Asbestos induces doxorubicin resistance in MM98 mesothelioma cells *via* HIF-1 α

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ABSTRACT: Human malignant mesothelioma (HMM), which is strongly related to asbestos exposure, exhibits high resistance to many anticancer drugs. Asbestos fibre deposition in the lung may cause hypoxia and iron chelation at the fibre surface. Hypoxia-inducible factor (HIF)-1 α , which is upregulated by a decreased availability of oxygen and iron, controls the expression of membrane transporters, such as P-glycoprotein (Pgp), which actively extrude the anticancer drugs. The present study aimed to assess whether asbestos may play a role in the induction of doxorubicin resistance in HMM cells through the activation of HIF-1 α and an increased expression of Pgp.

After 24-h incubation with crocidolite asbestos or with the iron chelator dexrazoxane, or under hypoxia, HMM cells were tested for HIF-1 α activation, Pgp expression, accumulation of doxorubicin and sensitivity to its toxic effect.

Crocidolite, dexrazoxane and hypoxia caused HIF-1 α activation, Pgp overexpression and increased resistance to doxorubicin accumulation and toxicity. These effects were prevented by the co-incubation with the cell-permeating iron salt ferric nitrilotriacetate, which caused an increase of intracellular iron bioavailability, measured as increased activity of the iron regulatory protein-1.

Crocidolite, dexrazoxane and hypoxia induce doxorubicin resistance in human malignant mesothelioma cells by increasing hypoxia-inducible factor-1 α activity, through an iron-sensitive mechanism.

KEYWORDS: Asbestos, doxorubicin resistance, hypoxia-inducible factor-1 α , iron, mesothelioma, P-glycoprotein

Human malignant mesothelioma (HMM) is an aggressive tumour of the serosal cavities, which is strongly related to the exposure to asbestos fibres [1]. It has a poor prognosis due to its resistance to many anticancer drugs [2] and to the difficult delivery of chemotherapeutic agents into the pleural tissue [3]. In several *in vitro* models of mesothelioma, it has been reported that the multidrug resistance (MDR) is caused by the overexpression of membrane transporters, such as P-glycoprotein (Pgp) and MDR-associated proteins (MRPs), which actively extrude the drugs, lowering their intracellular concentration and activity [4, 5]. The *Pgp* gene has a hypoxia-responsive enhancer in the promoter and is upregulated by the transcription factor hypoxia-inducible factor (HIF)-1 α [6]. HIF-1 α is composed of two subunits: β , which is constitutively expressed, and α , which is rapidly degraded under normal conditions but becomes stable when the oxygen or iron supply decreases, leading to a net increase in HIF-1 α [7, 8]. HIF-1 α is constitutively high in the hypoxic areas of tumours; moreover, many growth factors and cytokines

increase HIF-1 α synthesis under normoxic conditions [9]. When active, HIF-1 α upregulates several genes involved in processes such as cellular growth, glucose and iron metabolism, pH control, angiogenesis, matrix remodelling and drug resistance [10]. Since HIF-1 α promotes cellular proliferation, inhibition of apoptosis, invasion and MDR, its expression in tumours is related to poor prognosis [11]. Thus, different therapeutic approaches have been attempted in order to reduce HIF-1 α expression [10, 12]. In the lung, most cell types, including bronchial and alveolar epithelium, smooth muscle and vascular endothelium, overexpress HIF-1 α under hypoxic conditions [13]. High levels of HIF-1 α have been described in mesothelioma biopsies of patients, whereas mesothelial cells contain low amounts of HIF-1 α [14].

Asbestos may elicit both proliferation and apoptosis in mesothelial cells, thus representing a complete carcinogen [15]. Furthermore, crocidolite asbestos has been reported to act as an iron chelator and alter the intracellular availability of iron [16, 17]. In the present study, the authors investigated whether: 1) crocidolite asbestos may

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play a role in inducing doxorubicin resistance, which is observed in HMM cells; 2) such an effect may be mimicked by hypoxia and iron chelation; 3) the drug resistance eventually induced by asbestos, hypoxia and iron chelation is mediated by HIF-1 α activation and Pgp overexpression; and 4) iron supply to the cells may prevent these effects.

MATERIALS AND METHODS

Materials

Foetal bovine serum and Ham's F-12 medium were supplied by BioWhittaker (Verviers, Belgium); plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA, USA); MG132 and 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) were from Calbiochem (La Jolla, CA, USA). Electrophoresis reagents were obtained from Biorad Laboratories (Hercules, CA, USA) and the protein content of cell monolayers and cell lysates was assessed with the BCA Kit from Pierce (Rockford, IL, USA). Dexrazoxane (ICRF-187) was purchased from Chiron (Amsterdam, the Netherlands). When not otherwise specified, the other reagents were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Stock solutions of 3 mM ferric nitrilotriacetate (FeNTA) were prepared by mixing 1:1 6 mM nitrilotriacetic acid in 1 N NaOH, and 6 mM ferric chloride in 1 N HCl; the pH was adjusted to neutrality with NaOH [18].

