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Mitochondrial inside-out signalling during alkylating agent-induced anoikis

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1. ABSTRACT

Exposure of epithelial respiratory cells to the alkylating agent, mechlorethamine (HN2), induces anoikis initiated by mitochondrial depolarization and caspase-2 activation. The mechanisms of disruption of cell interactions were investigated and expression of integrins, E-cadherin, and actin were therefore studied after HN2 treatment. In the adherent cells, an early disruption of F-actin occurred associated with cell rounding. Inhibitors of caspase-2 resulted in attenuating of the decline of adhesion proteins and microfilaments. HN2-induced down-regulation of beta1 integrin, E-cadherin expression and F-actin pattern occurred in detached cells but were efficiently prevented by inhibitors of mitochondrial permeabilization. Moreover, inhibiting mitochondrial depolarization improved significantly both cell survival and capacity of detached cells to re-adhere. These findings confirm the pro-survival integrins and E-cadherin mediated signalling pathway. The central role of mitochondria in HN2-induced cell detachment is reinforced, suggesting that mitochondria acts as a key executor of reduced cell adherence during anoikis and could be responsible of an inside-out signalling. Present data support the potential of these therapeutics, generated via the inhibition of mitochondrial depolarization, as protectors against the alkylating agent lesions.

2. INTRODUCTION

The upper airway respiratory tract is the site of the most dramatic lesions of subjects exposed to the warfare agent, sulfur mustard yperite. We reported recently that mustard treatment, with sulfur mustard, yperite (SM) or nitrogen mustard mechlorethamine (HN2), an alkylating agent, induced cell detachment and subsequent apoptosis in human airway epithelial cell line 16HBE (1). Kinetics studies of HN2 0.1 mM effects demonstrated that cell detachment appeared after a latency phase of about 4h and at 24h over 35% of cells were detached and largely

apoptotic (32,4%) whereas the control cultures presented only minor detachment and only few apoptotic death. Apoptosis affecting only detached cells, was relevant of anoikis, characterized as a programmed cell death induced by the loss of interactions between cells and extracellular matrix (2). HN2-induced cell detachment was initiated by caspase-2 activation, down-regulation of Bcl-2 and loss of mitochondrial membrane potential, followed by apoptosis in detached cells with increase in p53 expression and Bax activation. Within recent years, the sequence of intracellular events during anoikis was characterized and it has been shown, in accordance with our previous findings in an epithelial cell line, that some of the Bcl-2 proteins family may participate in the regulation of anoikis and that caspase-2 was activated at the initiation of anoikis, followed by the activation of down-stream caspases (3,4).

Another central problem of anoikis is to clarify how integrin-mediated cell adhesion signals control the apoptotic machinery. Cell binding to extracellular matrix (ECM) and cell-cell contacts are required for the growth and survival of epithelial cells in *in vitro* culture as *in vivo*. Earlier studies in rabbit tracheal epithelial cells culture exposed to mustard had reported that morphological features of cell detachment showed the enlargement of intercellular spaces between basal cells associated with disorganization of the cytoskeleton (5,6). Therefore, we sought to explore whether matrix adhesion mechanisms and actin cytoskeleton were molecular targets of mustard toxic effects and what role could play mustard inhibitors in a protective attempt to antagonize toxic and blistering effects of mustard. Binding to ECM is mediated by transmembrane cell surface receptors involved in cell anchorage and signalling transduction, comprising integrins and E-cadherin, the most regulating molecules for cell-matrix and cell-cell attachment. The integrins heterodimers of alpha and beta subunits are expressed on whole cell surface and organized in basal focal contacts responsible of ECM anchoring. The pattern expression of integrins in healthy and injured pulmonary epithelial cells includes at least 8 different integrins, alpha2beta1, alpha3beta1, alpha6beta4, alpha9beta1,alpha5beta1,,alphavbeta5, alphavbeta6, and alphavbeta8 (7). The integrin alpha3beta1 participates in the formation of focal adhesion linked to the F-actin cytoskeleton. Cell-cell contacts are as well important in epithelial architecture and are sustained by organization of junction complexes, including the adherens junctions specialized in subapical junction and mediated by the homophilic partners cadherins (8). E-cadherin is present in all normal epithelia, and is expressed in well-defined adherens junctions in 16HBE14o- cells, that affords maintain of differentiated polarized morphology to these cells (9,10). To maintain anchoring and junction integrity, these transmembrane proteins must form links between the cytoskeleton of the contacting cells and the ECM elements. In cell-matrix anchorage, a protein adhesion complex connects integrin intracellular domain to actin stress fibres at site of focal contacts. The intracellular integrin-mediated signals involve key players such as integrin-associated non-receptor kinases including Focal-Adhesion-Kinase (FAK) and Integrin linked-Kinase (ILK) which convey survival signals, via the activation of PI-3K with its downstream target Akt/PKB. Overexpression of FAK or ILK inhibits anoikis (2,11). The role of cadherin in mediating cell survival versus apoptosis was also reported via the Akt/PKB signalling. The binding of E-cadherin leads to survival in a PI-3K manner and blockage of N-cadherin leads to apoptosis (12,13). The integrity of actin-skeleton has a crucial role in maintenance of cell-cell adhesion, since pharmacological agents that disrupt F-actin also disrupt cell-cell junctions. Polymerization of actin bundles is controlled by proteins that connect cadherins and promote actin assembly (14,15). They also stabilize the cadherin contacts in adherens junctions (16), whose phosphorylated state regulates Src and Rho family proteins or Rac1 GTPase (17-20).

In this study, we showed that HN2-induced anoikis in 16HBE14o- cells, consisted in the disruption of F-actin in the adherent cells, followed in the detached cells by of decrease of integrins and E-cadherin expression. Additionally, inhibitors of the mitochondrial depolarization, ebselen, melatonin and cyclosporine A, were shown to impair F-actin and beta1 integrin decrease and, rescued the survival and adhesive properties of the cells. HN2-induced molecular events are therefore suggested as an "inside-out" signalling pathway in which dissociation of focal contacts appear as a consequence of mitochondrial depolarization and not as a primarily cause.

3. MATERIAL AND METHODS

3.1. Cells and cell culture

The human bronchial epithelial 16HBE14o- cell line was a generous gift of Dr. Gruenert (San Francisco, USA) (21). Cells were grown in DMEM/Ham F12 medium, 2% Ultroser G, 1% glutamine, 0.5% fungizone and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) (Invitrogen, France), seeded at 30 000 cells/cm² in 6- or 12-well plates coated with type I collagen.

3.2. Chemical treatment

48 h after seeding, subconfluent monolayers were washed and treatments were carried out in DMEM/Ham F12 without Ultroser G, added with 0.1 mM mechlorethamine HN2, (N-methyl-2, 2'di (chloroethyl)amine) (Sigma, USA), a nitrogen structural analogous compound of the sulfur mustard yperite for an exposure time ranging from 6h to 24h. Different inhibitors of the opening of mitochondrial transition pore were tested: Ebselen (E) 10µM, Melatonin (M) 100 µM and Cyclosporin A (CsA) 1 µM (Sigma) were added 1 h before treatment with HN2. Alternatively, two inhibitors of matrix metalloproteinase were examined: doxycycline (20 µM) and GM6001 (15µM) (Calbiochem), both added 1 h before treatment. Cell-permeable inhibitors of caspases (R&D systems), pan-caspase z-VAD-fmk and caspase-2 inhibitor z-VDVAD-fmk, were stored at –20°C in 20 mM stock solutions in DMSO, diluted to 100 µM in medium before use and added in the medium 45 minutes before HN2 treatment. At the end of treatment, detached cells were collected in the medium, whereas adherent cells were dissociated in trypsin-EDTA (Invitrogen), inhibited by 10% FCS after 15 minutes or dissociated in Versene-EDTA

without trypsin (Invitrogen). Untreated cells were harvested as control. Both detached and adherent cells were counted using a hemacytometer.

