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Apoptosis and Release of CD44s in Bleomycin-Treated L132 Cells

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Abstract The anti-cancer drug bleomycin (BLM) induces lung injury and triggers apoptosis of alveolar epithelial cells. In epithelia, among other functions, the adhesion protein CD44 promotes the contact to components of the extracellular matrix like hyaluronate. A functional link between apoptosis and the loss of CD44 has been observed in colon carcinoma cells and involvement of CD44 in apoptosis of lung cells has been reported in several studies. The present in vitro study examined the expression of CD44s (CD44 standard) in two human epithelial lung cell lines, L132 and A549, during BLM-induced apoptosis. A loss of CD44s by lung epithelial cells and an increase of the soluble form of this adhesion protein in culture supernatants upon exposure to BLM were observed. Apoptosis was characterized by an activation of caspase-3 as well as by release of cytochrome C into the cytosol as shown for L132 cells. Inhibition of apoptosis by the broad-range caspase inhibitor Z-VAD-fmk reduced CD44 release by both cell lines demonstrating that CD44 release is a result of apoptotic processes. Kinetic experiments failed to discriminate between the initiation of apoptosis and CD44 release. Blocking experiments using antagonistic anti-CD95 receptor antibodies revealed that BLM may cause apoptosis and CD44 release in a CD95-independent manner. J. Cell. Biochem. 95: 1146–1156, 2005.

Key words: CD44; pulmonary epithelial cells; bleomycin; apoptosis; fibrosis

Bleomycin (BLM) is an anti-cancer drug inducing lung injury as its main side effect. BLM-induced lung injury includes different cell types of the lung leading to irreversible tissue destruction. Epithelial cells are among the targets of BLM [Ghosh et al., 2002]. The molecular mechanism of BLM-action involves induction of single and double strand breaks of the DNA in affected cells triggering repair mechanisms or, in case of extended damage, programmed cell death [Burger et al., 1986; Hagimoto et al., 1997; Jones et al., 2001; Kuwano et al., 2001].

Recent studies have shown that apoptosis in the alveolar epithelium is an important determinant in the pathogenesis of BLM-induced lung injury [Wang et al., 2000; Kuwano et al., 2001]; [for review see Sutherland et al., 2001]. BLM-induced experimental lung fibrosis in animals is well studied [Koslowski et al., 2001, 2003; Kuwano et al., 2001]. Exposure of lung epithelial cells to BLM induces apoptosis in vivo [Kuwano et al., 2000; Wang et al., 2000] and in vitro [Hong et al., 2003]. Studies with isolated cells and cell lines were conducted to determine the specific mechanisms of regulation of BLM-induced apoptosis in epithelial lung cells, alveolar macrophages, or cancer cells [Hug et al., 1997; Muller et al., 1997, 1998; Uhal et al., 1998; Ramp et al., 2000]. The involvement of reactive oxygen species (ROS) [Hong et al., 2003], p53 [Muller et al., 1998], and mitochondrial cytochrome C release [Gimonet et al., 2004], as well as regulation of BLM-induced apoptosis via activation of the FAS/FAS-ligand pathway [Muller et al., 1997, 1998] have been proposed.

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CD44 is a type I transmembrane glycoprotein involved in homo- and heterotypic cell adhesion and cell–matrix interaction. CD44 is abundant in many tissues and can bind the extracellular glycosaminoglycan hyaluronate as well as collagen subtypes and fibronectin [reviewed in Culty et al., 1990; Goodison et al., 1999]. Standard CD44 (CD44s) is involved in uptake and degradation of hyaluronate in lung and lymphoid tissue [Green et al., 1988; Menzel and Farr, 1998]. Higher molecular weight variants (CD44v isoforms) arise by alternative splicing of variant exons of the CD44 mRNA and exist predominantly in epithelia, malignant tissues, and activated lymphocytes [Mackay et al., 1994; Terpe et al., 1994]. The importance of CD44 for resolution of a BLM-induced inflammatory response was recently demonstrated in a study on mice showing CD44 deficiency after targeted disruption [Teder et al., 2002]. In the mentioned study these mice died and lung tissue showed accumulation of hyaluronan in the alveolar space, impaired clearance of apoptotic neutrophils, and insufficient TGF-β activation.