Cells

MM98 cells were HMM cells established from the pleural effusion of a male patient with histologically confirmed malignant mesothelioma; the mesothelial origin of the isolated cells was confirmed by positive immunostaining [19, 20]. Pgp overexpression and doxorubicin resistance of MM98 cells was performed as previously described [20]. Cells were cultured in Ham's F-12 medium (containing 3 μ M FeSO₄) supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine, and maintained in a humidified atmosphere at 37°C, 20% O₂ and 5% CO₂. When cultured under hypoxia, cells were maintained in a humidified atmosphere at 37°C, 3% O₂ and 5% CO₂ for 24 h, in an appropriate Heraeus incubator (Heraeus, Hanau, Germany), which can decrease the O₂ injection by increasing the N₂ flow injection. The O₂ tension was monitored throughout the incubation time by an O₂ sensor incorporated in the incubator, which automatically varies the N₂ flow injection in order to maintain a stable 3% O₂ level.

Cell seeding density may influence the activation of HIF-1 α and the resistance to doxorubicin, as previously described [21]. In preliminary seeding density-dependence experiments, it was observed that a decreasing cellular confluence was accompanied by decreased expression of HIF-1 α and Pgp protein and increased doxorubicin accumulation, both in normoxic and hypoxic cells (data not shown). For these reasons, all the experimental procedures reported in the present study were performed with a constant high cellular density (90% cellular confluence, corresponding to 1 $\times$ 10⁶ cells·mL⁻¹).

Asbestos fibres

Union International Contre le Cancer (International Union Against Cancer) crocidolite fibres were sonicated (Labsonic sonicator; Hielscher, Teltow, Germany; 100 W, 10 s) before incubation with cell cultures, in order to dissociate fibre bundles and allow better suspension and diffusion in the culture medium.

Electrophoretic mobility shift assay

Cells were plated in 60-mm-diameter dishes at confluence and all procedures for nuclear protein extraction were performed at 4°C using ice-cold reagents, as described previously [22]. The probe containing the HIF-1 α oligonucleotide consensus sequence was labelled with [γ -³²P]-adenosine triphosphate (ATP; 3,000 Ci·mmol⁻¹, 250 μ Ci; Amersham International, Little Chalfont, UK), using T4 polynucleotide kinase (Roche, Basel, Switzerland). The sequence of the oligonucleotide was 5'-TCTGTACGTGACCACACTCACCTC-3' (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For each extract, 10 μ g was incubated for 20 min with 20,000 counts·min⁻¹ (cpm) of [³²P]-labelled double-stranded oligonucleotide at 4°C. In the supershift assay, nuclear extracts were pre-incubated for 30 min at room temperature with 2 μ L of anti-HIF-1 α (Santa Cruz Biotechnology); the reaction mixture containing the [³²P]-labelled double-stranded oligonucleotide was then added. The DNA-protein complex was separated on a nondenaturing 4% polyacrylamide gel in Tris-borate-EDTA buffer (0.4 M Tris, 0.45 M boric acid and 0.5 M EDTA, pH 8.0). After electrophoresis, the gel was dried and autoradiographed by exposure to X-ray film for 24 h.

Iron regulatory protein-1 binding activity

To measure the iron regulatory protein (IRP)-1 activity, taken as an index of intracellular iron [8], the probe containing the iron-responsive element (IRE) sequence from ferritin mRNA was labelled with [γ -³²P]-ATP via T4 polynucleotide kinase. The sequence of the oligonucleotide was 5'-GUUCUUGCUU-CAACAGUGUUUGAACGGAAC-3'. For each extract, 10 μ g of cytosolic lysate proteins were incubated for 20 min with 20,000 cpm of [³²P]-labelled oligonucleotide at 4°C and subjected to electrophoretic mobility shift assay (EMSA). In competition assays, an excess of unlabelled (cold) IRP-1 oligonucleotide was added into the EMSA reaction mixture, then samples were processed as previously reported [8].

Western blot analysis

Western blot detection of Pgp and glyceraldehyde-3-phosphate dehydrogenase was performed as previously described [20]. To detect thioredoxin and glutathione reductase, the following antibodies were used: rabbit anti-thioredoxin-1/2 (sc-58439; diluted 1:250 in PBS/bovine serum albumin (BSA) 1%) and goat anti-glutathione reductase (diluted 1:200 in PBS/BSA 1%; all from Santa Cruz Biotechnology).