3.3. Immunofluorescence

Cells were plated in 8-well chamber slide (Labtek) coated with Type I collagen. After treatment, detached cells were collected with the medium, centrifuged and washed in PBS and, adherent cells were washed with PBS. The samples were fixed with paraformaldehyde 3% in PBS for 20 min, washed in PBS and kept at 4°C until labeling. The cells were permeabilized in PBS 0.1% Triton X-100 for 2 min, bloqued with 1% BSA in PBS for 20 min, stained for 1h with monoclonal antibodies anti-beta1 integrin, anti-alpha3 integrin, anti-alpha6 integrin, anti-E cadherin (R&D systems, UK). Cells were then revealed with Phycoerythrin (PE)-, TRITC- or FITC-conjugated IgG (1:100; Dako). F-actin was decorated using FITC-conjugated phalloidin (4:1000; Sigma). Nuclei were stained with propidium iodide (4 µg/mL) containing DNase-free RNase A (100 µg/mL) in PBS. Cells were examined under a fluorescence microscope (Leica; DMRB type).

3.4. Flow cytometry analysis

Fixed cells from adherent and detached populations were resuspended in 1 mL of PBS and immunolabeled with anti-integrin sub-units or E-cadherin antibodies and secondary fluorescent antibodies and labeled with fluorescent phalloidin or propidium iodide PI, then analyzed with an EPICS-Elite-ESP flow cytometer (Coultronic). Immunolabeling was also performed on unfixed cells and on adherent cells harvested without trypsin (Versen-EDTA without trypsin) and gave similar results. Controls of labeling are realized with cells failing incubation with primary antibody and cells incubated without fluorescent phalloidin that determined the threshold of positive specific labelling and the percentage of the positive labelled cells in a sample of about 10 000 cells.

3.5. Western blot analysis

Cells were recovered, rinsed in cold PBS, and lysed in lysis buffer (50 mM HEPES–KOH, pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5 mM DTT, protease inhibitors) for 30 min at 4°C. Standardization of protein loading was achieved by protein measurements using the bicinchoninic acid assay and loading of equal protein amounts (10 µg per lane). After SDS–PAGE and blotting to PVDF nitrocellulose membranes (Millipore, Billerica, Massachusetts, US), membranes were blocked for 1 h with 5% non-fat dry milk in PBS, 0.1% Tween-20 and incubated 1h with anti-actin antibody. Secondary antibody, goat anti-mouse IgG (1:10,000) conjugated with horseradish peroxidase (Santa Cruz) for 1 h, was detected using ECL method (Amersham, UK).

3.6. Cell counts

After treatment, detached cells were collected in the medium and adherent cells were dissociated using trypsin-EDTA (Invitrogen) inhibited by 10% FCS. Both detached and adherent cells were counted using a hemocytometer and the percentage of detachment was calculated.

3.7. Cell attachment assays

Cells were treated for 20h with HN2 0.1 mM alone or in the presence of the different protectors: z-VAD, a combination of ebselen, melatonin and cyclosporin A, doxycycline or GM6001. Untreated and treated detached cells for 10h were collected in supernatant, centrifuged and counted. Then they were replated on collagen with fresh medium DMEM added with 2% UG. Capacity of re-adhering was evaluated after 24H by the percentage of live adherent cells reported to the number of replated cells.

3.8. Discrimination between live, apoptotic and necrotic cells

FDA-EtBr double labeling: After HN2 treatment, cells were incubated for 20 min at 37 °C with fluorescein diacetate (FDA, 1 µg/mL) and ethidium bromide (EtBr, 20 µg/mL) in DMEM/Ham F12, as previously described (1,22), observed and counted on a conventional epifluorescence microscope.

3.9. Statistical analysis

All results are representative of at least three separate experiments. Data were analyzed using one-way analysis of variance. The Student-Newman-Keuls test was used for all pairwise comparisons of mean responses among the different treatment groups (SigmaStat). Differences between groups were considered significant if the p value was less than 0.05. Data are presented as the mean +/- standard error of the mean for three replicates.

4. RESULTS

In a prior study we showed that HN2 treatment resulted, in a time- and concentration-dependent manner, in cell detachment followed by apoptosis. Thus, in the present study, in order to elucidate the mechanisms of HN2-induced anoikis, expression and localization of membrane adhesion proteins and organization of actin cytoskeleton were examined in control untreated and treated cells. The evaluation of these parameters was performed separately in the adherent cells and the detached cells harvested from 6h to 24h of HN2 0,1mM treatment or in control culture.

4.1. Localization and expression of integrins and E-cadherin are not affected in adherent cells by HN2 treatment.

Investigation of localisation of integrins and E-cadherin was carried out by indirect immunolabeling with monoclonal antibodies anti-beta1 and -alpha3 integrin subunits and anti-E-cadherin (Figure 1Aa-f). Integrins were expressed at cell surface of the totality of untreated cells and the pattern of immunolabeling for beta1 and alpha3 was equally fluorescent (Figure 1Aa, c). The labeling of E-cadherin lined the cell boundary of contacting cells (Figure 1Ae), but was negative in the isolated cells of sub-confluent cultures (non illustrated). Immunolabeling of beta1 and alpha3 integrins and E-cadherin was performed after 6h, 10h, 18h and 24h of HN2 0.1 mM treatment. After HN2 treatment as long as 24h, which led to the detachment of numerous cells from the matrix, the cell surface pattern of beta1 and alpha3 integrins and E-cadherin was unchanged in the treated adherent cells labeled *in situ* (Figure 1Ab, d, f).