During CD95 (APO-1/Fas)-mediated apoptosis of colon carcinoma cells, a shedding of CD44 from the cell surface was reported [Gunthert et al., 1996]. This observation was discussed as a functional link between apoptosis and the loss of cell contact to extracellular matrix or neighboring cells. We have shown previously that in glyoxal-induced apoptosis of epithelial lung cell spheroids, detached cells have lost CD44s and were committed to die [Roehlecke et al., 2000]. Implication of CD44 in apoptosis has been reported in several studies [Ayroldi et al., 1995; Takazoe et al., 2000; Lisignoli et al., 2001; Yasuda et al., 2001]. In a previous investigation on human, rat, and porcine lung fibrosis we described the loss of CD44, particularly its epithelial isoforms, from non-proliferating alveolar epithelium [Kasper et al., 1995, 1996].

The present in vitro study was addressed to evaluate the relationship of BLM-induced apoptosis in human epithelial lung cells to the expression of CD44s.

MATERIALS AND METHODS

Cell Culture and BLM-Treatment

Human L132 embryonic lung cells (ATCC, Rockville, MD) were grown in DMEM/F12 medium supplemented with 5% fetal calf serum (FCS), 50 IU penicillin/ml, 50 μg streptomycin/ml, and 6.4 mg gentamycin/ml at 37°C in 5% CO_2 (medium and supplements from Biochrom, Berlin, Germany). A549 cells of a human epithelial-like carcinoma line (ATCC, Rockville, MD) were grown in DMEM (10% FCS, all other supplements as described above). BLM (Bleocell, cell pharm GmbH, Hannover, Germany) at a concentration of 100 mU/ml was added to the culture medium for 24 h.

Inhibition Experiments

For inhibition of apoptosis, broad-spectrum caspase inhibitor Z-VAD-fmk (*N*-benzyl-carboxy-Val-Ala-Asp-fluoromethylketone; R&D Systems, Wiesbaden, Germany) was added at a concentration of 40 μM for 24 h. For inhibition of CD95R-mediated apoptosis, the antagonistic anti-CD95 antibody (clone ZB4, Upstate Lake Placid, NY) was used at a concentration of 50 ng/ml, and added to the cultures 1 h before exposure to BLM. The effectiveness of this antibody as antagonist has been tested in preliminary FACS analysis experiments using an agonistic anti-CD95 antibody (clone CH11, Upstate, 50 ng/ml) for induction of apoptosis in L132 cells. In these experiments, done in triplicate, antagonistic anti-CD95 completely abolished activation of caspase-3 induced by the agonistic anti-CD95 antibody (data not shown).

Immunocytochemistry

For detection of apoptotic markers in the cells by immunocytochemistry, cells were clotted in fibrin, fixed in 4% neutralized formaldehyde solution, and embedded in paraffin as described previously [Schulze et al., 1999]. Sections (4 μm) were dewaxed, dried overnight, and treated with microwaves in 10 mM sodium citrate buffer. After incubation with the primary anti-active caspase-3 antibody (Becton Dickinson Biosciences, Heidelberg, Germany; diluted 1:200 with background reducing component S3022; DAKO, Hamburg, Germany) and the detection with the Vectastain Elite Kit (development with DAB) endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in distilled water for 10 min. After washing in PBS (pH 7.4), sections were incubated with monoclonal mouse anti-CD44s (clone Hermes-3; kindly provided by Dr. U. Guenthert, Basel, Switzerland, undiluted hybridoma supernatant) for 30 min at 37°C followed by detection with the Vectastain Elite Kit. For the development of peroxidase, the HistoGreen Kit (Linaris, E109; Bettingen,

Germany) was employed, followed by counterstaining with hematoxylin (8–30 s).

Immunoelectron microscopy

For immunoelectron microscopy, fixed L132 cells were embedded in Lowicryl HM20 [Polysciences Europe, Hamburg, Germany; for details see Schulze et al., 1999]. Ultrathin sections were mounted on pioloform-coated mesh nickel grids, preincubated with 10% normal goat serum in Tris-buffered saline (TBS, pH 7.6) for 45 min, and incubated overnight with the monoclonal mouse anti-CD44s antibody (undiluted) at 4°C. After washing with buffer (TBS with 0.2% BSA), the incubation with 10 nm gold-conjugated anti-mouse IgG followed (Biocell, Cardiff, UK; dilution 1:50). After a further rinse in buffer, the sections were stained with 2% aqueous uranyl acetate (8 min) and lead citrate (2 min). A Zeiss EM 906 transmission electron microscope (Oberkochen, Germany) operated at 80 kV was used for ultrastructural analysis.

