Galectin-3 overexpression protects from cell damage and death by influencing mitochondrial homeostasis

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Abstract Galectins are a family of proteins involved in several cell processes, including their survival and death. Galectin-3 has in particular been described as an anti-apoptotic molecule entangled with a number of subcellular activities including anoikis resistance. In this work we partially address the mechanisms underlying this activity pointing at two key factors in injury progression: the alteration of mitochondrial membrane potential and the formation of reactive oxygen species. Overexpression of galectin-3 appears in fact to exert a protective effect towards both these events. On the basis of these data, we propose a reappraisal of the role of galectin-3 as a regulator of mitochondrial homeostasis.

Key words: Galectin-3; Oxidative balance; Cell injury; Cell death

1. Introduction

Galectins are a growing family of evolutionarily conserved proteins widely distributed in nature from lower invertebrates to mammals [1]. It is composed of at least 10 different members [2]. Among them, galectin-3 was extensively studied. This is a monomer with M_r 30 000 with a high affinity for β-galactosides mainly entangled in cell adhesion processes through recognition of, and combination with, complementary glycoconjugates [3,4]. In addition, galectin-3 was also described as a versatile multifunctional protein involved in multiple biological processes including either cell growth or cell death [4]. In fact, intracellular synthesis of galectin-3 is relevant to the proliferation state [5] as well as to pre-mRNA splicing [6] or to cell cycle and negative regulation of apoptotic mechanisms [7–8]. More in general, galectin-3 appears to be involved in several human pathologic processes including cancer as well as infectious diseases. In fact, galectin-3, being involved in cell–cell and cell–matrix interactions, has been proposed to play a role in the pathogenic mechanisms of metastasis [9]. On the other hand, a role for such a molecule has also been hypothesized in the initial stages of the human immunodeficiency virus (HIV) infection process, most likely due to its activity on the transport and/or splicing of HIV mRNA [10].

To address the role of galectin-3 in cell pathology, an experimental study on galectin-3 overexpressing cells has thus been undertaken. We have studied the effects of tumor necrosis factor alpha (TNF-α), a cytokine which provokes receptor-mediated damage and apoptosis [11], and menadione, a quinone able to induce necrosis by cell lysis [12]. Redox imbalance is implicated in the mechanism of action of both compounds [13,14]. Our results show that galectin-3 overexpression is able to protect the cells from injury and death induced by both menadione and TNF-α by reducing intracellular ROS generation and influencing mitochondrial membrane potential ($\Delta\Psi$).

2. Materials and methods

2.1. Production and maintenance of stable galectin-3 transfectants

The human breast cancer cell line Evsa-T was obtained from Dr. J.A. Foekens (Rotterdam, The Netherlands). Purified plasmid DNA was used to transfect Evsa-T cells by the calcium phosphate method as previously reported [15]. Two days after transfection, cells were selected by culturing them in the presence of 1 mg/ml of G418 for at least 2 weeks. Different clones were maintained at 37°C in Dulbecco's modified Eagle's medium containing 2% glutamine, 10% fetal calf serum and antibiotics (Flow, Irvine, UK). Three of these clones, termed EV-M13, EV-M5 and EV-M10, were selected. The expression of galectin-3 in the EV-M13 clone, as well as in Evsa-T parental cells, was very low. By contrast, clone EV-M10 expressed high levels of this lectin [15]. We, thus, decided to use EV-M13 cells as appropriate controls. Moreover, the EV-M5 clone expressing intermediate levels of galectin-3 also displayed an intermediate 'behavior' with respect to other cell clones considered. Hence, these results, although of importance, have been omitted.

2.2. Cell treatments

2.2.1. Menadione treatments. The culture medium was changed with a buffer solution (phosphate-buffered saline containing 1 mM $MgCl_2$ and $CaCl_2$, pH 7.3) and the cells were treated with 200 μM menadione (Sigma, St. Louis, MO, USA) diluted in dimethylsulfoxide (DMSO, Sigma). Cells treated with an equal volume of DMSO for the same period of time but without menadione, were considered as controls.

2.2.2. TNF-α treatments. Cells were exposed to sequential treatment of 4 mM cycloheximide (CHX, Sigma) for 2 h followed by 50 IU TNF-α (Sigma, final purity > 95%) as previously described [11].

For qualitative and quantitative studies of cell injury and death, cell treatments were performed for 4 h. For the analysis of early events ($\Delta\Psi$, reactive oxygen species (ROS) evaluation and glutathione (GSH) intracellular levels) treatments were performed for 3 h.