Doxorubicin accumulation

Cells were grown in 60-mm diameter dishes, incubated for 24 h in fresh medium containing 5 μ M doxorubicin, washed twice in ice-cold PBS and detached with trypsin/EDTA (0.05/0.02% v/v). Intracellular doxorubicin accumulation was measured as described elsewhere [23].

Annexin V and propidium iodide assays

MM98 cells were incubated for 24 h in medium containing 5 μ M doxorubicin, in the absence or presence of crocidolite asbestos fibres (25 μ g·cm⁻²), dexrazoxane (100 μ M), FeNTA (60 μ M), YC-1 (5 μ M), verapamil (50 μ M) or MG132 (10 μ M), under normoxic (20% O₂) or hypoxic (3% O₂) conditions. The cells were then washed twice with fresh PBS and incubated for 10 min at room temperature in 1 mL of binding buffer

(100 mM hydroxyethyl piperazine ethane sulphonic acid/NaOH (pH 7.5), 140 mM NaCl and 25 mM CaCl₂) containing 10 μ M annexin V/fluorescein isothiocyanate (FITC) conjugate or 2.5 μ M propidium iodide (PI). The cell suspensions were washed three times with fresh PBS and rinsed with 1 mL of binding buffer. An aliquot of cell suspension was used for cell counting by toluidine blue staining. In each assay, 0.5×10^6 cells were employed. The fluorescence of each sample was recorded using a PerkinElmer LS-5 spectrofluorimeter (PerkinElmer, Shelton, CT, USA). Excitation and emission wavelengths were 488 and 530 nm for annexin V/FITC, and 536 and 617 nm for PI, respectively. FeNTA, dexrazoxane, hypoxia, YC-1 and verapamil did not exert any change in cellular viability *versus* control cells in the absence of doxorubicin, whereas 15% of cells were positive for annexin V and PI when incubated with crocidolite asbestos (data not shown). To assess the cytotoxicity of doxorubicin, the doxorubicin-dependent apoptosis was evaluated: the fluorescence obtained under each experimental condition in the absence of doxorubicin was taken as a blank and was subtracted from the fluorescence obtained under the same experimental conditions in the presence of doxorubicin. Results were expressed as fluorescence mU per 10^6 cells.

Statistical analysis

All data in text and figures are provided as mean $\pm$ SE. The results were analysed using a one-way ANOVA and Tukey's test. Values of $p < 0.05$ were considered significant.

RESULTS

Crocidolite asbestos, dexrazoxane and hypoxia enhanced doxorubicin resistance and Pgp expression

MM98 cells, which exhibit high Pgp expression and doxorubicin resistance *per se* [20], were incubated for 24 h in a medium containing 5 μ M doxorubicin and ~ 3 μ M FeSO₄, in the absence or presence of crocidolite asbestos, dexrazoxane (a well-known iron chelator [24]) or FeNTA (a cell-permeating compound which increases the intracellular iron [18]), or under hypoxia (3% O₂). In preliminary dose-dependence experiments, 60 μ M FeNTA and 10 μ M dexrazoxane were found to be the minimal doses able to significantly increase and decrease, respectively, the intracellular doxorubicin accumulation *versus* the respective control (data not shown). Thus, for subsequent experiments, the concentrations of 60 μ M FeNTA and 100 μ M dexrazoxane were used, the latter in order to be sure of chelating all the iron released from FeNTA. At these concentrations, both compounds did not exert any cytotoxic effect on MM98 cells *per se* (data not shown). The presence of crocidolite asbestos significantly lowered the intracellular accumulation of doxorubicin (fig. 1a) and clearly enhanced the expression of Pgp (fig. 1b), compared with control. Both dexrazoxane and hypoxia exerted the same effects. Conversely, FeNTA completely reversed the effects of each experimental condition, increasing the cell drug content and decreasing the level of Pgp protein (fig. 1). When used alone, FeNTA increased the doxorubicin accumulation above the control levels (fig. 1a). The doxorubicin-induced cell death in MM98 cells was due to apoptosis, and dexrazoxane, crocidolite and hypoxia significantly reduced the number of MM98 cells positive for both annexin V and PI (fig. 1a). In contrast, FeNTA significantly increased the cytotoxicity of

doxorubicin and prevented the effects of dexrazoxane, crocidolite and hypoxia (fig. 1a).