ELISA for Soluble CD44

For determination of the concentration of soluble CD44, cells were treated with 100 mU BLM/ml or with BLM in combination with Z-VAD-fmk as described above. L132 cells and A549 cells were used. The dependence of soluble CD44 concentration on the cell density was tested by seeding 5×10^4, 7.5×10^4, or 10×10^4 L132 cells/ml and treatment with 100 mU BLM/ml. For the following experiments, the cell density of 10×10^4/ml was used for both cell lines. Supernatants were obtained after 24 h of incubation with BLM, centrifuged for 4 min at 500*g* and stored at −80°C. The concentration of soluble CD44 was analyzed using an enzyme-linked immunosorbant assay kit (Bender MedSystems, Germany). Absorption was determined at 450 and 620 nm reference wavelength, respectively, using a TECAN Sunrise ELISA reader (Vienna, Austria).

Immunoblotting

For determination of the kinetics of apoptosis and of CD44 release, BLM-treated L132 cells were harvested after 2, 3, 4.5, 6, 8, and 12 h. Non-adherent cells were collected by centrifugation at 500*g*, 4°C for 8 min, (cells from three culture dishes each) and homogenates were prepared by dispersion of the cells in 62.5 mM Tris/HCl buffer, pH 6.8 containing 2% sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 50 mM dithiothreitole, and 0.01% bromophenol blue. Adherent cells were scraped after addition of lysis buffer mentioned above and combined with non-adherent cells. Samples were stored at −80°C, sonicated after thawing and centrifuged at 8,000*g*, 4°C for 20 min. Protein concentration was determined by the Micro BCA protein assay (Pierce, Rockford, IL). Cell culture supernatants were collected and submitted to determination of soluble CD44 concentration by ELISA as described above. All experiments were done in triplicate for each condition. Proteins (50 μg/lane) were separated by SDS polyacrylamide gel electrophoresis under reducing conditions and blotted onto nitrocellulose membrane. After blocking in 20 mM Tris/HCl, pH 7.6 containing 140 mM NaCl and 5% non-fat milk powder (BioRad, Hercules, CA) at 25°C for 2 h, cleaved caspase-3 and γ-tubulin were detected using polyclonal rabbit anti-cleaved caspase-3 antibody (dilution 1:500, New England Biolabs, Frankfurt a.M., Germany) and monoclonal anti-γ-tubulin (dilution 1:2,000, Sigma, Deisenhofen, Germany). Horseradish peroxidase conjugated anti-rabbit and anti-mouse immunoglobulins (New England Biolabs) were used as secondary antibodies. Protein bands were visualized by enhanced chemiluminescence (ECL, New England Biolabs) and analyzed with a Genegnom chemiluminescence detection system (Synoptics, Ltd., UK).

Determination of Cytochrome C

L132 cells cultured as described above were treated with BLM (100 mU/ml) and mitochondrial release of cytochrome C was proved by determination of this protein in cytosolic fractions after 2 and 4 h by ELISA. Preparation of cytosolic samples and measurement of cytochrome C followed the instructions of the manufacturer's of a commercial kit (Zymed® Cytochrome C ELISA, Zymed, Berlin, Germany).

Measurement of Intracellular ROS Levels

Intracellular ROS levels were measured by flow cytometry in L132 cells loaded with the redox-sensitive dye DCFH-DA. The non-fluorescent DCFH-DA readily diffuses into the cells where it is hydrolyzed to the polar derivative DCFH, which is oxidized in the presence of H_2O_2 to the highly fluorescent DCF. After exposure to BLM (100 mU/ml) or H_2O_2 for 6 h, approximately 1×10^5 cells were incubated

for 60 min at 25°C with 7.5 μM DCFH-DA, harvested, and then washed with PBS. The cells were resuspended in 250 μl PBS and the fluorescence was recorded on FL-1 channel of FACSCalibur (Becton Dickinson, San Jose, CA).

Flow Cytometry

Cells treated as described above were detached from culture dishes and washed in PBS. For assessment of cell surface CD44s expression, cells were preincubated for 30 min on ice with monoclonal mouse antibody against human CD44s (clone F10 44-2, Novocastra, Newcastle, UK; dilution 1:20) and then incubated with phycoerythrine (PE)-conjugate of anti-mouse Ig (Dianova, Hamburg, Germany) for 30 min on ice. After three washes, cells were fixed in 2% (w/v) formaldehyde in PBS for 20 min and centrifuged. The cells were resuspended in PBS containing 0.5% BSA and permeabilized for 20 min using 0.5% (w/v) saponin (Sigma) in the same buffer. After incubation of cells with polyclonal anti-active caspase-3 antibody for 1 h, the cells were washed twice in PBS containing 0.5% BSA and 0.5% (w/v) saponin and incubated with a fluorescein isothiocyanate (FITC)-coupled anti-rabbit IgG (Sigma) for an additional hour at 25°C. Cells were washed again and resuspended in 400 μl PBS, and analyzed by flow cytometry (FACSCalibur; Becton Dickinson). For each analysis, 10,000 events were recorded.

