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Hsp90 cleavage by an oxidative stress leads to its client proteins degradation and cancer cell death

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ABSTRACT

The heat shock protein 90 (Hsp90) plays a crucial role in the stability of several proteins that are essential for malignant transformation. Hsp90 is therefore an interesting therapeutic target for cancer therapy. In this paper, we investigated whether an oxidative stress generated during ascorbate-driven menadione redox cycling (ascorbate/menadione), affects Hsp90 leading to the degradation of some critical proteins and cell death. Unlike 17-AAG, which inhibits Hsp90 but enhances Hsp70 levels, ascorbate/menadione-treated cells present an additional Hsp90 protein band of about 70 kDa as shown by Western blot analysis, suggesting Hsp90 cleavage. This Hsp90 cleavage seems to be a selective phenomenon since it was observed in a large panel of cancer cell lines but not in non-transformed cells. Antibodies raised against either the N-terminus or the C-terminus domains of Hsp90 suggest that the site of cleavage should be located at its N-terminal part. Furthermore, antibodies raised against either the α- or the β-Hsp90 isoform show that Hsp90β is cleaved while the α isoform is down-regulated. We have further shown that different Hsp90 client proteins like Bcr-Abl (a chimerical protein expressed in K562 leukemia cells), RIP and Akt, were degraded when K562 cells were exposed to an oxidative stress. Both Hsp90 cleavage and Bcr-Abl degradation were observed by incubating K562 cells with another H_2O_2-generating system (glucose/glucose oxidase) and by incubating KU812 cells (another leukemia cell line) with ascorbate/menadione. Due to the major role of Hsp90 in stabilizing oncogenic and mutated proteins, these results may have potential clinical applications.

1. Introduction

The heat shock protein 90 (Hsp90) plays a crucial role in the stability of several proteins (like Bcr-Abl, Akt, RIP, mutated p53, etc.) that are essential for malignant transformation [1,2]. Hsp90 is a conformational flexible protein that associates with a distinct set of co-chaperones depending on nucleotide (ADP or ATP) occupancy of an amino-terminal binding pocket. Nucleotide exchange and ATP hydrolysis drive the so-called Hsp90 chaperone machine to bind co-chaperones as well as client proteins, protecting these latter from proteasomal degradation [3]. The ability of Hsp90 to interact with multiple signaling networks is exploited by cancer cells, in which the expression of Hsp90 is increased [4]. Given the number of key nodal proteins that are Hsp90 clients, its inhibition represents an interesting target for cancer therapies. For instance, the

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safety evaluation of a small molecule inhibitor of Hsp90, the benzoquinone ansamycin antibiotic 17-allylamino-17-demethoxygeldanamycin (17-AAG), has been recently completed in four phase I clinical studies [5–8]. In fact, 17-AAG appears to act by suppressing the chaperone function of Hsp90, which causes the degradation of several client proteins.

On the other hand, it has been recently reported that the chaperoning function of Hsp90 may be disrupted by protein cleavage induced by hydrogen peroxide [9,10] or other reactive oxygen species (ROS) generated by arsenate [11]. The mechanisms underlying such a protein cleavage are still elusive, but due to the major role of Hsp90 in stabilizing key proteins involved in cancer cell survival, we decided to investigate whether an oxidative stress affects Hsp90, inducing the degradation of its client proteins and leading to cancer cell death. The rationale of our approach was that in K562 cells, a human leukemia cell line expressing Bcr-Abl (an Hsp90 client protein), oxidative stress generated by ascorbate-driven menadione redox cycling (ascorbate/menadione), inhibits glycolysis leading to an ATP depletion [12]. Since the assembly of the Hsp90–client protein complexes requires ATP [13,14], this could represent a new strategy to inhibit Hsp90.

The main finding of this work is that ascorbate/menadione induces an oxidative stress that causes a cleavage of Hsp90, which appears to preferentially affect cancer cells rather than normal cells. Indeed, by comparing a large panel of normal and cancer cell lines, the Hsp90 cleavage by ascorbate/menadione was observed in all tumor cell lines tested but in none of the non-transformed cells. This partial proteolysis disrupts the chaperoning function of Hsp90 leading to degradation of its client proteins: Bcr-Abl, RIP and Akt. The major role of oxidative stress in this process is supported by the fact that both Hsp90 cleavage and Bcr-Abl degradation were observed in K562 cells incubated with another H_2O_2-generating system (glucose/glucose oxidase) and by the effects of redox-modulators (N-acetylcysteine and aminotriazole). Due to the critical function played by Hsp90 in stabilizing oncogenic proteins in cancer cells, these data suggest that ascorbate/menadione might be of interest in anticancer therapy.