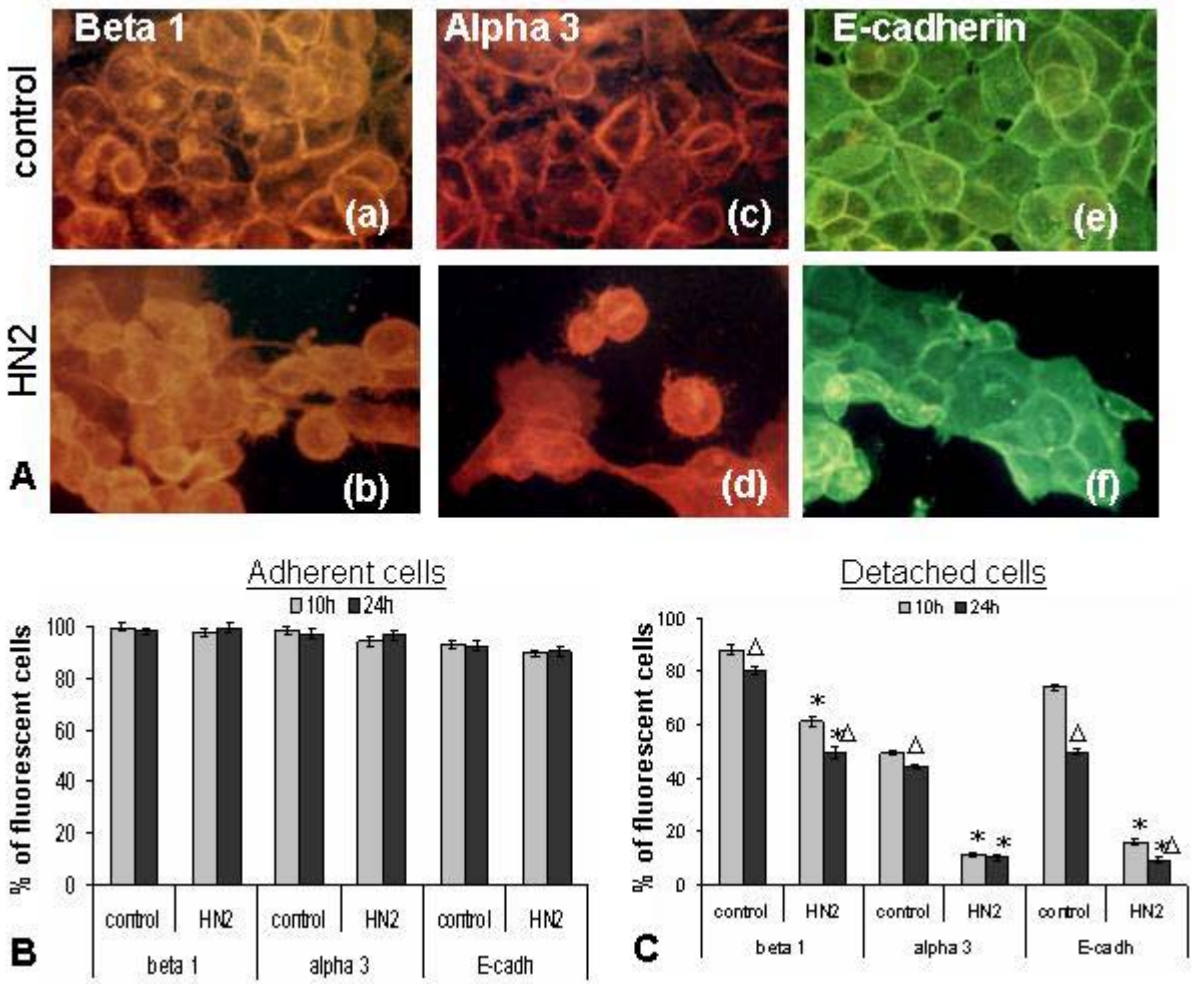

Figure 1. A: Adherent cells: Cell surface distribution of integrins and E-cadherin is not disturbed after HN2 0.1 mM treatment for 24h. *In situ* immunolabeling with monoclonal antibodies against sub-unit integrin beta1 (a, b), alpha3 (c, d) and E-cadherin (e, f), followed with PE- or FITC-stained secondary antibodies, show that beta1 and alpha3 integrins are expressed at level of cell-matrix and cell-cell contacts in all the cells in untreated (a, c, e) and HN2-treated cells (b, d, f). B and C: FACS analysis of beta1, alpha 3 integrins and E-cadherin expression. Untreated (control) or HN2 treated cells for 10h and 24h, were collected, fixed with 3% paraformaldehyde and immunolabeled. Levels of beta1, alpha3 integrins and E-cadherin expression at cell surface were evaluated by percentage of integration of fluorescence after indirect immunolabeling performed in adherent (B) and detached (C) cells. * Values significantly different from control ($p < 0.05$). Δ Values significantly different from previous exposure time ($p < 0.05$).

The level of beta1 and alpha3 integrins and E-cadherin expression was then assayed quantitatively by flow cytometry in the adherent cells dissociated from the matrix without trypsin to avoid proteolytic cleavage of adhesion proteins. After 10h or 24h only a slight decrease was noted in the percentage of positive labelled adherent cells, but without significant differences between untreated and treated cells (for example: 92.8± 1.7% and 90.2 ± 1.7% of E-cadherin fluorescence integration respectively for untreated cells and after HN2 0.1 mM for 24h treatment, Figure 1B).

4.2. Integrins and E-cadherin cell surface expression is reduced in HN2 treated detached cells

Immunolabeling of cell surface proteins in the detached cells of treated and untreated populations appeared heterogeneous. Untreated and HN2-treated cells comprised dead cells and numerous apoptotic cells that presented an irregular pattern of labeling of integrins (Figure 5A, c, e and g) and E-cadherin (non illustrated). The level of beta1 and alpha3 integrins and E-cadherin expression was therefore assayed quantitatively by flow cytometry in the detached cells collected in the supernatant. In contrast with adherent cells, the levels of expression of beta1, alpha3 and E-cadherin, measured after 10h to 24h of treatment, decreased in HN2-untreated and treated detached cells. This decrease was significantly more dramatic in HN2 treated cells than in untreated cells. For example after 24 h treatment, 0.1 mM HN2 led to 10.0 ± 0.9% of alpha-3 fluorescence integration in detached cells, whereas control cultures presented 44.2 ± 1.0% of alpha-3 fluorescence integration (Figure 1C).