2.3. Scanning electron microscopy (SEM)

Control and treated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. Following post fixation in 1% OsO_4 for 30 min, cells were dehydrated through graded ethanols, critical point dried in CO_2 and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope.

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2.4. Evaluation of apoptosis and necrosis

Both untreated and treated cells were harvested, washed and double stained by using an annexin V-FITC apoptosis detection kit (Eppendorf s.r.l., Mi, Italy). This kit is based on the observation that soon after initiating apoptosis most cell types translocate the membrane phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Annexin V has a strong Ca^{2+} dependent affinity for PS and therefore serves as a probe for detecting apoptosis. Cells which have lost membrane integrity (therefore considered as necrotic cells) will show red staining (PI) throughout the nucleus and therefore will be easily distinguishable from the apoptotic cells. Samples were incubated for 10 min in the dark with annexin V and PI (40 μg/ml).

2.5. Measurements of intracellular ROS

Cells (5×10^5) were incubated in 490 μl of Hanks balanced salt solution (HBSS, pH 7.4) with 5 μl of hydroetidine (HE, Molecular Probes, Eugene, OR, USA) or dihydrorhodamine 123 (DHR 123, Molecular Probes) in polypropylene test tubes for 5 min at 37°C [16]. The final concentration of HE and DHR 123 were 1 and 10 μM, respectively. HE is a chemically reduced ethidium derivative. It is a non-fluorescent membrane-permeable dye that can be oxidized directly to the red fluorescent ethidium bromide by $O_2^{\bullet}$ generated inside the cells after different treatments. DHR 123 is a dye freely diffusing into cells, oxidized primarily by H_2O_2 in a myeloperoxidase dependent reaction to green fluorescence [17]. As DHR 123 is accumulated by mitochondria, we excluded possible interference in ROS evaluation by using carbonyl cyanide *p*-(trifluoro-methoxy) phenylhydrazone (FCCP, 20 μM Sigma) a protonophore able to depolarize mitochondrial membrane, as previously described [18]. Furthermore, according to literature data [19] we also confirmed the results concerning H_2O_2 production by using 2′,7′-dichloro-dihydrofluorescein diacetate (H_2DCFDA, Molecular Probes).

To exclude PI-positive cells from the analysis of H_2O_2 production we performed a double staining procedure by using H_2DCFDA/PI, whereas, to estimate the percentage of dead cells in samples stained with HE (which emits in FL2 channel like PI) we incubated parallel tubes with PI for 15 min at 37°C. However, in consideration of the low rate of dead cells (between 3 and 5%), these data will not be discussed further.

2.6. Determination of total intracellular GSH levels

Intracellular GSH content was evaluated by using 5-chloromethyl-2′,7′-dichloro-dihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes). CM-H_2DCFDA passively diffuses into living cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular GSH. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cells. Untreated and treated cells (5×10^5) were incubated with 10 μM of CM-H_2DCFDA for 15 min at 37°C in HBSS supplemented with 5 mg/ml of bovine serum albumin. Cells exposed to L-buthionine-[*S*,*R*]-sulfoximine, 7.5 μM (Sigma), a GSH depleting drug, for 16 h were considered as negative controls. PI-positive cells were excluded from our analysis.

2.7. ΔΨ

The $\Delta\Psi$ of control and treated Evsa-T cells was studied by using the probe JC-1. According to the method, cells were stained with 10 μg/ml of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1, Molecular Probes). JC-1 is a metachromatic probe able to enter the mitochondria selectively. It exists in a monomeric form (in the green channel, FL1) but, depending on the membrane potential, JC-1 can form J-aggregates that are associated with a large shift in the emission range (in the orange channel, FL2) [11]. JC-1 is both qualitative (considering the shift from green to orange) and quantitative (considering the 'pure' fluorescence intensity). As a methodological control, cells were treated with increasing concentrations (from 0.1 to 10 μg/ml) of K^+ ionophore valinomycin (Sigma) which dissipates $\Delta\Psi$ but not pH gradient. To verify cell viability parallel tubes were incubated with PI for 15 min at 37°C before analyses.