2. Materials and methods

2.1. Chemicals and antibodies

Menadione sodium bisulfite, sodium ascorbate, dimethylsulfoxide, N-acetyl-cysteine, glucose oxidase, 3-methyladenine, pepstatin, 17-allylamino-17-demethoxygeldanamycin (17-AAG), nitro-blue-tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), hydrogen peroxide, antipain and cycloheximide were purchased from Sigma (St. Louis, MO). Iodoacetate was purchased from Acros Organics (Geel, Belgium). Calpeptin was purchased from Calbiochem (San Diego, CA). Polyclonal rabbit primary antibodies against c-abl (#2862) and Akt (#4685) were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal primary antibodies against Hsp90α/β C-terminus were purchased from either Santa Cruz Biotechnology (F-8) (Santa Cruz, CA), Pharmingen (clone 68) (San Jose, CA), or StressGen (clone AC-88) (Ann Harbor, MI). Unless otherwise indicated, the Santa Cruz antibody was used for detection of Hsp90. Mouse monoclonal antibodies against specific isoforms of Hsp90 were from Abcam (Cambridge, UK) for Hsp90α (clone D7a) and Zymed (San Francisco, CA) for Hsp90β (clone H9010). Goat polyclonal antibody directed against Hsp90 N-terminus (N-17) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal primary antibody against β-actin (clone AC-15) was purchased from Abcam (Cambridge, UK), mouse monoclonal primary antibody against Hsp70 (clone C92F3A-5) was purchased from StressGen (Ann Harbor, MI). Mouse monoclonal primary antibody against RIP (clone 38) was purchased from Pharmingen (San Jose, CA). Mouse monoclonal primary antibody against LC3 (clone 5F10) was purchased from Nanotools (Teningen, Germany). Rabbit secondary antibodies were purchased from Chemicon (Billerica, MA). Mouse secondary antibodies were purchased from Dako (Glostrup, Denmark). Complete Mini protease inhibitor cocktail was purchased from Roche Applied System (Mannheim, Germany). All other chemicals were ACS reagent grade.

2.2. Cell culture conditions

The CML cell line K562 was a gift of Dr. F. Brasseur (Ludwig Institute for Cancer Research-LICR-Brussels) and maintained in RPMI medium supplemented with 10% foetal calf serum, streptomycin 100 μg/ml, penicillin 100 IU/ml, and gentamicin (50 μg/ml) at 37 °C in humidified 5% CO_2. Cells were treated with ascorbate (2 mM) and menadione (10 μM) either separately or in association for the indicated length of time.

K562 cells were incubated at a concentration of 1 million cells per ml. Inhibitors and/or ascorbate/menadione were added directly to the incubation medium at the indicated times. When required, preincubation of 1 h was used for some of the inhibitors. Glucose oxidase was added directly (0.25 U/ml) to the medium, which was supplemented with 25 mM of glucose to avoid its depletion.

The additional cancer cell lines FSAII (human fibrosarcoma), LLC (human lung adenocarcinoma) and B16 melanoma (murine melanoma), were a gift of Dr. O. Feron (FATH, UCL, Belgium). The MCF7 (human breast carcinoma) cell line was a gift of Dr. F. Brasseur (Ludwig Institute for Cancer Research-LICR-Brussels). The Ishikawa (human endometrial adenocarcinoma) cell line was obtained from the European Collection of Cell Cultures (ECACC, UK). The KU812 (chronic myelogenous leukemia) cell line was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). In addition, we also employed the TLT (murine hepatoma) cell line. The panel of non-transformed cell lines includes Balb/c3T3 (mouse fibroblasts) from the European Collection of Cell Cultures (ECACC, UK), HUVEC (human endothelial cells), freshly isolated mouse hepatocytes and human peripheral blood leucocytes.

2.3. Cell survival and ATP measurement

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH) both in the culture medium and