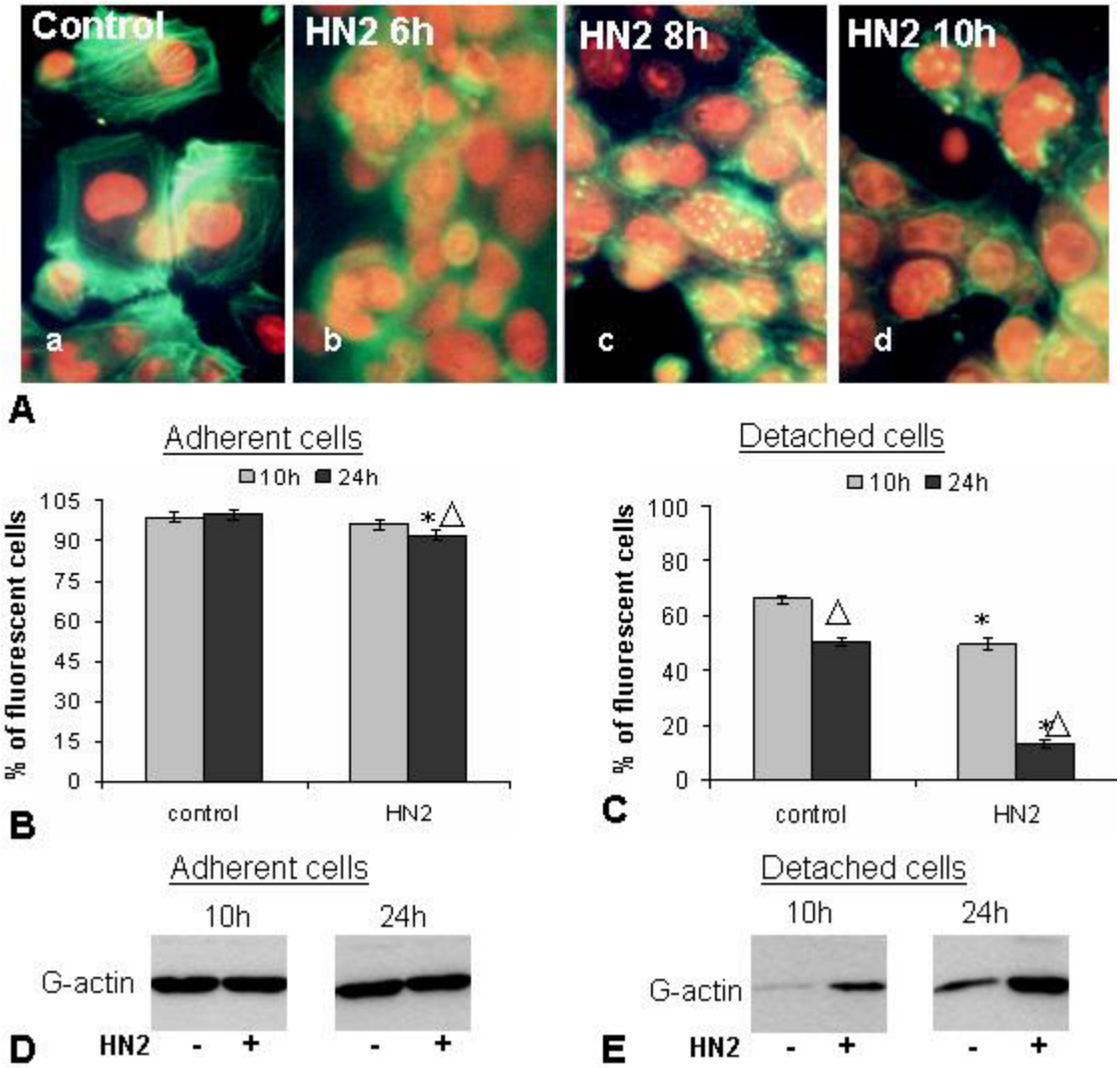

Figure 2. A: Adherent cells: HN2 treatment disrupts the microfilament network. FITC-phalloidin *in situ* staining of F-actin. a: untreated cells exhibit a developed microfilament network consisting of peripheral adherens junctions in contacting cells and stress fibres particularly developed in scattered isolated cells. b: HN2 0.1mM treatment 6h: adherens junctions diminish, stress fibres begin to disrupt and peripheral filipodes become thinner. c: HN2 8h, adherens junctions disappear and stress fibers are reduced to bright patches at basal cell pole. d: HN2 10h, basal focal contacts have disappeared, cells are rounded. B and C: FACS analysis of microfilament content. F-actin content was evaluated by percentage of integration of fluorescence of FITC-phalloidin in untreated (control) or HN2 treated cells for 10h and 24h. Adherent (B) and detached (C) cells were collected, fixed with 3% paraformaldehyde and stained with FITC-phalloidin. Nuclei are counterstained with propidium iodide. D and E: Analysis of G-actin protein expression in adherent (D) and detached (E) cells, untreated or HN2 treated for 10h and 24h. Total cells extracts were prepared, followed by Western blot analysis of protein equivalent amounts using a monoclonal anti-actin antibody. * Values significantly different from control ($p < 0.05$). Δ Values significantly different from previous exposure time ($p < 0.05$).

4.3. HN2 treatment induces the disorganization of F-actin in both adherent and detached cells

F-actin organization and content were investigated during HN2 treatment. In adherent cells, actin cytoskeleton visualized with fluorescent phalloidin, was markedly developed in untreated cells, either in adherens junctions or stress fibers (Figure 2Aa), but it appeared progressively disorganized after increasing time of HN2 treatment (Figure 2Ab-d). Soon after 6h of HN2 treatment, the stress fibers disrupted and peripheral filipodes became thinner (Figure 2Ab). After 8h, actin fibers shortened and resolved as bright dotted points at cellular basal sites and, adherens junctions became fuzzy (Figure 2Ac). After 10 to 24h, microfilaments were collapsed around the nucleus and this was associated with the rounding of the cells (Figure 2Ad). In the detached HN2-treated cells, phalloidin-labeling of F-actin was negative in the numerous apoptotic fragments, whereas few non-fragmented rounded cells still

contained labeled perinuclear microfilaments (non illustrated).

The content of F-actin was investigated quantitatively by fluorescent phalloidin staining and FACS analysis. A rapid slight decrease of microfilaments content could be noted in the adherent HN2-treated cells, which progressed from 10h until 24h compared to untreated cells (Figure 2B). In the detached cells, F-actin content was low and decreased more significantly in HN2 treated than in untreated cells (65.9 ± 1.3% and 50.4 ± 2.1% of F-actin fluorescence integration after respectively 10 and 24h of control culture in comparison with 49.3± 1.3% and 12.9 ± 1.5% of F-actin fluorescence integration after respectively 10 and 24h of HN2 0.1 mM, Figure 2C).

In order to determine whether the HN2-induced decrease in F-actin in the cells could be related to a down-regulation of G-actin, cell lysate fractions were resolved on SDS-PAGE and probed for the presence of actin. Immunoblotting analysis showed no changes of G-actin in adherent cells after HN2 treatment (Figure 2D). In the detached cells, a lower actin content was detected, but either, after 10h or 24h, the HN2 treated detached cells still contained a higher content in G-actin than the untreated cells (Figure 2E). This suggested that the decrease in F-actin observed after HN2 treatment did not result of a G-actin down-regulation but corresponded to a marked depolymerization of F-actin.

4.4. Effect of caspase inhibitors

The effects induced by HN2 on cell adhesion mechanisms and actin cytoskeleton were then examined in correlation to the activation of caspases. Caspase inhibitors were used and their subsequent effect was analyzed. The pan-caspase inhibitor z-VAD allowed maintaining of a higher content in F-actin, with an efficacy of action more noticeable after 10h of treatment, but then after declining until 24h (+52.0±2.4% of F-actin expression after 10h and +16.0±2.2% after 24h in detached cells pre-treated with z-VAD, Figure 3A). Z-VAD also promoted a higher level of expression of integrins beta¡alpha□ and of E-cadherin in detached treated cells after 10h (+75.9±2.7% of alpha3 expression, +56.2±2.7% of beta1 expression and +75.1±2.7% of E-cadherin expression after 10h treatment in detached cells pretreated with z-VAD, Figure 3B). This protective effect, noticed after 10h of treatment, also decreased after 24h (+29.1±2.8% of alpha3 expression, +30.5±2.8% of beta1 expression and +31.9±2.9% of E-cadherin expression after 24h treatment in detached cells pretreated with z-VAD (Figure 3B).

The specific inhibitor of the initiator caspase-2, z-VDVAD, was then tested. Caspase-2 inhibitor was particularly efficient to maintain a high level of expression of alpha3 (41.1% of alpha3 immunolabeling in presence of caspase-2 inhibitor versus 11.5% in cells treated only with HN2 0.1mM and 49.3% in control cultures) and beta1 (90.7% beta1 immunolabeling in presence of caspase-2 inhibitor versus 61.3% in cells treated only with HN2 0.1mM and 87.9% in control cultures), but exerted a moderate protection on E-cadherin expression level after 24h of treatment (37.7% of E-cadherin immunolabeling in presence of caspase-2 inhibitor versus 16.1% in cells treated only with HN2 0.1mM and 73.9% in control cultures) (Figure 3C). A partially protective effect was also provided by inhibitors of caspase-1 and -3, but remained weaker (non illustrated). The distribution of betaı and E-cadherin was examined at the cell surface of HN2 detached treated cells in the presence of caspase-2 inhibitor, which resulted in a more regular surface immunofluorescent labeling and in the maintain of small clusters of cells (Figure 3D).

4.5. Effect of Inhibitors of mitochondrial potential disruption

We have previously demonstrated that the presence of protectors such as ebselen (E), melatonin (M) and cyclosporin A (CsA), reduced HN2-induced anoikis via the inhibition of the opening of the mitochondrial transition pore (1). The effect of HN2 on the F-actin content, integrins and cadherin expression was thus assayed in detached cells in the presence of these inhibitors.

Ebselen (E, 10µM) alone re-established a microfilament contents in detached HN2-treated cells comparable to control cultures even after 24h of treatment (Figure 4A). Since the association of the three protectors exerted a more efficient protection against HN2-induced anoikis, their effect was measured when present in combination (E ˜ı□□ + M 100□M + CsA 1□M). The rescue in microfilament content promoted by ebselen was confirmed in the presence of the combination of these three protectors (data not shown). Moreover, the pre-treatment with the 3 protectors maintained a much higher level in expression of adhesion proteins (alpha3, beta1 and E-cadherin) in the detached cells, compared to HN2 treated cells for 10h (Figure 4B). This protective effect was particularly significant concerning beta1 and alpha3 integrins, whereas E-cadherin expression was less modified (Figure 4B).