Control experiments using L132 cells and double immunofluorescence techniques exhibited identity of active caspase-3 positive cells and apoptotic cells containing the caspase-cleavage fragment of cytokeratin-18 (data not shown).

Statistical Analysis

For statistical analysis of ELISA-data, multiple comparisons of means using ŠIDAK-procedure were employed. Statistical analysis of flow cytometry data was performed by analysis of variance with ANOVA. For statistical analysis of kinetic experiments, values of active caspase-3 and of released CD44 were compared as follows: each level of the factor except the first was compared to the mean of previous levels.

RESULTS

Immunocytochemical Detection of the Loss of Surface CD44s by Apoptotic Cells

Immunocytochemical double label detection of apoptosis and CD44s surface expression in paraffin sections of clotted L132 cells was performed using antiserum against active caspase-3 and anti-CD44s antibodies, respectively. CD44s surface expression was remarkably reduced on BLM-treated, apoptotic cells, which are positive for active caspase-3 (Fig. 1B). In contrast, in CD44s-positive cells activated caspase-3 was not detectable (Fig. 1A,B). Surface expression of CD44s by A549 was examined by immunocytochemical techniques demonstrating a strong staining for this adhesion molecule (data not shown).

Immunoelectron Microscopy

The immunoelectron microscopy confirmed the changes of CD44s expression in apoptotic L132 cells as compared with non-apoptotic cells (Fig. 2). Immunoreactivity for CD44s was detected by immunoelectron microscopy at the cell

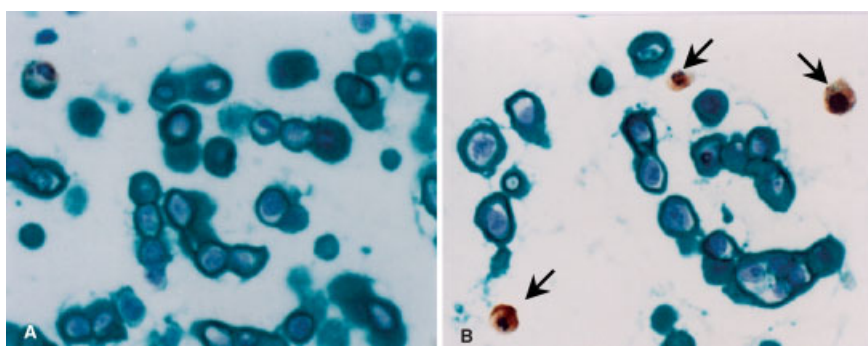

Fig. 1. Detection of CD44s and active caspase-3 as apoptosis marker in cultured L132 cells. Double staining for CD44s (green color) and active caspase-3 (brown color) in untreated (**A**) and in BLM-treated (**B**) cells. Note the loss of CD44s immunoreactivity in active caspase-3 positive cells (arrows). Immunoperoxidase staining, original magnification 300×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

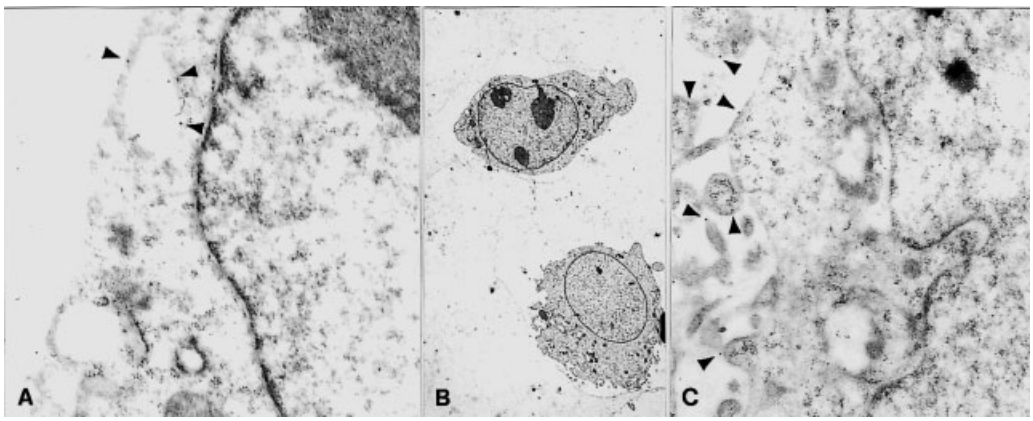

Fig. 2. Postembedding immunoelectron microscopy for CD44s in Lowicryl HM20 embedded L132 cells. Arrowheads indicate the immunogold localization of CD44s. **A**: It represents a higher magnification of the upper, apoptotic cell in (**B**) and (**C**) represents a higher magnification of the lower, non-apoptotic cell in (B). Original magnification 1,293× (B); 16,700× (A, C).