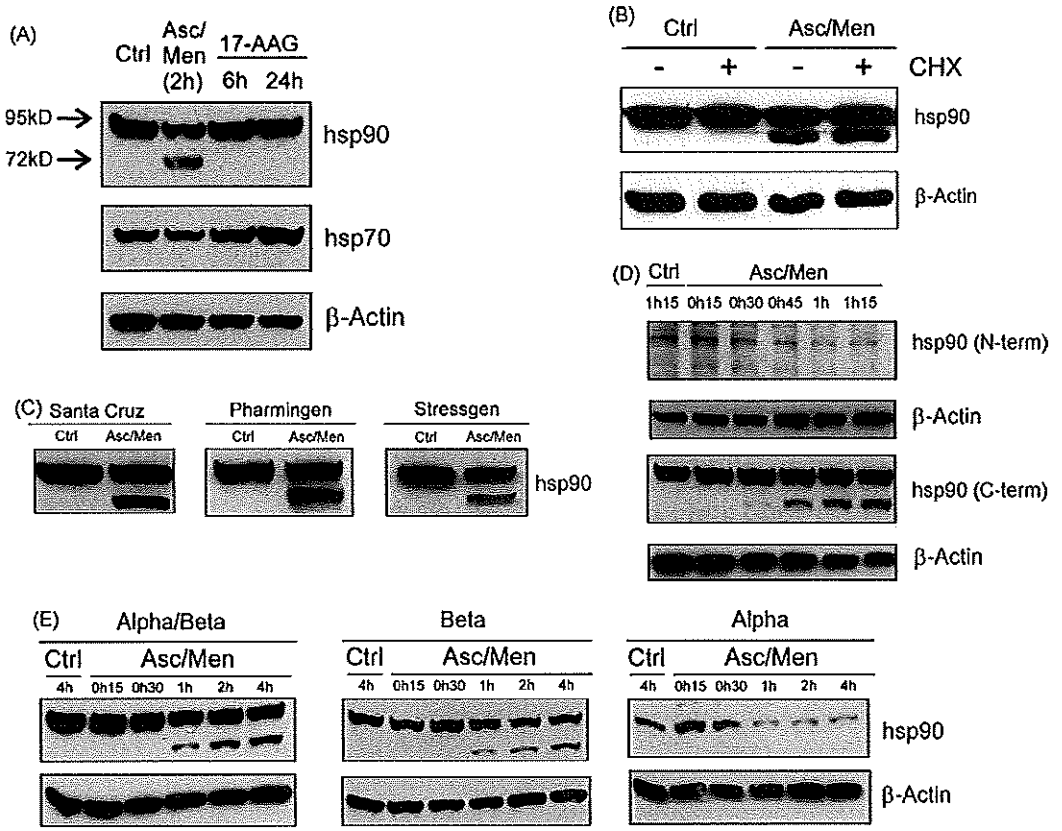

Fig. 1 – Ascorbate/menadione induces Hsp90 cleavage. K562 cells were incubated in the absence (Ctrl) or in the presence of ascorbate (Asc, 2 mM) and menadione (Men, 10 μM) either in combination (Asc/Men) or added separately. Aliquots of cell suspension were taken at the indicated times and Western blots were performed as described under Section 2. Typical results out of three separate experiments are represented. (A) Cells were incubated for 2 h in the presence of Asc/Men or for 6 and 24 h with 17-AAG at 5 μM. (B) Cycloheximide (CHX) was preincubated for 30 min at a concentration of 10 μg/ml. Cells wee further incubated for 2 h in the absence or in the presence of Asc/Men. (C) Cells were incubated in the presence or the absence of Asc/Men for 2 h. The resulting membranes were probed against three different commercial antibodies directed against both Hsp90 isoforms. (D) Cells were incubated for different times in the presence of Asc/Men. Membranes were probed against antibodies raised against either the N-terminus or the C-terminus part of Hsp90. (E) Cells were incubated in the absence or in the presence of Asc/Men for different incubation times. At the indicated times, aliquots of cells suspension were taken and processed as indicated above. The membranes were probed against antibodies raised against both hsp90 isoforms (α and β), Hsp90α isoform or Hsp90β isoform.

in the cell pellet obtained after centrifugation, as previously described [12]. The results are expressed as a ratio of released activity to the total activity.

ATP content was determined by using the Roche ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany) according to the procedures described by the suppliers. The results are expressed as nmol/mg of proteins. The amount of proteins was determined by the method of Bradford using BSA as reference.

2.4. *Western blot analysis*

Appropriate protein amounts (40–50 μg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membranes were blocked in blocking-buffer (5% non-fat dry milk, Tris 20 mmol/L, pH 7.6, NaCl 150 mmol/L, 0.01% Tween-20) for 1 h at room temperature, which was followed by incubation with primary antibody overnight at 4 °C. The membranes were washed and incubated for 60 min with a secondary antibody. Immunodetection was performed using the ECL detection kit (Amersham, UK) for HRP-coupled secondary antibodies or a buffer containing NBT/BCIP for alkaline phosphatase-coupled secondary antibodies. β-actin served as a loading control.

2.5. *Statistical analysis*

Data were analyzed by using ANOVA followed by a post hoc Bonferroni test to determine the statistical significance among the different groups. The level of significance was set at $p \leq 0.05$ ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$).

3. Results

3.1. *Oxidative stress by ascorbate/menadione induces Hsp90 cleavage*

Fig. 1A shows the appearance of an additional Hsp90 protein band of approximately 70 kDa in K562 cells treated with ascorbate/menadione (2 mM/10 μM) suggesting Hsp90 cleavage. In contrast to the ansamycin antibiotic 17-AAG, a well-known Hsp90 inhibitor, the cleavage of Hsp90 induced by ascorbate/menadione was not accompanied by an increase in the Hsp70 levels. In addition, the incubation of cells with cycloheximide (a protein synthesis inhibitor), did not affect the appearance of the low molecular weight species of Hsp90 (Fig. 1B), ruling out a new synthesis of a truncated form of Hsp90. The appearance of this second protein band was not an experimental artifact and it was not specific for one particular antibody, since three different commercial antibodies detected the low molecular weight species of Hsp90 in ascorbate/menadione-treated cells (Fig. 1C). By using antibodies raised against epitopes located either at the N-terminal or C-terminal part of Hsp90, it was shown that the appearance of the second protein band was only observed with antibodies addressed to the C-terminal part, suggesting that the site of cleavage should be located at the N-terminus of Hsp90 (Fig. 1D). Furthermore, the use of antibodies raised against either the α- or β-Hsp90 isoform, allows the detection of the cleaved protein band only in Hsp90β while the α isoform is not cleaved but rather seems to be down-regulated (Fig. 1E).