4.6. Effect of inhibitors of matrix metalloproteinase

The distribution of cell surface adherent proteins was examined under exposure to HN2 in presence of the inhibitor of metalloproteinases, GM 6001. After HN2 treatment for 24 hours in the presence or absence of GM 6001, detached and adherent cells were harvested and immunolabeled for alpha3 and beta1 integrins. Control detached cells mainly contained dead fragmented cells poor in integrins alpha3 and beta1 (Figure 5a, e). GM 6001 alone did not modify this pattern (Figure 5b, f). HN2-treated detached cells included fragmented and cells non-fragmented cells with irregular alpha3 and beta1 labeled patches at their surface (Figure 5c, g). After HN2 treatment in the presence of GM 6001, the immunolabeling of integrins remained more uniform at cell surface (Figure 5d, h). On the contrary to the detached cells, the labeling of adherent cells exhibited an unchanged pattern for beta1 and alpha3 at cell surface either after HN2 treatment and in the absence or in the presence of GM 6001 (non illustrated).

4.7. Cell attachment assays

We next examined the capacity of inhibitors of

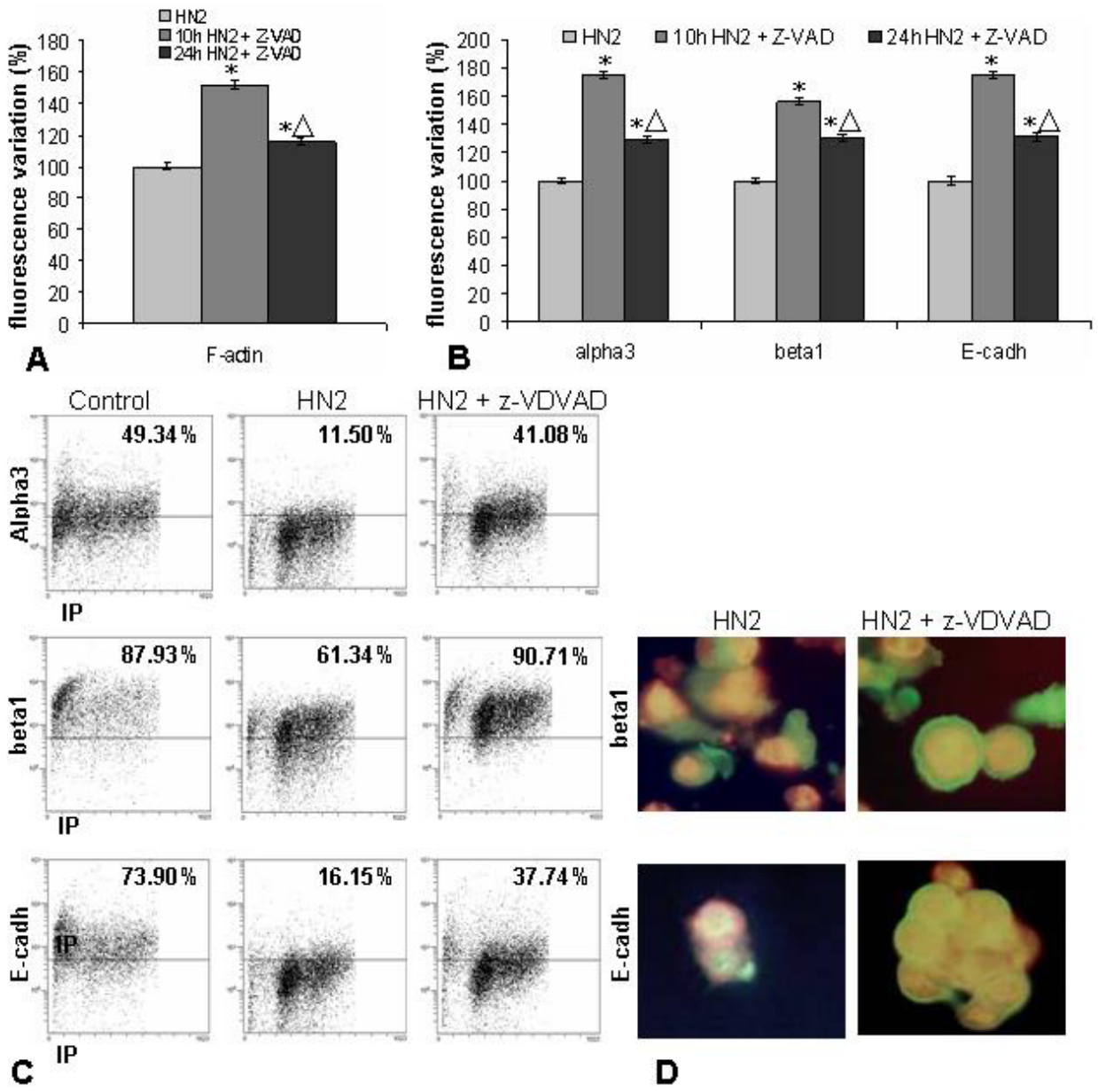

Figure 3. Effect of caspase inhibitors on microfilament network and adhesion proteins expression in detached HN2-treated cells. A: F-actin content was evaluated by FITC-phalloidin staining and FACS analysis after 10h, and 24h of HN2 treatment in the absence or presence of z-VAD (100 μM). The results are expressed as the percentage of the variation of F-actin content in the presence of z-VAD reported to HN2 treatment in the absence of z-VAD (100%). B: Expression of beta1 and alpha3 integrins and E-cadherin was evaluated by immunolabeling and FACS analysis after 10h and 24h of HN2 treatment in the absence or presence of z-VAD (100 μM). The results are expressed as the percentage of the variation of expression levels of adhesion proteins in the presence of z-VAD reported to HN2 treatment in the absence of z-VAD (100%) at the indicative times. C: Integrin beta1, and alpha3 and E-cadherin expression levels after 24h of HN2 treatment in the absence or presence of inhibitor of caspase-2 z-VDVAD (100 μM) in detached HN2-treated cells. Dual parameters dot plots of integration of immunofluorescent labeling of adhesion proteins versus propidium iodide integration (PI). Percentages of cells labeled for beta1, and alpha3 and E-cadherin quantified above the line are indicated. (FL1, IP channel; FL2, PE channel). D: Immunofluorescent labeling of beta1 and E-cadherin in detached cells treated with HN2 for 24h in the absence or presence of caspase-2 inhibitor z-VDVAD and nuclear staining with PI. In the presence of caspase-2 inhibitor, the detached cells exhibit less numerous apoptotic nuclei, a more regular pattern of integrin beta1 and E-cadherin localization and remained frequently associated in small clusters. * Values significantly different from HN2 treated cells ($p < 0.05$). Δ Values significantly different from previous exposure time ($p < 0.05$).