Crocidolite asbestos and dexrazoxane increased IRP-1 activity

IRP-1 activity is strictly dependent on the labile pool of intracellular iron, and its increased binding to the IRE sequence of ferritin mRNA is considered a sensitive index of decreased intracellular iron levels [25]. This is more useful than the measurement of the total amount of iron, with other tools, for providing information about the actual bioavailability of iron. Resting MM98 cells exhibited a detectable IRP-1 binding activity (fig. 2), which was decreased by FeNTA and enhanced by dexrazoxane. Interestingly, crocidolite asbestos also increased IRP-1 binding activity when compared with control. FeNTA reversed the effects of both dexrazoxane and crocidolite (fig. 2). MM98 cells incubated under hypoxic conditions (3% O₂) for 24 h did not exhibit an IRP-1 binding activity

FIGURE 1. Effects of crocidolite asbestos (Cro), dexrazoxane (Dxr), hypoxia (Hyp) and ferric nitrilotriacetate (FeN) on intracellular doxorubicin accumulation, cell survival and P-glycoprotein (Pgp) expression in MM98 cells. Cells were incubated for 24 h in the absence (C) or presence of FeN (60 μ M), Dxr (100 μ M) or Cro fibres (25 μ g·cm⁻²). Hypoxic conditions: 3% (instead of 20%) O₂. a) Intracellular doxorubicin accumulation (□), annexin V (■) and propidium iodide (PI; ■) assays. MM98 cells were incubated in the presence of 5 μ M doxorubicin, then detached and lysed in ethanol/HCl. The intracellular content of the drug was measured as described in the Materials and methods section. In parallel, an aliquot of MM98 cells was used for the quantitative measurement of annexin V or PI fluorescence (fluor). Measurements were performed in triplicate and data are presented as mean $\pm$ SE (n=4). b) Pgp was immunoprecipitated from lysates of MM98 cells and then detected by Western blotting. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in the same cell lysates as a control for equal loading. The figure is representative of three experiments with similar results. *: $p < 0.05$; ***: $p < 0.001$ *versus* control.

different from control cells, although the addition of FeNTA reduced IRP-1 binding activity elicited by hypoxia (fig. 2).

Crocidolite asbestos, dexrazoxane and hypoxia induced HIF-1 α activation

The expression of the *Pgp* gene is strictly regulated by the transcription factor HIF-1 α [6], whose activity is very sensitive to decreased intracellular iron and to hypoxia [8]. A basal nuclear translocation of HIF-1 α was detectable in control MM98 cells, and crocidolite asbestos, dexrazoxane and hypoxia (3% O₂) induced a marked increase in HIF-1 α level in nuclear extracts (fig. 3). In contrast, FeNTA repressed HIF-1 α activity, both basal and elicited by crocidolite, dexrazoxane and hypoxia (fig. 3).

Activation of HIF-1 α increased *Pgp* expression and doxorubicin resistance

To verify whether the increase in HIF-1 α was responsible for *Pgp* overexpression and doxorubicin resistance, these parameters were measured in the presence of 5 μ M YC-1, a specific inhibitor of HIF-1 α [26]. In preliminary experiments, it was observed that the addition of 5 μ M YC-1 for 24 h efficiently reduced the HIF-1 α nuclear translocation under any experimental conditions (data not shown). This concentration of YC-1 was chosen for subsequent experiments. YC-1 prevented *Pgp* induction in all experimental conditions (fig. 4a), increased the intracellular doxorubicin accumulation and cytotoxicity *per se*,

and abolished the effects of crocidolite, dexrazoxane and hypoxia (fig. 4b). Interestingly, the effects of YC-1 on the content and cytotoxicity of doxorubicin were similar to those evoked by FeNTA, which reduced HIF-1 α activity (fig. 3). The different expression of *Pgp* was crucial in regulating the doxorubicin accumulation in MM98 cells. To correlate the doxorubicin resistance with the induction of *Pgp* in the present experimental model, the intracellular accumulation and the cytotoxic effect of the drug were measured after a 24-h incubation with dexrazoxane, crocidolite or hypoxia, alone or together with FeNTA, in the presence of 50 μ M verapamil, a well-known *Pgp* inhibitor [27]. Preliminary dose-dependence experiments showed that 50 μ M verapamil was the minimal dose able to significantly increase intracellular doxorubicin *versus* the respective control (data not shown). Verapamil lowered the *Pgp* activity and increased the doxorubicin accumulation and toxicity under each experimental condition (fig. 5). In a similar manner to YC-1, verapamil mimicked the effect of FeNTA, which reduced the *Pgp* expression in MM98 cells (fig. 1).

The effects of hypoxia on HIF-1 α activation and doxorubicin resistance were reverted by subsequent normoxia

MM98 cells were cultured for 24 h in hypoxic conditions, then they were left to grow in 20% O₂ for 1, 3, 6 and 24 h. At each time-point, HIF-1 α nuclear translocation, *Pgp* expression, doxorubicin accumulation and cytotoxicity were assessed. Hypoxia induced a marked nuclear translocation of HIF-1 α , increased the *Pgp* expression and significantly reduced the doxorubicin accumulation and cytotoxicity (fig. 6). After a 3-h incubation in normoxic conditions, the amounts of HIF-1 α and *Pgp* were markedly reduced and returned to baseline levels after 6 h of normoxia (fig. 6a and b). After this time, the accumulation and toxicity of doxorubicin were also similar to those observed under constant normoxic conditions (fig. 6c).