3.2. *The Hsp90 cleavage is a selective process occurring only in cancer cells*

We decided to further examine whether Hsp90 cleavage is also observed in other cancer cell lines as well as in non-transformed cells challenged with ascorbate/menadione. Fig. 2 shows immunoblots against Hsp90 in cell lysates obtained in a wide panel of cell lines after incubation in the absence or in the presence of ascorbate/menadione. They include both normal (HUVEC, Balb/c3T3, freshly prepared human leucocytes and freshly prepared murine hepatocytes) and transformed cells (TLT, Ishikawa, MCF-7, melanoma B16, FSAII and LLC). Irrespective of species (rodents or human) or tissue origin (breast, liver, skin, endometrial), ascorbate/menadione caused a cleavage of Hsp90 in transformed cell lines, while no Hsp90 cleavage was observed in any normal cell line tested so far, suggesting a rather selective effect by ascorbate/menadione.

3.3. *The Hsp90 cleavage leads to degradation of its client proteins*

Fig. 3A shows that in ascorbate/menadione-treated K562 cells, the time-dependent appearance of a low molecular weight species of Hsp90 is accompanied by the degradation of the Bcr-Abl protein. Neither ascorbate nor menadione induced Hsp90 cleavage and Bcr-Abl degradation when added separately. Meanwhile, the levels of c-Abl protein were unaffected. Since the half-life of Bcr-Abl protein in K562 cells is about 40 h [15], it is unlikely that such a decrease in the amount of protein may occur through a translational inhibition of Bcr-Abl but it is rather due to protein degradation. Moreover, no cytotoxicity was observed up to 6 h of incubation, but after 24 h of incubation, cell death in ascorbate/menadione-treated cells significantly increased to reach 87% (assessed by LDH leakage). Once again, when administered separately, neither ascorbate (2 mM) nor menadione (10 μM) were cytotoxic after 24 h of incubation (data not shown). To confirm that Hsp90 cleavage by ascorbate/menadione would impair the stability of other Hsp90 client proteins, we looked for the stability of RIP and Akt. The incubation of the cells in the presence of ascorbate/menadione also induced the degradation of both RIP and Akt (Fig. 3B). It is tempting, therefore, to suggest that both processes, namely Hsp90 cleavage and degradation of its client proteins, may be linked in a cause–effect manner.

Since ascorbate/menadione inhibits glycolysis leading to a strong and rapid depletion of ATP [12] and because this

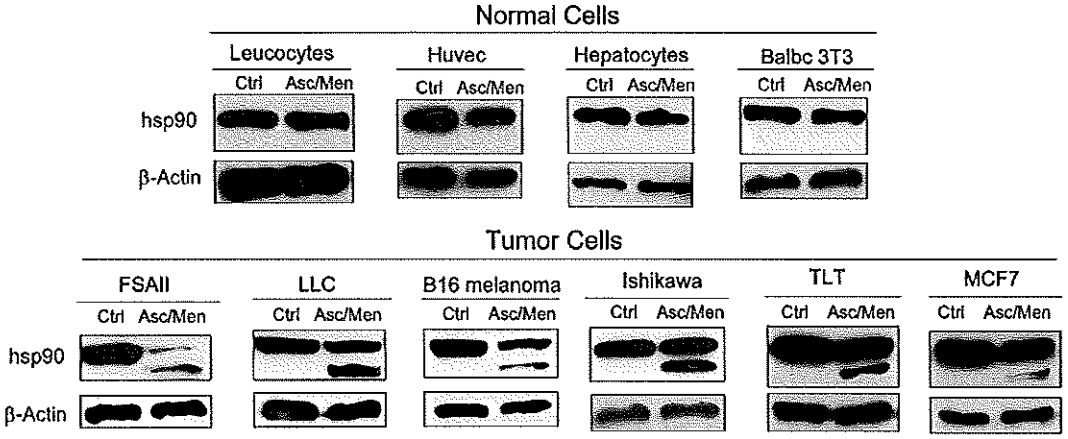

Fig. 2 – Effect of ascorbate/menadione on Hsp90 cleavage in both transformed and non-transformed cells. Transformed and non-transformed cells were incubated for 2 h in the absence (Ctrl) or in the presence of ascorbate/menadione (Asc/Men, 2 mM/10 μM). Cells were harvested and immunoblotting was performed using antibodies against Hsp90 and β-actin as described under Section 2. Typical results out of three separate experiments are represented.