2.8. Data analysis and statistics

All samples were washed, cooled on an ice bath and immediately analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 488 argon laser. At least 20 000 events have been acquired. Data were recorded and statistically analyzed by a Hewlett Packard computer using the Lysys II Software. All data reported are the mean of four separate experiments ± S.D. Statistical analysis was performed by using Student's *t*-test. Concerning flow cytometry experiments, calculation of the fluorescence was carried out after the conversion of logarithmically amplified signals into values on a linear scale and the statistical significance was calculated by using the parametric Kolmogorov–Smirnov (K/S) test or non-parametric tests to analyze unimodal or bimodal curves, respectively. Only *P* values of less than 0.01 were considered as significant.

3. Results

3.1. Cell injury analysis

We have recently reported that galectin-3 overexpression is able to protect Evsa-T cells from apoptotic injury [15]. In this work we compared the effects of two different experimental toxins (TNF-α and menadione) by considering (i) $\Delta\Psi$ and (ii) by evaluating some parameters indicative of the redox state of the cells, i.e. ROS generation and GSH content. TNF-α is a cytokine which provokes receptor-mediated apoptosis while menadione is a superoxide producing quinone mainly inducing necrosis [11,14]. In Fig. 1a–f qualitative–descriptive analyses of this phenomenon performed by SEM are reported. With respect to controls (Fig. 3a,b), cells exposed to both CHX/TNF-α (Fig. 1c,d) or menadione (Fig. 1e,f) showed the typical sequence of changes finally resulting in cell detachment and death. However, although similar in principle, remarkable differences were detected between the two cell clones. In fact, cell retraction, rounding, blebbing and, finally, detachment from the substrate (not shown) were easily detected in EV-M13 cells (Fig. 1a,c,e; left panel) while these alterations were only partially visible in the EV-M10 clone (Fig. 1b,d,f; right panel). In these cells, expressing high levels of galectin-3, stable cell–cell and cell–substrate interactions were in fact maintained without any sign of rounding or detachment (Fig. 1d,f). For a quantitative evaluation of this phenomenon, a cytometric analysis of cells double stained with annexin V-FITC/PI to distinguish apoptosis from necrosis, was then performed (Fig. 1g). In fact, confirming SEM observations, the EV-M13 clone (low levels of galectin-3), with respect to the EV-M10 clone (overexpressing galectin-3), underwent significantly higher ($P<0.01$) rates of apoptotic (annexin V single positive) as well as of necrotic (annexin V/PI double positive) cell death after both CHX/TNF-α and menadione.

3.2. ΔΨ

In view of the importance of mitochondrial integrity in the apoptotic as well as in the necrotic cell death process, specific analyses on $\Delta\Psi$ by using a JC-1 probe were carried out. In particular, we performed a flow cytometry study to verify the possible influence of galectin-3 expression on mitochondrial homeostasis. As a positive control of this series of experiments we used the same cell lines (EV-M13 and EV-M10) treated with different concentrations (0.1, 1 and 10 μg/ml) of K^+ ionophore valinomycin as specified in Section 2. According to literature data [11], we observed a dose dependent decrease of FL2 signals. In particular, we revealed 60 ± 7%, 75 ± 5% and 89 ± 4% of cells with depolarized mitochondria after 0.1, 1 and 10 μg/ml valinomycin exposure, respectively. After both TNF-α and menadione treatments, the EV-M13 clone (left panel) showed a marked decrease in mitochondrial membrane polarization. This is represented by the alteration of the