Crocidolite and FeNTA regulated the levels of HIF-1 α by affecting proteasomal degradation

To clarify the mechanisms of crocidolite-dependent HIF-1 α induction in MM98 cells, the expression of thioredoxin, which is known to regulate HIF-1 α protein synthesis [28, 29], was

FIGURE 2. Effects of crocidolite asbestos (Cro), dexrazoxane (Dxr), hypoxia (Hyp) and ferric nitrilotriacetate (FeN) on the iron regulatory protein (IRP)-1 binding activity. Cells were incubated for 24 h in the absence (C) or presence of FeN (60 μ M), Dxr (100 μ M) or Cro fibres (25 μ g·cm⁻²). Hypoxic conditions: 3% (instead of 20%) O₂. a) Cytosolic extracts were subjected to electrophoretic mobility shift assays. The figure is representative of three experiments with similar results. In each experiment, one lane (-) was loaded with bidistilled water in place of cellular extracts. b) The relative band intensity, calculated by ImageJ software (National Institutes of Health, Research Services Branch, Bethesda, MD, USA; available from <http://rsb.info.nih.gov/ij/>) and expressed as arbitrary units, is presented as mean $\pm$ SE (n=3). *: p<0.05; #: p<0.05 *versus* control.

FIGURE 3. Effects of crocidolite asbestos (Cro), dexrazoxane (Dxr), hypoxia (Hyp) and ferric nitrilotriacetate (FeN) on hypoxia-inducible factor (HIF)-1 α nuclear translocation in MM98 cells. Cells were incubated for 24 h in the absence (C) or presence of FeN (60 μ M), Dxr (100 μ M) or Cro fibres (25 μ g·cm⁻²). Hypoxic conditions: 3% (instead of 20%) O₂. Detection of HIF-1 α activation was performed on nuclear extracts by electrophoretic mobility shift assays. The figure is representative of three experiments with similar results. In each experiment, one lane (-) was loaded with bidistilled water in place of cellular extracts. A supershift assay (SSA) was performed on MM98 cells previously incubated for 24 h under 3% O₂, to assess the specificity of the HIF-1 α binding.

FIGURE 4. Effects of crocidolite asbestos (Cro), dexrazoxane (Dxr), hypoxia (Hyp) and ferric nitrilotriacetate (FeN) on P-glycoprotein (Pgp) expression and doxorubicin efficacy, in the presence of 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1). MM98 cells were incubated for 24 h in the absence (C) or presence of FeN (60 μ M), Dxr (100 μ M) or Cro fibres (25 μ g·cm⁻²). Hypoxic conditions: 3% (instead of 20%) O₂. YC-1 (5 μ M for 24 h) was added in all the experimental conditions. a) Cells were lysed and Pgp was immunoprecipitated from lysates of MM98 cells and detected by Western blotting. MM98 cells incubated under hypoxic conditions in the absence of YC-1 were used as a positive control (Hyp) for hypoxia-inducible factor-1 α activation. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in the same cell lysates as a control for equal loading. The figure is representative of three experiments with similar results. b) Intracellular doxorubicin accumulation (□), annexin V (■) and propidium iodide (PI; ■) assays were performed on MM98 cells incubated in the presence of YC-1 (5 μ M for 24 h). Cells were cultured in a medium containing 5 μ M doxorubicin, then detached and lysed in ethanol/HCl. The intracellular content of the drug was measured as described in the Materials and methods section. In parallel, an aliquot of MM98 cells was used for the quantitative measurement of annexin V or PI fluorescence (fluo). Measurements were performed in triplicate and data are presented as mean ± SE (n=3). ***: p<0.001 versus control without YC-1.

assessed. Thioredoxin was constitutively expressed in MM98 cells and was unchanged under each experimental condition (fig. 7). Additionally, increased oxidative stress, due to the decrease of antioxidant enzymes such as glutathione reductase, may activate HIF-1 α [30], and indeed, crocidolite fibres decreased the amount of glutathione reductase in MM98 cells (fig. 7). However, when FeNTA was added together with crocidolite, the decrease in glutathione reductase was not reversed (fig. 7), while the HIF-1 α activation was diminished (fig. 3). Thus, the effect of crocidolite, dexrazoxane, hypoxia and FeNTA on HIF-1 α was not related to changes in thioredoxin or glutathione reductase in MM98 cells.