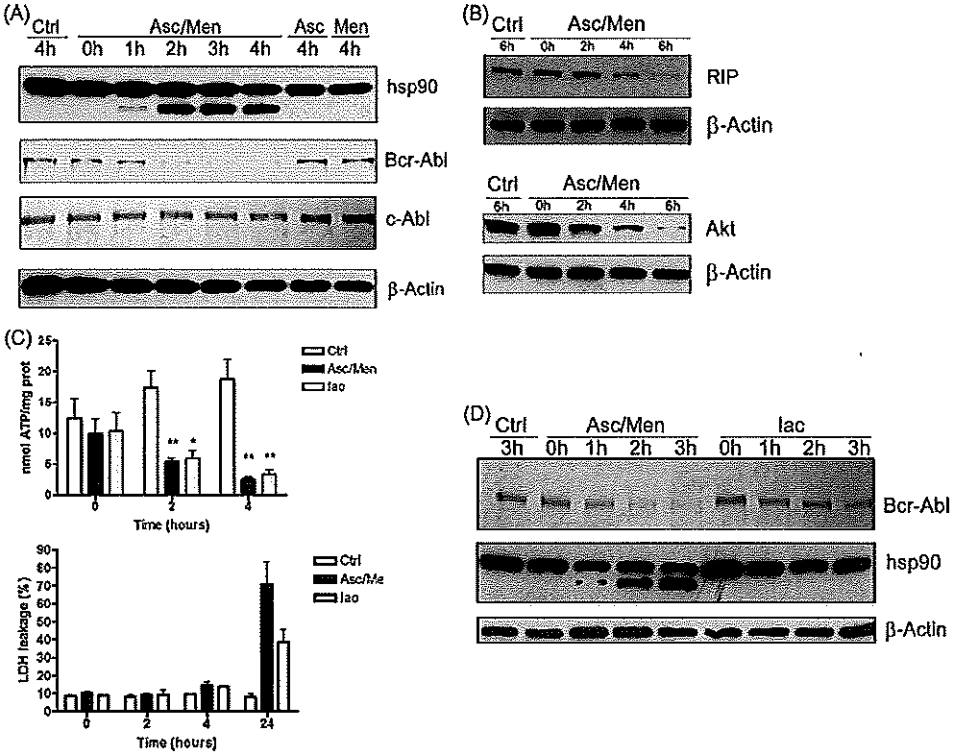

Fig. 3 – The Hsp90 cleavage leads to the degradation of its client proteins. (A) K562 cells were incubated for 4 h in the absence (Ctrl) or in the presence of ascorbate and menadione either in combination (Asc/Men) or added separately, or iodoacetate (Iac, 100 μM). Aliquots of cell suspension were taken at the indicated times. The resulting membranes were probed against Hsp90 and c-Abl/Bcr-Abl. (B) Cells were incubated for 6 h in the absence or in the presence of Asc/Men. At the indicated times, and after SDS/PAGE and protein transfer, the membranes were probed against both RIP1 and Akt proteins. (C) ATP content of cells is expressed as nmol/mg protein and LDH leakage is expressed as % of released enzyme. These results represent mean values ± S.E.M. of three separate experiments. Bcr-Abl and Hsp90 contents were assessed by immunoblotting. (D) Cells were incubated for 3 h in the absence or in the presence of Asc/Men. At the indicated times, and after SDS/PAGE and protein transfer, the membranes were probed against both Hsp90 and c-Abl/Bcr-Abl proteins. For Western blots, typical results out of three separate experiments are represented.

latter may affect the activity of Hsp90 [16], a potential link between the content of ATP and the stability of both Hsp90 and Bcr-Abl proteins was further examined. First, we compared the effect of both ascorbate/menadione and iodoacetate (100 μM), a well-known metabolic inhibitor of glycolysis [17], on K562 intracellular ATP levels (Fig. 3C). Both ascorbate/menadione and iodoacetate provoked a similar decrease of ATP levels, by about 70%, after only 2 h of incubation. Since the cellular viability was unaffected by either ascorbate/menadione or iodoacetate after 4 h of incubation (Fig. 3C), such a drop of ATP is unlikely due to cell death but is rather the consequence of glycolysis inhibition. Thus, if ATP depletion is involved in Hsp90 cleavage leading to Bcr-Abl degradation, it would be expected that the incubation of K562 cells with iodoacetate should give the same result as that shown by ascorbate/menadione. Nevertheless, the incubation of K562 cells in the presence of iodoacetate did not result in either Hsp90 cleavage or Bcr-Abl degradation, as was the case in the presence of ascorbate/menadione (Fig. 3D).