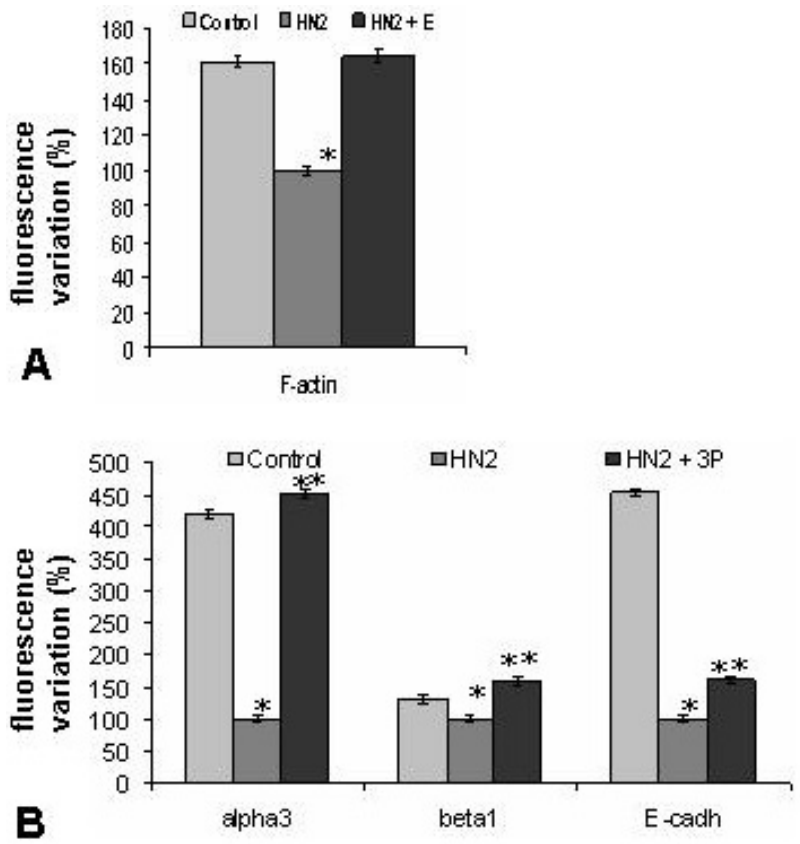

Figure 4. Inhibitors of the disruption of mitochondrial potential preserves the expression of beta1, alpha3 integrins, E-cadherin and microfilament content in HN2 treated cells: A, Effect of Ebselen (E) on microfilament content in HN2-treated cells. FACS analytic diagrams of integration of fluorescent phalloidin in total (adherent and detached) cells, untreated cells (control), HN2 0.1 mM or HN2 + E treated cells for 24h. B, Effect of 3 protectors in combination (ebselen E + melatonin M + cyclosporin CsA). Expression of adhesion proteins quantified after immunolabeling of beta1, alpha3 subunits and E-cadherin. Untreated cells (control), treated cells for 18h with HN2 0.1 mM (HN2) or with HN2 in the presence of E 10 μM + M 100 μM + CsA 1 μM (= HN2+3P). Results are expressed as the percentage of the variation of expression levels of F-actin and adhesion proteins in the presence of inhibitors reported to HN2 treatment in the absence of protectors. * Values significantly different from control ($p < 0.05$). Among treatment conditions, values not significantly different have the same number of asterisks.

caspases, mitochondrial depolarisation and metalloproteinases, that maintained a high level of adhesion proteins and F-actin and a normal integrin distribution, to contribute to the survival of the detached cells and allow them to re-adhere to the matrix after removal of the toxic. Thus, after treatment with HN2 for 10h in the absence or presence of z-VAD, z-DVAD, mitochondrial protectors in combination (E+M+CsA=3P), metalloproteinase inhibitors GM 6001 or doxycycline (23), the detached cells were collected in the supernatant, centrifuged and counted to evaluate the cell detachment (Figure 6A). They were rinsed and plated on collagen in fresh medium DMEM added with 2% ultroserG. Cell attachment to the matrix after 24h in fresh medium was estimated by the percentage of live cells as determined by FDA-EtBr double labeling which discriminates the green fluoro-diacetate fluorescent live cells from the dead cells with nuclei stained in orange with ethidium bromide. All these protectors significantly increased the percentage of re-adhered live cells with respectively 43.6±1.9%, 48.2±2.2%, 29.1±1.7% and 35.2±2.9% of re-adhered live cells for z-VAD, three mitochondrial protectors combination, doxycycline and GM6001 in comparison with percentage of re-adhered live HN2-treated cells (18.1±1.2%) (Figure 6B). This result emphasizes the deleterious role of mitochondrial depolarization in the down-regulation of cell adherens mechanisms and cell survival.

5. DISCUSSION

Our data showed that anoikis promoted by HN2 treatment corresponded to the prompt disorganization of actin-F in adherent cells, with the disruption of stress fibres and adherens belt junctions leading to cell rounding and cell detachment. Then, changes in the expression and distribution of integrins and E-cadherin occurred at cell surface of the detached cells, thus after cell detachment and during apoptosis.

5.1. Disruption of actin-F and subsequent cell rounding may be closely related to onset of cell detachment

This was observed in airway epithelial cell lines

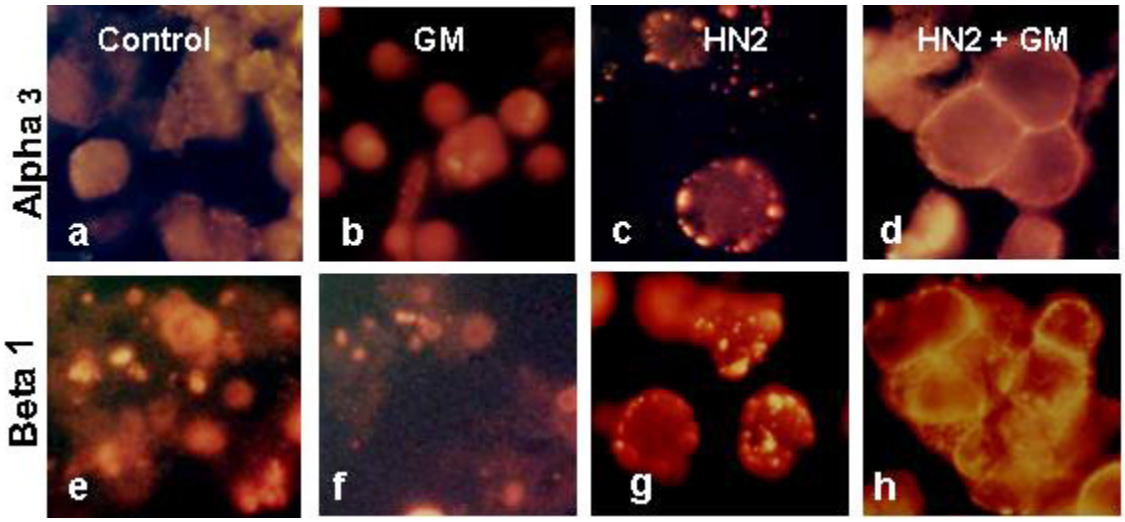

Figure 5. Effect of metalloproteinase inhibitor GM6001 on integrin localization in untreated and HN2-treated detached cells. Immunolabeling of alpha3 (a-d) and beta1 (e-h) integrin subunits in detached cells after 24h: in control cells (a and e), GM 6001 15 µM alone (b and f), HN2 0.1 mM (c and g) or HN2 + GM 6001 (d and h). Detached cells collected with the medium were immunolabeled prior fixation with anti-beta1 and anti-alpha3 and secondary PE-stained antibody, and postfixed with 3% paraformaldehyde.

16HBE and 1HAEo$^-$ and other epithelial cell types, endothelial cells and keratinocytes (6,24-26). FACS analysis of the actin-F content in HN2-treated cells confirmed the down-regulation of polymerized state of actin which is initiated before detachment and carried on until cell death. Finally, using immunoblotting to analyze actin-G levels, we confirmed here that HN2-induced F-actin depolymerization was not related to proteolytic cleavage of G-actin (27). HN2-induced disorganization of actin-F pattern may engender the loss of cytoskeletal coupling and the reduction of focal adhesion plaques. The actin cytoskeleton participation in the anoikis pathways has been proposed, including the peripheral reorganization of both focal adhesions and actin-myosin system. For instances, the rapid loss of vinculin and taline from focal adhesion was noted with cell rounding and detachment (28). Actin-cytoskeleton-based structures are regulated by the Rho family GTPases, such as Rac1, that is essential in cell survival and protection from anoikis (29,30). Finally, the src family of tyrosine kinases, also contributes to anoikis resistance via a PI3-K- or FAK-pathway (31,32).