FIGURE 5. Effects of crocidolite asbestos (Cro), dexrazoxane (Dxr), hypoxia (Hyp) and ferric nitrilotriacetate (FeN) on doxorubicin efficacy, in the presence of verapamil. MM98 cells were incubated for 24 h in the absence (C) or presence of FeN (60 μ M), Dxr (100 μ M) or Cro fibres (25 μ g·cm⁻²). Hypoxic conditions: 3% (instead of 20%) O₂. In each experimental condition, verapamil (50 μ M for 24 h) was added, then cells were assayed for intracellular doxorubicin content (□), and annexin V (■) and propidium iodide (PI; ■) fluorescence (fluo). Measurements were performed in triplicate and data are presented as mean ± SE (n=3). ***: p<0.001 versus control without verapamil.

To assess whether FeNTA may repress the activation of HIF-1 α by increasing its proteasomal degradation, MM98 cells were incubated with the proteasome inhibitor MG132 for 24 h. MG132 alone increased the basal HIF-1 α translocation to the nucleus and completely prevented the FeNTA-induced decrease of HIF-1 α activation observed in the presence of crocidolite (fig. 8a). In parallel, MG132 increased the expression of Pgp (fig. 8b) and decreased the intracellular accumulation and toxicity of doxorubicin (fig. 8c), under each experimental condition.

DISCUSSION

HMM exhibits a constitutive resistance to many common anticancer drugs [31], mainly due to the overexpression of membrane transporters, such as Pgp and MRPs [5]. The efficacy of doxorubicin, as well as platinum-derived compounds and antifolate drugs (which are commonly used in mesothelioma therapy [2]), is often reduced in mesothelioma cells. Indeed, these chemotherapeutic agents are poorly deliverable in pleural tissue [3]. Moreover, they are all substrates of Pgp and MRPs [2]. In a previous study, it was observed that MM98 cells have prominent constitutive Pgp expression and are poorly sensitive to doxorubicin [20]. Since the *Pgp* gene has a hypoxia-responsive enhancer in the promoter and is upregulated by HIF-1 α [6], the current authors investigated whether HIF-1 α could play a role in doxorubicin resistance in MM98 cells and whether asbestos, which is the major pathogenetic agent of mesothelioma, may play a role in the induction of drug resistance *via* HIF-1 α modulation.

Crocidolite asbestos significantly lowered the intracellular accumulation and the pro-apoptotic effect of doxorubicin, and in parallel enhanced the expression of Pgp. As far as the

FIGURE 6. Effects of hypoxia followed by re-exposure to normoxia on hypoxia-inducible factor (HIF)-1 α activation, P-glycoprotein (Pgp) expression and doxorubicin accumulation and cytotoxicity. MM98 cells were incubated for 24 h under normoxic (20% O₂; C) or hypoxic (3% O₂; Hyp) conditions. Some samples, after 24 h in hypoxia, were cultured under normoxic conditions (+N) for 1, 3, 6 and 24 h. At each time-point, cells were subjected to the following investigations. Each figure is representative of three experiments with similar results. a) Cells were lysed and 10 μ g of nuclear extracts were used for electrophoretic mobility shift assays. In each experiment, one lane (-) was loaded with bidistilled water in place of cellular extracts. A supershift assay (SSA) was performed on MM98 cells previously incubated for 24 h under 3% O₂, to assess the specificity of the HIF-1 α binding. b) Pgp was immunoprecipitated from the lysates of MM98 cells and then detected by Western blotting. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in the same cell lysates as a control for equal loading. c) Intracellular doxorubicin accumulation (□), annexin V (■) and propidium iodide (PI; ■) assays. MM98 cells were incubated with 5 μ M doxorubicin, then detached and lysed in ethanol/HCl. The intracellular content of the drug was measured as described in the Materials and methods section. In parallel, an aliquot of MM98 cells was used for the quantitative measurement of annexin V or PI fluorescence (fluor). Data are presented as mean $\pm$ SE (n=3). #: p < 0.05 versus hypoxia alone; *: p < 0.005 versus control.

FIGURE 7. Effects of crocidolite asbestos (Cro), dexrazoxane (Dxr), hypoxia (Hyp) and ferric nitrilotriacetate (FeN) on thioredoxin (Trx) and glutathione reductase (Grx) in MM98 cells. Cells were incubated for 24 h in the absence (C) or presence of FeN (60 μ M), Dxr (100 μ M) or Cro fibres (25 μ g·cm⁻²). Hypoxic conditions: 3% (instead of 20%) O₂. Cells were lysed and the detection of Trx and Grx was performed. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in the same cell lysates as a control for equal loading. The figure is representative of three experiments with similar results.