3.4. *Role of oxidative stress on the ascorbate/menadione-induced Hsp90 cleavage and Bcr-Abl degradation in two different leukemia cell lines*

To confirm that the effects of oxidative stress on Hsp90 cleavage and the subsequent Bcr-Abl degradation were not depending on the hydrogen peroxide-generating system, K562 cells were exposed to another H_2O_2-generating system (Fig. 4A). The incubation of cells in the presence of 0.25 U/ml of glucose oxidase and 25 mM glucose (Glox) caused both Hsp90 cleavage and Bcr-Abl degradation in a similar way but to a smaller extent as observed by using ascorbate/menadione. Interestingly, the addition of H_2O_2 (1 mM), as a bolus and not in a sustained way, as produced by an H_2O_2-generating system, did not affect either Hsp90 cleavage or Bcr-Abl degradation. Moreover, such effects were not specific for one leukemia cell line, since both the Hsp90 cleavage and Bcr-Abl degradation were also observed in KU812 cells (another leukemia cell line expressing Bcr-Abl) exposed to ascorbate/menadione (Fig. 4B). Given that we have previously shown that the cytotoxicity by

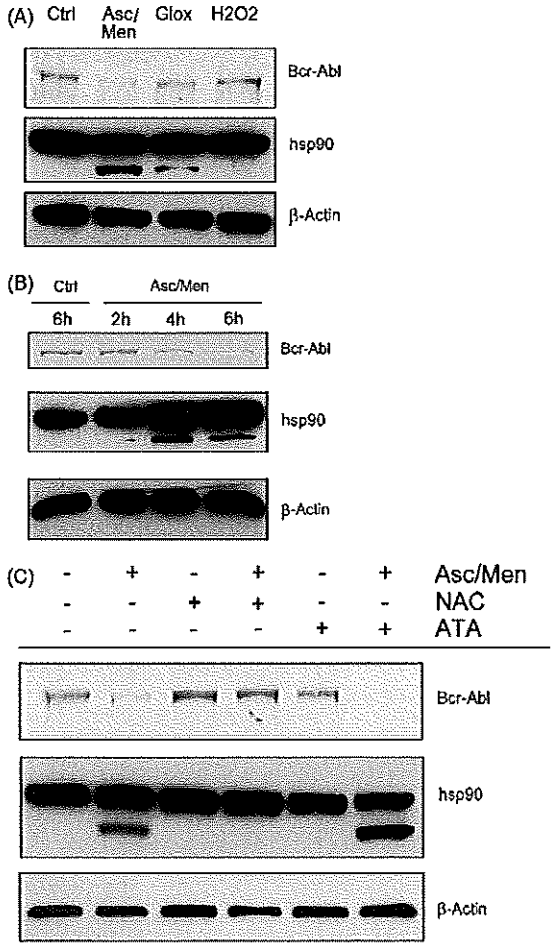

Fig. 4 – Role of oxidative stress on ascorbate/menadione-induced Hsp90 cleavage and Bcr-Abl degradation in two different leukemia cell lines. (A) K562 cells were incubated for 2 h in the absence (Ctrl) or in the presence of ascorbate/menadione (Asc/Men, 2 mM/10 μM), glucose (25 mM)/glucose oxidase (Glox, 0.25 U/ml), and H_2O_2 (1 mM); (B) KU812 cells were incubated in the presence (Asc/Men) or the absence (Ctrl) of 2 mM/10 μM of ascorbate/menadione; (C) K562 cells were incubated for 2 h in the presence or the absence of ascorbate/menadione (Asc/Men, 2 mM/10 μM), 3 mM of N-acetylcysteine (NAC) and 5 mM of 3-aminotriazole preincubated for 1 h (ATA). Aliquots of cell suspension were taken at the indicated times. Cells were washed, lysed and proteins were separated by SDS/PAGE followed by electroblot to nitrocellulose membranes. The different proteins were probed with their respective antibodies as indicated under Section 2. Typical results out of three separate experiments are represented.

ascorbate/menadione was either suppressed by the antioxidant molecule NAC [18,19], or enhanced by inhibiting catalase with aminotriazole [20], the effects of these compounds on Hsp90 cleavage and Bcr-Abl degradation were further examined. The incubation of K562 cells with ascorbate/menadione and NAC (3 mM) totally suppressed both Hsp90 cleavage and Bcr-Abl degradation, whereas in ascorbate/menadione-treated cells preincubated with aminotriazole (5 mM), an increased cleavage of Hsp90 and a complete degradation of Bcr-Abl were observed (Fig. 4C).

3.5. *Potential effects of some metabolic inhibitors on the ascorbate/menadione-induced Hsp90 cleavage and Bcr-Abl degradation*

The incubation of cells with 3-methyladenine (an inhibitor of macroautophagy), while having a very weak effect against Bcr-Abl degradation, did not protect against either Hsp90 cleavage (Fig. 5A) or cytotoxicity (Fig. 5B) induced by ascorbate/menadione. In spite of these results, the potential role of macroautophagy induction by ascorbate/menadione was further examined. In agreement with a previous report [21], we also observed that in K562 cells LC3 is already processed under control conditions (Fig. 5C). Moreover, the oxidative stress induced by ascorbate/menadione did not modify the amounts of both LC3I and LC3II protein bands. Taken together, these results suggest that macroautophagy is unlikely involved in both processes (protein cleavage and oncogene degradation).

Regarding the potential role for proteases, neither a pan-caspase inhibitor, namely Z-VAD-FMK, nor a cathepsin inhibitor (E64d) or several protease inhibitors (calpeptin, pepstatin, antipain) were able to modify both Hsp90 cleavage and the subsequent Bcr-Abl degradation induced by ascorbate/menadione (data not shown).