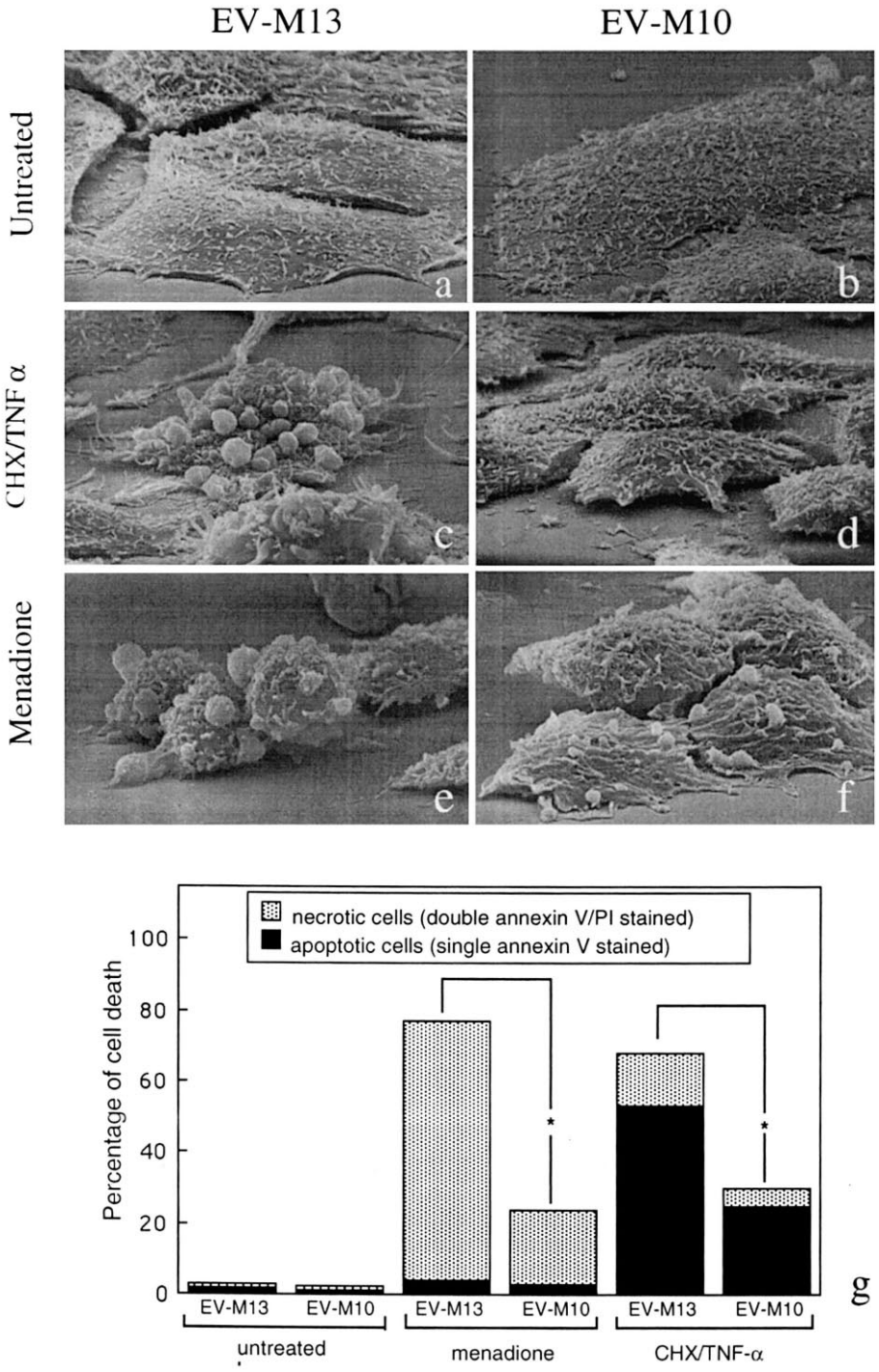

Fig. 1. Qualitative (a–f) and quantitative (g) analysis of cell injury. Scanning electron micrographs showing: (a) EV-M13 and (b) EV-M10 control cells; (c) EV-M13 and (d) EV-M10 cells treated with CHX/TNF-α; (e) EV-M13 and (f) EV-M10 cells treated with menadione. Magnification 2700×. (g) Quantification of necrosis and apoptosis of cells double stained with annexin V-FITC/PI. * in (g) indicates $P < 0.01$.

contour plot as shown in Fig. 3c,e, respectively. In fact, according to the above method, the cells with depolarized mitochondria are those moving from quadrant I to quadrant II as they lose greenish orange fluorescence (in FL2). This effect was consistently reduced in the EV-M10 clone (right panel), which expresses a high level of galectin-3, after both TNF-α (Fig. 3d) and menadione (Fig. 3f) administration. By contrast, $\Delta\Psi$ of EV-M13 and EV-M10 untreated cells were comparable (Fig. 2a,b). Accordingly, quantitative evaluation of the percentage of cells with depolarized mitochondria indicated a significant difference between the two cell clones ($P < 0.01$, Fig. 3g).

