indicated time points and cultured in the presence or absence of FC (FC+ or FC−, respectively). Cell lysates were subjected to western blotting analyses as described in "Materials and methods." Representative blots of three independent experiments are shown. Tubulin levels were assayed to monitor protein-loading levels between samples. **a** Bax expression in FC+ pMø cells was strongly induced from 2 to 6 h. **b** Bcl-x_L showed a sustained overexpression in FC+ pMø cells from 18 h of incubation until the end of the experience. Results represent mean ± SEM of three separate experiments. *Asterisk* depicts treatment significantly different from the control; *$p<0.05$, **$p<0.01$, ***$p < 0.001$

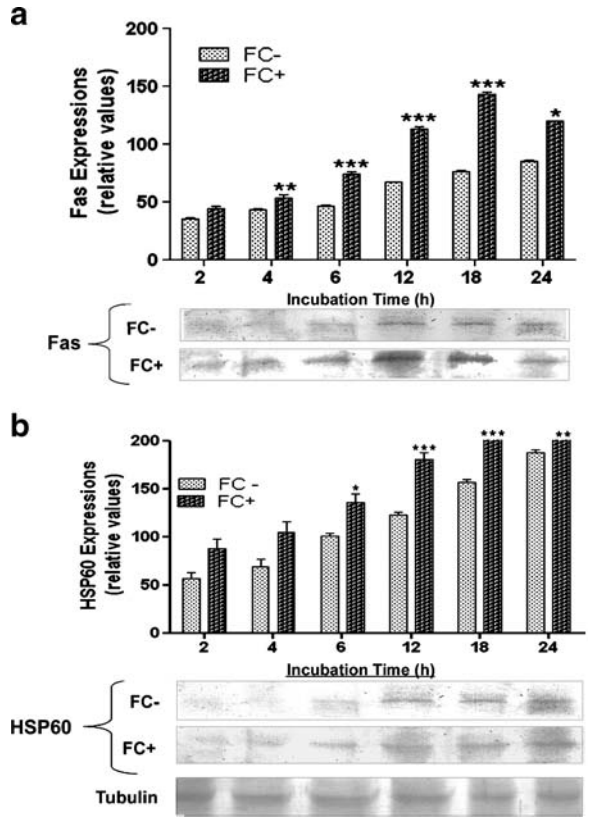

Fig. 6 Fas and Hsp60 expressions in pMø cells cultured with FC. Peritoneal macrophages were obtained at indicated time points and cultured in the presence or absence of FC (FC+ or FC−, respectively). Cell lysates were subjected to western blotting analyses as described in "Materials and methods." Representative blots of three independent experiments are shown. **a** Fas expression in FC+ pMø cells was noticeable from 4 h, showing maximum levels at 18 h of incubation. **b** Hsp60 expression in FC+ pMø cells enhanced at 6 h, peaked at 12–18 h, and remained elevated until 24 h. Results represent mean ± SEM of three separate experiments. Representative blots of three independent experiments are shown. Tubulin levels were assayed to monitor protein-loading levels between samples. *Asterisk* depicts treatment significantly different from the control; *$p<0.05$, **$p<0.01$, ***$p<0.001$

It is well known that, at morphological level, necrosis is quite different from apoptosis. During this process, the cells first swell, then the plasma membrane collapses, and subsequently, the cells are rapidly lysed. Our observations were concurrent with the necrotic pictures of FC-treated pMø, between 4 to 18 h (MGG stained cells), and similar results were seen with AO/EB staining from 4 to 24 h. This fact might indicate that FC induces in vitro apoptosis and necrosis in pMø as it was demonstrated by SEM, MGG, and AO/EB.

The necrotic and apoptotic cell response may depend on a balance between pro- and anti-apoptotic members of the Bcl-2 family. It is widely known that the relationship between survival factors and death factors dictates the fate of the cell. Particularly, the increased expression of Bax is known to stimulate

apoptosis. Variations in Bax/Bcl-x_L ratio are indicative of the predominance between apoptosis and survival processes. It has been communicated that this ratio is mainly affected by Bcl-x_L up regulation rather than substantial decreases in Bax expression after cytotoxic administration (Zhang et al. 2000). However, depending on the cell type, cellular context, or stimuli, a cell dies by apoptosis or necrosis (Hetz et al. 2002a).

Our findings showed that, after FC exposure, reduced cell viability in pMø cells is concomitant with an increased expression of pro-apoptotic proteins such as Bax, which is also in agreement with a higher apoptotic rate. Furthermore, the absence of the activation of the Bcl-x_L pathway implies an activation of this process.

To the best of our knowledge, FC induces in pMø the apoptotic process through Bax expression. In addition, our results revealed that a significant increase of necrosis, as a late cell process in pMø cells, was observed following 4 to 24 h FC treatment, in agreement with a previous communication (Sant' Anna 2008).

Simultaneous activation of necrotic and apoptotic pathways leading to cell death within the same pMø population cell was observed from 4 to 6 h, and this process is likely to occur fairly frequently as previously described (Hetz et al. 2002b). It has been shown that a depletion of intracellular ATP concentration may serve as one possible mechanism permitting the switch from apoptotic to necrotic cell death (Leist et al. 1997).

These results clearly show the coexistence of increased expressions of Fas and Hsp60 from 6 to 18 h with similar patterns. Like Fas, the Hsp60 overexpression was coincident with maximal necrosis period, suggesting that Fas receptors could be related to the induction of necrosis, as it was also demonstrated for lymphoid cells (Holler et al. 2000). The initiation of signaling pathways via Fas that lead to both apoptotic and necrotic cell death has been described in murine L929 fibrosarcoma cells, whereas necrosis was observed downstream of Fas (Vercammen et al. 1998).

These findings suggest that in pMø cells, FC induces necrosis in a coordinated process linked to the extrinsic factor Fas, with another danger signal, Hsp60. In addition, the overexpression of the anti-apoptotic protein Bcl-x_L from 12 to 24 h of incubation could be associated to macrophage differential function and survival, in accordance with the viability observed at the same incubation time points (Lakics et al. 2000). Interestingly, we observed that the co-expression of Bcl-x_L cannot suppress the necrotic programs, in agreement with previous reports (Adams and Cory 1998).

In summary, experimental data show that two different pathways emerge in pMø exposed to FC, one leading Bax-dependent apoptosis (2–6 h) and the other one favoring necrosis (4–18 h) related to Fas-receptor activation and the overexpression of Hsp60.

This study reveals that the pro-apoptotic proteins that were substantially elevated, at the particular period of time, dictate the faith of cell death. Therefore, apoptosis along with necrosis may be considered as a form of the execution phase of programmed cell death and could provide the dangerous signal that potentiates the Mø response. Thus, FC treatment in pediatric dentistry must be reconsidered.

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