4. Discussion

The major finding of this study is that an oxidative stress induces Hsp90 cleavage in cancer cells leading to the degradation of its client proteins. This lethal oxidative injury for cancer cells is caused by the formation of ROS (specially H_2O_2) that are generated during the menadione redox cycling enhanced by ascorbate [19,22]. Given that oncogenic transformation is associated with an increased generation of ROS, which renders transformed cells more sensitive to oxidative stress, this approach may serve as a biochemical basis to selectively kill cancer cells, as previously described by Trachootham et al. [23].

By degrading Hsp90, ascorbate/menadione may overcome a well-known cause of cancer drug resistance, namely the appearance of mutated proteins (i.e. mutations of Bcr-Abl that render cells resistant to imatinib). This represents an advantage over drugs interacting specifically with the targeted protein and appears as a different mechanism from the previously described Hsp90 inhibitors. Indeed, the phenomenon reported here involves the cleavage of the Hsp90 protein, disrupting its molecular chaperone function, as shown by the degradation of its client proteins. This cleavage is a rapid process that is not accompanied by an increase in the amount of Hsp70 protein, an important undesirable side effect in classical Hsp90 inhibition [24]. Different commercial antibodies were able to detect the low molecular weight species of Hsp90, ruling out a potential artifact. Moreover, our results show that the site of cleavage is likely located at the

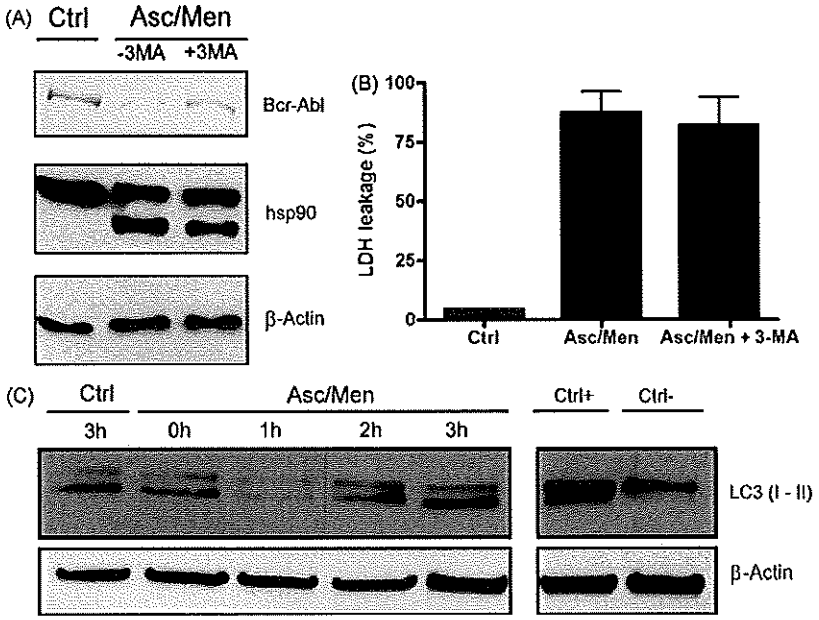

Fig. 5 – Potential role of macroautophagy on the ascorbate/menadione-induced Hsp90 cleavage and Bcr-Abl degradation. K562 cells were preincubated (1 h) or not with 3-methyladenine (3-MA, 10 mM). (A) Afterwards, they were exposed for 2 h to ascorbate/menadione (Asc/Men, 2 mM/10 μM). Aliquots of cell suspension were taken and cells were washed, lysed and proteins were separated by SDS/PAGE followed by electroblot to nitrocellulose membranes. The membranes were probed against Hsp90 and c-Abl/Bcr-Abl as indicated under Section 2. Typical results out of three separate experiments are represented. (B) After preincubation with 3-MA, cells were incubated for 24 h in the absence (Ctrl) or in the presence of ascorbate/menadione (Asc/Men, 2 mM/10 μM). LDH leakage is expressed as % of released enzyme. (C) The conversion of LC3-I to LC3-II was assessed by immunoblotting against LC3 protein of cell lysates from K562 cells exposed to ascorbate/menadione (Asc/Men, 2 mM/10 μM) for different times. Positive control (Ctrl+) consisted of WT MEF cells, well known to express both LC3-I and LC3-II. Negative control (Ctrl−) consisted of ATG5$^{-/-}$ MEF cells which express only LC3-I.