3.3. Redox balance

Mitochondrial membrane depolarization has been reported to enhance the generation superoxide anion in a number of cells [20]. In turn, these products are implicated in the initiation of both necrosis and apoptosis. In light of this, we per-

formed a flow cytometry analysis to investigate the ROS generation in our clones after both TNF-α (Fig. 3a–c, left panel) and menadione (Fig. 3d–f, right panel) addition by using HE and DHR 123 as fluorogenic substrates. These probes are penetrating cations which are accumulated by mitochondria. To exclude the possibility that $\Delta\Psi$ could obscurate the monitoring of ROS, we performed these experiment both in the presence or absence of FCCP protonophore as reported in Section 2. The production of $O_2^{\bullet}$ and H_2O_2 was significantly increased after treatment with both TNF-α (Fig. 3a,b) and menadione (Fig. 3d,e) but not after FCCP addition (not shown) in both cell lines. However, as clearly shown in Fig. 3, this increase was more evident in the EV-M13 clone, expressing low levels of galectin-3, with respect to EV-M10 cells expressing high levels of this lectin. This difference between EV-M13 (full lines) and EV-M10 (dashed lines) clones was highly significant ($P < 0.01$) for both $O_2^{\bullet}$ and H_2O_2. It is important to note the correspondence between the increased ROS production and the proportional decrease of $\Delta\Psi$ (Fig. 2).

Since the role of GSH depletion in cell injury is well known [21], intracellular reduced GSH in living cells was also evaluated by flow cytometry analysis. We found that intracellular levels of GSH were strongly reduced in EV-M13 cells treated with menadione as well as with TNF-α (Fig. 3c,f, respectively, full lines). By contrast, this reduction was significantly ($P < 0.01$) less pronounced in EV-M10 cells, expressing high

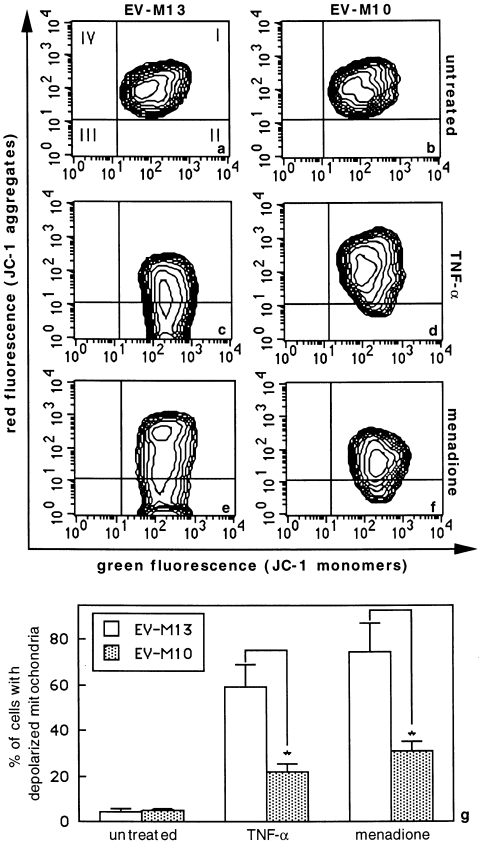

Fig. 2. Cytofluorimetric analysis of $\Delta\Psi$. Left panel, EV-M13 cells; right panel, EV-M10 cells. Abscissa, FL1 (green fluorescence, log scale); ordinate, FL2 (orange fluorescence, log scale). In quadrant II, cells with depolarized mitochondria. A representative of four experiments is shown in (a)–(f). Quantification of data concerning four different experiments is reported in (g). * indicates $P < 0.01$.

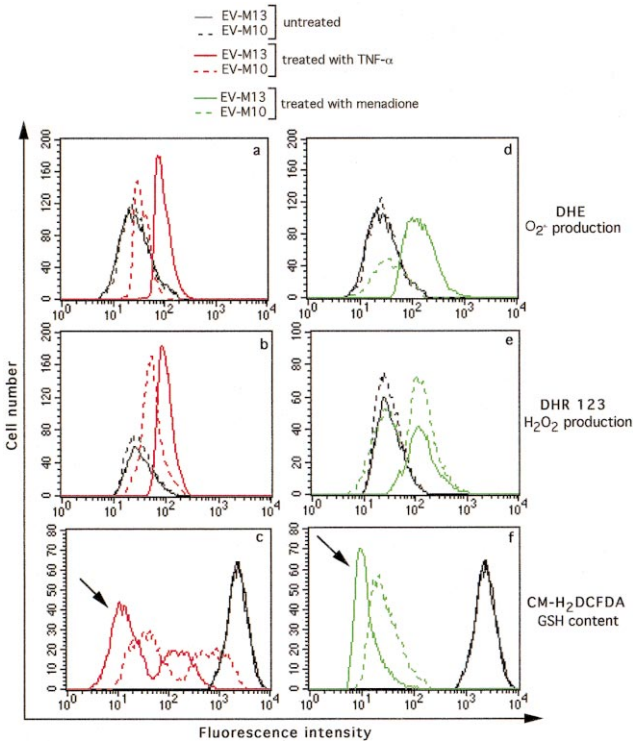

Fig. 3. Cytofluorimetric analysis of superoxide and peroxide production and GSH content in living cells. On the abscissa the fluorescence intensity (log. scale) is reported; on the ordinata, the relative cell number. Full lines represent the EV-M13 clone, dashed lines represent the EV-M10 clone. A representative of four experiments is shown.