mechanism is concerned, it is known that: 1) crocidolite asbestos alters intracellular iron availability, impairing several metabolic pathways involved in survival or damage repair [17, 32]; 2) asbestos fibres bind iron from solutions [17] and adsorb iron from intracellular ferritin [3, 16]; and 3) crocidolite induces nitric oxide synthesis in murine alveolar macrophages by decreasing iron bioavailability. The latter effect is inhibited by iron supplementation and enhanced by the iron chelator deferoxamine [33]. To investigate whether iron availability may affect drug resistance in MM98 cells, intracellular iron was manipulated using FeNTA, an iron salt that permeates the cell membrane increasing intracellular iron [18], and dexrazoxane, a potent and specific iron chelator [24]. In a similar manner to asbestos, dexrazoxane and hypoxia made MM98 cells more resistant to doxorubicin, and in parallel increased the expression of Pgp. FeNTA reversed such effects, suggesting that crocidolite and dexrazoxane are likely to increase doxorubicin resistance by decreasing the intracellular iron availability. Notably, when used alone, FeNTA increased the doxorubicin accumulation and cytotoxicity compared with controls. Indeed, the basal levels of HIF-1 α and Pgp became undetectable in the presence of FeNTA, owing to the accelerated degradation of HIF-1 α . Thus, FeNTA-treated MM98 cells may accumulate more intracellular doxorubicin than the control cells and decrease their constitutive resistance to the drug. A sensitive marker of the intracellular iron availability, particularly of the labile iron pool, is the activation of IRP-1 [8]. IRP-1 is an RNA-binding protein that regulates the translation of several mRNAs in response to cellular iron [25]. In MM98 cells, FeNTA decreased and crocidolite and dexrazoxane increased IRP-1 binding to ferritin mRNA. These effects strongly suggest that FeNTA increases and crocidolite and dexrazoxane decrease the labile pool of intracellular iron. Crocidolite and dexrazoxane lost their ability to activate IRP-1 when incubated together with FeNTA, which is similar to the observations for the doxorubicin accumulation. Hypoxia *per se* did not significantly change IRP-1 activity, as it is not expected to affect the intracellular iron pool in MM98 cells. Since increased bioavailability of iron can reverse the effect of hypoxia, both iron and oxygen levels could modulate drug resistance by interacting at the same target, which could

FIGURE 8. Effects of crocidolite asbestos (Cro), ferric nitrilotriacetate (FeN) and MG132 (MG) on hypoxia-inducible factor (HIF)-1 α nuclear translocation, P-glycoprotein (Pgp) expression and intracellular doxorubicin accumulation and toxicity in MM98 cells. Each figure is representative of three experiments with similar results. Cells were incubated for 24 h in the absence (C) or presence of FeN (60 μ M), MG (10 μ M) or Cro fibres (25 μ g·cm⁻²). a) Electrophoretic mobility shift assays for the detection of HIF-1 α activation were performed on nuclear extracts. In each experiment, one lane (-) was loaded with bidistilled water in place of cellular extracts. A supershift assay (SSA) was performed on MM98 cells previously incubated for 24 h with 10 μ M MG, to assess the specificity of the HIF-1 α binding. b) Pgp was immunoprecipitated from lysates of MM98 cells and then detected by Western blotting. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in the same cell lysates as a control for equal loading. c) Intracellular doxorubicin accumulation ($\square$), annexin V ($\blacksquare$) and propidium iodide (PI; $\blacksquare$) assays. MM98 cells were incubated with 5 μ M doxorubicin, then detached and lysed in ethanol/HCl. The intracellular content of the drug was measured as described in the Materials and methods section. In parallel, an aliquot of MM98 cells was used for the quantitative measurement of annexin V or PI fluorescence (fluor). Data are presented as mean $\pm$ SE (n=3). *: p<0.05 versus Cro; ***: p<0.001 versus control; #: p<0.001 versus FeN.

be HIF-1 α . HIF-1 α is known to be activated by a decrease in intracellular iron availability [7, 8] and by hypoxia [6]. In its turn, activated HIF-1 α enhances Pgp expression and plays a central role in inducing drug resistance in tumours [6, 10].

A basal level of HIF-1 α nuclear translocation was detected in resting MM98 cells, and this could be responsible for the constitutive expression of Pgp observed in these cells.

Furthermore, Pgp and HIF-1 α appeared to be modulated in the same way. HIF-1 α translocation to the nucleus was significantly reduced by FeNTA and increased by crocidolite and dextrazoxane. The effect of crocidolite and dextrazoxane on HIF-1 α activity was abolished by FeNTA. This result suggests that crocidolite asbestos and dextrazoxane regulate both HIF-1 α activation and Pgp levels by decreasing the intracellular iron availability. When HIF-1 α activation was diminished by the specific HIF-1 α inhibitor YC-1, the Pgp expression was low and the doxorubicin accumulation and toxicity were high under all experimental conditions. Similarly, when the Pgp activity was inhibited by verapamil, none of the stimuli reduced the content or the cytotoxic effect of doxorubicin. YC-1 and verapamil mimicked the effects of FeNTA, which reduced the HIF-1 α activity and Pgp expression in MM98 cells. On the basis of these results, it was hypothesised that crocidolite, dextrazoxane, hypoxia and FeNTA exert their effects on doxorubicin efficacy by affecting the activity of HIF-1 α . HIF-1 α in turn regulates the expression of Pgp, which represents a crucial factor for doxorubicin accumulation in MM98 cells.