N-terminal extremity and probably involves only the Hsp90β isoform. Although this is not the first report about Hsp90 cleavage, several differences may be outlined between our results and those previously reported. They include: the proposed molecular size of the cleaved fragment, the protein domain where the cleavage seems to occur, the putative Hsp90 isoform involved, and the experimental model employed (cell types, free-cellular systems, nature of the stress, etc.). For instance, some authors did the assays by exposing either lung epithelial cells [9] or Hela and H-60 cells [10] to H_2O_2 as a bolus. While both groups report Hsp90 cleavage and RIP degradation, a molecular size of about 45 kDa for the cleaved fragment was reported, instead of about 70 kDa as we observed. Recently, other authors [11] reported that apoptosis induced by arsenite (10 μM) in NIH3T3 fibroblasts is mediated by a ROS-dependent Hsp90 degradation which becomes evident after 8–12 h of treatment. At that time, however, most cells are already dead. By using either recombinant Hsp90 protein or K562 cytoplasmic extracts exposed to 50 nM to 1 μM human granzymeB [25], some authors report a proteolysis of Hsp90β isoform which generates bands of about 70–75 kDa. Although both the Hsp90 isoform and the molecular size of the cleaved fragments correspond to what we observed, the sites of cleavage by granzymeB are located at the C-terminal part of Hsp90, while under our experimental conditions the site of cleavage is presumably located at the N-terminal part of Hsp90. Finally, some authors performed the assays in a free-cell system using purified rat liver Hsp90 exposed to a hydroxyl radical-generating system, namely a mixture of $FeCl_3$ (0.5 mM) ascorbate (30 mM) and 1 mM of nucleotide [26]. By mapping the N-terminal ATP-binding site, the authors reported cleaved fragments of about 70–75 kDa, that correspond to cleavages observed in the Hsp90 binding site of nucleotides. Given its molecular size and the localization of the putative site of cleavage at the N-terminus, it is tempting to hypothesize that it corresponds to the cleaved fragment we detected in whole cells exposed to ascorbate/menadione. Studies are now in progress in order to elucidate the site of cleavage.

Since ATP plays a role in Hsp90 activity [1], we raised the question whether a drop of ATP, besides the subsequent energetic failure, would affect Hsp90. Our results did not totally exclude a role of ATP, but since the metabolic inhibitor iodoacetate did not affect either Hsp90 or Bcr-Abl, the ATP depletion is presumably not the crucial event in the early steps leading to Hsp90 cleavage and the subsequent Bcr-Abl degradation. Furthermore, a putative role of macroautophagy in the mechanism leading to Hsp90 cleavage was not established. Indeed, due to the high basal levels of autophagy in K562 cells [21] and the lack of effect of 3-methyladenine, the involvement of macroautophagy in Hsp90 cleavage is rather unlikely. Two sets of experiments outline a major role for oxidative stress. First, the incubation of K562 cells in the

presence of an H_2O_2-generating system like glucose/glucose oxidase, leads to both Hsp90 cleavage and Bcr-Abl degradation. Second, the use of redox-modulators (NAC and aminotriazole) strongly affected these latter processes.

Since numerous key proteins can be stabilized by Hsp90, namely Bcr-Abl, v-Src, Raf-1, HER2/Neu (ErbB2), HIF-1α, and the mutated p53 protein [1,2], we postulate that the degradation of these crucial proteins following Hsp90 cleavage by ascorbate/menadione would be a major component of the efficacy of this combination with regard to a large number of cancer cell lines [27–31]. Importantly, while for cancer cells the consequence of these targeted protein degradations is the rapid and irreversible induction of cell death, the treatment by ascorbate/menadione does not interfere with non-transformed cells. One explanation relies on the fact that cancer cells readily take up ascorbate (vitamin C). Indeed, due to its structural similarity with glucose [32], dehydroascorbic acid is transported by the facilitative glucose transporters (GLUTs) and is then intracellularly reduced to ascorbate. Due to the high glycolytic metabolism of cancer cells, most tumors overexpress GLUTs, leading to the accumulation of ascorbate in tumors [33]. An additional explanation may be the enhanced sensitivity of cancer cells towards an oxidative stress due to their deficiency in antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase [34].

Actually, two sets of data reinforce the idea about a selective effect by ascorbate/menadione: firstly, the cleavage of Hsp90 was only observed in tumor cells and not in normal cells; secondly, ascorbate/menadione was cytotoxic against K562 cells but without effect on human hematopoietic stem cells [22]. The greater sensitivity of cancer cells toward the ascorbate/menadione-induced oxidative stress is certainly one element that explains the preferential cleavage of Hsp90 in these cells. Another explanation may be that Hsp90 itself is more susceptible to an oxidative degradation in cancer cells since Hsp90 conformation is different in tumor cells as compared to normal cells. Indeed, tumor cells contain Hsp90 complexes in an activated high-affinity conformation that are highly sensitive to Hsp90 inhibitors [35]. Since conformation has been reported to influence the susceptibility of Hsp90 to proteolytic degradation [36], this could explain its preferential cleavage in cancer cells following ascorbate/menadione exposure.

By considering the low toxicities of each compound administered separately as shown by previous clinical trials [37,38], and on the basis of the in vivo results we obtained in K562-bearing mice [20], we postulate that ascorbate/menadione may have potential clinical applications. Within this frame, a similar approach for treatment of metastatic or locally advanced, inoperable transitional cell carcinoma of the urothelium (stage III and IV bladder cancer) has been granted by the FDA and the results of a first clinical trial have been recently published [39].

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