5.2. Loss of adherent proteins follows cell detachment

A significant decline in integrin subunits beta1 and alpha3 and E-cadherin expression was measured at the surface of the detached HN2-treated cells following F-actin disintegration. This is consistent with recent reports which show that signalling through PI3K leads to changes associated with cell adhesion, reorganization of actin filaments, thus contributing to dislocalization of integrins and E-cadherin and reduced focal adhesion plaques (33,34). The loss of integrins from the cell surface after HN2 treatment may block several survival pathways. Integrin alpha3beta1 is the major receptor for laminin-5 of the basal membrane and its role in cell survival after cell detachment evidenced the prime importance of beta1 subunit, via activation of FAK and the PI3-K/Akt signalling pathway (35-37). A main role for beta1 cytoplasmic domain was demonstrated, with its interaction with pp125^{FAK} and now appeared crucial to mediate the pro-survival regulation, via a direct PI3-K mediated stimulation of Akt activity (35,38-40). The beta1 signalling can be modulated by the alpha subunit cytoplasmic domains and, the loss of beta1 binding partners, alpha3 or alpha6, was reported associated to the induction of apoptosis. However, the alpha3 subunit, as the alpha2 subunit, was demonstrated to exert specific roles in morphological differentiation and their inhibition prevented differentiation without altering cell attachment (41-43).

The loss of cadherin-mediated adhesion may depend on several causes, including changes in the actin cytoskeleton , internalization via a clathrin-dependent pathway or ubiquitination and proteasome-mediated degradation (44,45). Cleavage and shedding of E-cadherin are also implicated after induction of apoptosis (46). E-cadherin decrease in HN2-treated detached cells appears involved in the onset of anoikis or induction of apoptosis, as in enterocytes or keratinocytes (47,48).

5.3. The role of caspases in down-regulation of F-actin and adherent proteins

We had previously reported a rapid activation of caspases, notably the caspase-2, in initiation of HN2-induced cell detachment and continued activation during apoptosis, and their inhibitors, z-VAD and caspase-2 inhibitor z-VDVAD, reduced markedly cell detachment (↓ 30%) and apoptosis (↓ 99%) (1). F-actin decrease was partly impaired by z-VAD and, integrin expression was also shown to be preserved by z-VAD and z-VDVAD, implying a role for caspases, and particularly caspase-2, activation in signalling pathways leading to anoikis as in other models (4,49,50). However the protective effect was

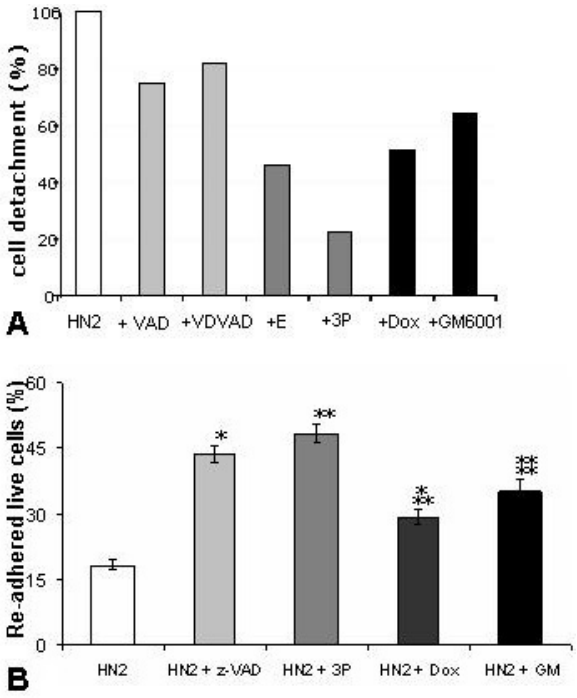

Figure 6. Cell attachment assays. A: Detached untreated and treated cells for 10h with HN2 0.1 mM alone, or with z-VAD, 3 protectors 3P (E+M+CsA), doxycyline DOX, GM 6001, were collected in supernatant. Percentage of detachment was calculated from the number of detached cells reported to total cell number. B: Detached cells were rinced and replated on collagen with fresh medium DMEM added with 2% UG. Cell attachment capacity and survival were evaluated after 24H by the percentage of live adherent cells to the matrix reported to the number of replated cells. Survival was determined by FDA-EtBr labeling that stains live cells with fluoro-diacetate in green and dead cells with ethidium bromide in orange. * Values significantly different from HN2 treated cells ($p < 0.05$). Among treatment conditions, values not significantly different have the same number of asterisks.

transitory and only delayed the disruption of actin cytoskeleton and expression decrease of adherent proteins. Thus, caspase activation does not appear to be the unique pathway responsible of HN2-induced F-actin depolymerization and adherent proteins down-regulation.

5.4. The role of mitochondria in anoikis

HN2-induced cell detachment initiation and following apoptosis had also been shown to correlate with loss of mitochondrial membrane potential, associated to down-regulation of Bcl-2 (1). The role of Bcl-2 family proteins was recently evoked in regulation of anoikis (51). The overexpression of Bcl-2 can prevent epithelial cells upon disruption of extracellular matrix and protection from anoikis occurs via beta1 integrin signalling pathway in a Bcl-2 dependent manner (52,53). On the contrary, cell adhesive properties and apoptosis are correlated with decrease of Bcl-2 and reduced expression of integrins (54,55). A decrease in Akt function and down-regulation of genes of the Bcl-2 family was observed under exposure to a sulfur mustard vesicant, and thus appeared as a key factor for the mustard-induced apoptosis (56). More recently, relationships between PI3-K/Akt kinase pathway, integrins and mitochondrial Bcl-2 family proteins were considered constitute a key decisional checkpoint in cell survival and death (36,51,57,58). Consequently, in an attempt to clarify a correlation between the involvement of HN2-induced mitochondrial depolarization and down-regulation of actin-F, integrins and E-cadherin, we explored whether inhibitors of loss of mitochondrial potential might modulate F-actin pattern, integrins and E-cadherin decrease during HN2-induced anoikis.

5.5. Mitochondria depolarization correlates with down regulation of F-actin and adherent proteins

HN2-treated cells in the presence of inhibitors of mitochondrial depolarization, ebselen E, melatonin M and cyclosporin A CsA, were previously shown less susceptible to detach (↓ 50 to 70%) and to undergo apoptosis (↓ 30 to 45%) (1,59, 60). Here we observed that, in the presence of these protectors, E, CsA and M, the actin cytoskeletal organization was significantly improved and, the expression levels of integrin beta1 and E-cadherin were markedly rescued in the detached cells. These findings are consistent with the increase in expression of beta1 integrin and E-cadherin at the cell surface on MCF-7 human breast cancer cells due to the action of melatonin (61). These data confirm that the maintain of beta1 sub-unit, in a high-affinity active conformation at cell surface, plays a primordial role in cell anchorage and cell survival signalling, and support a main role for mitochondrial depolarization in their dramatic decrease. F-actin content and expression of E-cadherin were also improved in the presence of inhibitors of mitochondrial depolarization, in agreement with anoikis resistance promoted by activation of E-cadherin (47). E-cadherin-mediated EGFR activation enhances the cell survival in the aggregates in a cooperative colony, through a process called synoikis and, anoikis of non-malignant intestinal epithelial cells is driven by detachment-induced down-regulation of Bcl-$X_{(L)}$ (62,63).