Interestingly, all the effects of hypoxia were reversed by a subsequent exposure to normoxia: after a 24-h incubation under hypoxic conditions, MM98 cells showed an increased expression of HIF-1 α and Pgp and accumulated less doxorubicin; however, when cells were left to grow in normoxic conditions after the incubation under hypoxia, HIF-1 α and Pgp decreased and the doxorubicin content and toxicity increased as a function of time. The different rates of degradation of HIF-1 α under hypoxic and normoxic conditions may explain both the acquisition of a drug-resistant phenotype in hypoxia and the reversal of the resistance observed during the subsequent normoxia.

Acting as an iron chelator, crocidolite may directly activate HIF-1 α in cells: oxygen and iron decrease the stability of HIF-1 α by promoting its hydroxylation and subsequent proteasomal degradation [7]. Furthermore, crocidolite may increase HIF-1 α levels *via* several different mechanisms. In mesothelial cell lines, crocidolite asbestos induces mRNA transcription of thioredoxin proteins [34]. Thioredoxins regulate the rate of HIF-1 α synthesis [29]. In MM98 cells, none of the agents that modulated the activity of HIF-1 α had any effect on the levels of thioredoxin. Therefore, the increased HIF-1 α activity observed after dextrazoxane, crocidolite or hypoxia was not mediated by thioredoxin overexpression.

HIF-1 α can be activated in cells subjected to oxidative stress [30]. Crocidolite may induce severe oxidative stress in mesothelioma cells in several different ways [1]. Asbestos fibres contain iron [17], which may generate reactive oxygen species (ROS) *via* a Fenton-like reaction [32]. Moreover, following the phagocytosis of asbestos fibres, macrophages may produce ROS by activating nicotinamide adenine dinucleotide phosphate oxidase [32]. Asbestos may also evoke oxidative stress *via* other mechanisms, such as the inhibition of the pentose phosphate pathway [35], the decrease of several antioxidant enzymes (glutathione peroxidase, glutathione reductase and catalase) [36, 37] and the enhanced leakage of reduced glutathione [38]. Asbestos-induced oxidative stress may directly damage DNA, impair the DNA repairing systems and activate some redox-sensitive transcription factors, such as

nuclear factor- κ B [1] and HIF-1 α [30]. In MM98 cells, crocidolite markedly reduced the amount of glutathione reductase. The increased HIF-1 α nuclear translocation elicited by crocidolite might be a consequence of the oxidative stress caused by the decrease in glutathione reductase. However, when FeNTA was added together with crocidolite, the decrease in glutathione reductase was not reversed, while, under the same experimental conditions, the crocidolite-induced increase in HIF-1 α activity was prevented. This suggests that glutathione reductase decrease and HIF-1 α increase are not related in crocidolite-treated cells. It cannot be excluded that at least part of the crocidolite effect is mediated by oxidative stress, but results with the proteasome inhibitor MG132 suggest that proteasomal degradation is the critical step that regulates the levels of HIF-1 α in mesothelioma cells. Indeed, MG132 enhanced HIF-1 α stability, Pgp expression and doxorubicin resistance in MM98 cells, and completely reversed the effects of FeNTA.

In conclusion, the results of the present study suggest that the exposure of MM98 cells to crocidolite asbestos induces the activation of hypoxia-inducible factor-1 α , which is accompanied by increased expression of P-glycoprotein and, in parallel, by increased resistance to doxorubicin accumulation and toxicity. Crocidolite is likely to activate hypoxia-inducible factor-1 α by decreasing the iron availability in the cell, as inferred by the ability of the iron chelator dexrazoxane to elicit analogous effects and by the reversing action of the cell-permeant iron salt ferric nitrilotriacetate. It may be supposed that crocidolite, besides increasing cellular proliferation and inhibiting apoptosis in mesothelial cells [15], may play a role in the induction of multidrug resistance in MM98 cells. Also, hypoxia promptly induces hypoxia-inducible factor-1 α in MM98 cells. Such an activation might mimic the *in vivo* situation, and indeed hypoxia-inducible factor-1 α is often high in patients affected by malignant mesothelioma [14]. Moreover, the current data suggest that the increased intracellular iron content may decrease doxorubicin resistance even in the presence of crocidolite fibres or hypoxia. Although the present results were obtained in an *in vitro* model, they may open a new therapeutic strategy to reverse doxorubicin resistance in malignant mesothelioma.

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