levels of galectin-3 (Fig. 3c,f, respectively, dashed lines). Furthermore, after TNF-α treatment, additional alterations were appreciated. In fact, two distinct cell sub-populations containing different levels of GSH, in both EV-M13 (full line) and EV-M10 (dashed line) clones, as indicated by bimodal curves, were detected (Fig. 3c). In particular, in the TNF-α-treated EV-M13 clone, one of these cell sub-populations (see arrow in Fig. 3c) showed a dramatic reduction in the intracellular GSH levels (similar to that observed in necrotic cells, see arrow in Fig. 3f). This cell sub-population, being PI-positive dead cells excluded from our analysis, has to be considered in the late phases of apoptosis.

4. Discussion

As a general rule, there are conflicting reports describing the biological role of galectins, e.g. their pro- and anti-apoptotic activity. In particular, several reports indicated that galectin-3, probably the most studied of these lectins, can prevent zww cell death in human cells of different histotypes [7,15,20]. The anti-apoptotic effect of galectin-3 appeared to result from the ability of this molecule to heterodimerize with bcl-2, a well known suppressor of apoptosis [22]. In addition, very recent works point out that transfection of galectin-3 cDNA in human breast carcinoma cells can confer anoikis-resistance acting on cell cycle progression [7] and increasing integrin dependent adhesion capability [15]. In particular, we reported that the apoptotic phenomenon induced by ultraviolet radiation B (UVB) or by TNF-α in Evsa-T cells can be counteracted by overexpression of galectin-3 [15]. Since both

UVB and TNF-α can induce oxidative imbalance [13,23], we hypothesized that galectin-3 could interfere with ROS generation. In fact, in the present work we partially address this point by considering two different stimuli whose mechanism of action implicates different redox alterations: TNF-α and menadione. Our data revealed that ROS generation induced by these treatments was significantly prevented by overexpression of galectin-3. This effect was consistent with the inhibition of cell death offered by the overexpression of this lectin independently from the mode of cell death, i.e. by apoptosis or necrosis. Both of these include in fact an oxidative imbalance as the mandatory mechanism. This suggests that galectin-3 might interfere with very early stages of cell death that are associated with perturbation of mitochondrial homeostasis and subsequent formation of ROS and GSH depletion. This is evinced by the fact that EV-M10 cells, expressing high levels of galectin-3 and 'preserved' $\Delta\Psi$, after TNF-α and menadione treatments, displayed decreased ROS generation and higher levels of thiols, with respect to EV-M13 clone. Furthermore, considering the significant sequence similarity with bcl-2 [22] and the close relationships between bcl-2 and mitochondria in apoptosis progression [24], the present data represent the first indication that galectin-3 could be an additional mitochondrial-associated apoptotic regulator.

The results reported here also suggest a more general role exerted by galectins in certain pathologic conditions, including infectious and non-infectious diseases. For instance, Tat protein from HIV, an essential pro-oxidant molecule involved in viral replication [25], is capable of inducing an overexpression of galectin-3 [10]. From a final point of view, this could be exerted by a direct action on mitochondria as well as on ROS formation, leading to the hindering of infected cell suicide and allowing viral production. On the other hand, a role for galectins in non-infectious disease, i.e. tumor progression, has also been proposed [1]. Particularly, increased galectin-3 expression in more invasive tumor cells has been demonstrated, e.g. in colon carcinoma [26]. This increase could favor cell survival and subsequently, thanks to its demonstrated effect on cytoskeleton [15,27], tumor cell spreading. Moreover, in the same vein, we cannot rule out the possibility that the increased cell resistance to oxidative imbalance due to galectin-3 overexpression could lead to an increased resistance towards certain chemotherapic drugs which exert their activity by modifying the redox state of the cells. As a general rule, on the basis of our results, we can hypothesize that galectin-3, by influencing mitochondrial homeostasis, can specifically interfere with redox pathways controlling cell survival and death, playing a role in the pathogenesis of several diseases.

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