5.6. Effect of matrix metalloproteinase.

In addition, based on the fact that metalloproteinases were activated in HN2 treatment, and that pre-treatment with metalloproteinase inhibitor such as doxycycline, reduced HN2-induced apoptosis and anoikis, the effect of HN2 in the presence of metalloproteinase inhibitors doxycycline and GM 6001 was examined in respect of cell adhesive properties (23,64). Pre-treatment with GM 6001 impaired HN2-induced redistribution of integrin subunits beta1 and alpha3 at cell surface of detached cells, that might increase the adhesive responses.

5.7. Maintain of cell adhesiveness and survival is actively sustained by inhibition of mitochondrial depolarization

In order to test the relevance of these findings, we next tested the adhesive capacity of HN2-treated cells in the presence of different protectors. Adheseviness to the matrix and survival of detached cells treated with HN2 alone or added with protectors, z-VAD, metalloproteinase

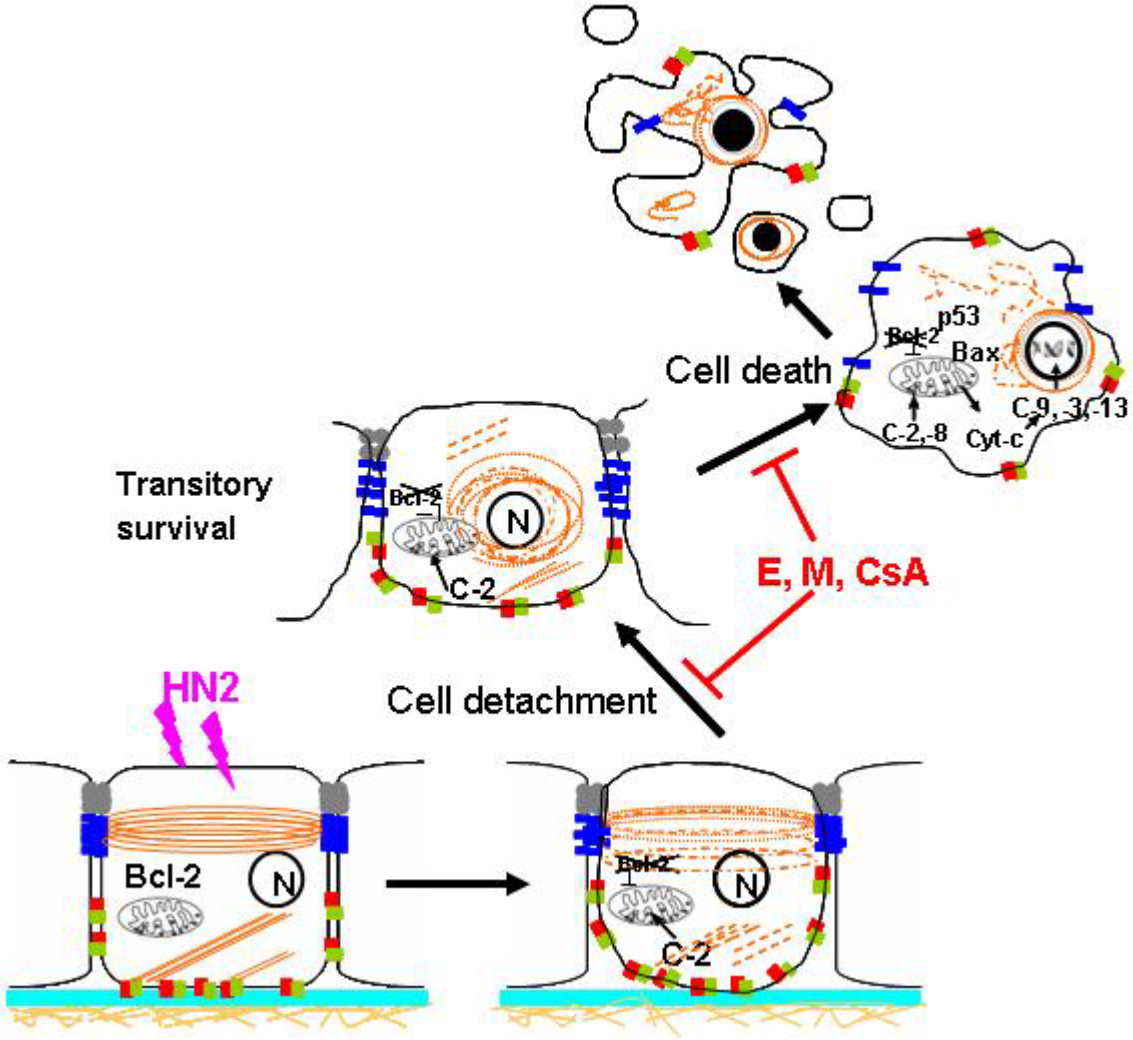

Figure 7. Schematic diagram of cellular events during HN2-induced anoikis. HN2 exposure triggers rapid caspase-2 activation and Bcl-2 decrease, associated with F-actin (orange) cytoskeleton disruption and consecutive cell rounding. Disorganization of cell-cell and cell-matrix contacts are followed by cell detachment of transitory living cells. In detached cells, activation of caspase-2 and effector caspases -9, -3 and -13, activation of p53, mitochondrial tranlocation of Bax and loss of mitochondrial potential are associated with the decrease of membrane adherent proteins integrins β1 (red), α3 (green) and E-cadherin (blue). Apoptotic cell death occurs in detached cells unable to reattach to the matrix.

inhibitors or E, M and CsA were compared after removal of the toxic. Cell attachment assays of HN2-treated cells showed that all of the inhibitors enhanced cell adherence properties and cell survival of the detached cells, suggesting that HN2-detachment and apoptosis are driven by several simultaneous pathways. The metalloproteinase inhibitors resulted in the less protective effects. Metalloproteinase activity on ECM and/or association with integrins may promote apoptosis/anoikis by loss of contact with ECM and inhibitors of metalloproteinase such as TIMP-1 may inhibit exert apoptosis inhibition involving the FAK survival signal transduction pathway (65,66). However, present data minimize the putative role of metalloproteinase activation in HN2-induced anoikis. The mitochondria protectors displayed better improvement than caspase inhibitor. The inhibition of loss of mitochondrial potential correlated well with the preservation of F-actin and integrins.The preservation of beta1, rendering the cells more resistant to apoptosis, was reported to increase the capacity in survival and adherence capacity of the cells, as well as mediating repair after mechanical injury (67-69). A prerequisite for effective ligand binding and subsequent intracellular events resides in the activation of integrins by cytosolic signals that confer a correct conformation of the extracellular domain (70,71). We thus envisage that HN2-induced suppression of integrin-mediated adhesion and signal transduction can be due to an inside-out apoptosis-promoting mechanism. It was recently demonstrated that DAP death-associated protein-kinase activation resulted in the down-regulation of integrins through an inside-out signal mechanism (72). Decreasing adhesion associated with loss of actin-F pattern and integrin and E-cadherin

expression can be restored by inhibition of mitochondrial depolarization. Consequently, HN2-induced anoikis could be triggered by an inside-out signal emanating from mitochondrial depolarization that modulates the functions of focal contacts and cell expression surface of integrins and E-cadherin (Figure 7).

In summary, the present study provides new insight into the expression of integrins and E-cadherin in the airway epithelial cells under exposure to blistering mustard and sheds light on how the mitochondrial depolarization may play determinant role in induction of cell detachment. Identifying such pathway is a critical step in developing efficient therapies for the treatment of mustard-destruction of airway epithelial tissue.

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