membrane only in non-apoptotic cells (Fig. 2C). In non-apoptotic cells CD44s was found to be associated with microvilli at the cell surface (Fig. 2C; arrowheads), whereas apoptotic cells contained some immunogold labeling of intracellular vesicles located near the cell surface (Fig. 2A; arrowheads).

Detection of Soluble CD44

To detect CD44-release, the concentration of soluble CD44 in culture supernatants was analyzed with or without BLM-treatment of cells. To determine if release of CD44 upon BLM-treatment is a common feature of cells related to alveolar epithelial cells, we employed L132 and A549 cells. There was a significant 2.5-fold increase in the concentration of soluble CD44 in supernatants of BLM-treated L132 cells compared to the controls (Fig. 3A). For A549 cells, the CD44 level was also increased to about 1.5-fold (statistically significant, Fig. 3B). By addition of the broad range caspase-inhibitor Z-VAD-fmk, the BLM-induced increase in the concentration of soluble CD44 was reduced to the control level for L132 cells (Fig. 3A). For A549 cells, the release of CD44 by BLM was also reduced by Z-VAD-fmk (statistically significant), although the level of the soluble CD44 remained increased compared to controls (Fig. 3B).

Quantification of CD44s Surface Expression and Correlation to Apoptosis

For quantification of changes in CD44s surface expression and determination of the

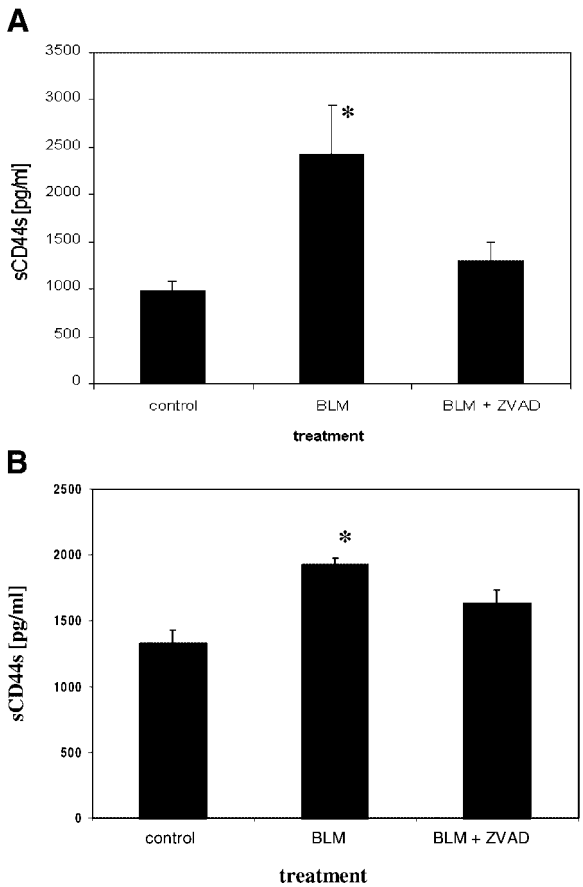

Fig. 3. Changes of the concentration of soluble CD44 in culture supernatants of L132 cells (**A**) and A549 cells (**B**) in response to BLM and Z-VAD-fmk. The cells were exposed to BLM (100 mU/ml) or to BLM + Z-VAD-fmk (40 μM) for 24 h and the concentration of soluble CD44 was quantified by ELISA in supernatants after separation of detached cells by centrifugation. Data represent the mean ± SD (n = 5) (* values are significantly different from controls and from samples obtained from cells treated with BLM + Z-VAD-fmk, $P < 0.05$).

TABLE I. Flow Cytometric Detection of CD44s Surface Expression and Active Caspase-3 in L132 Cells Incubated With 100 mU BLM/ml for 24 h (Data Represent Means and 95% CI in Bracklets, n = 6)

Treatment	Percentage of apoptotic cells	Percentage of CD44-negative cells	Percentage of apoptotic, CD44-negative cells
Untreated (control)	1.4 (−0.16; 2.64)	2.7 (1.61; 3.79)	0.5 (−0.1; 1.1)
BLM	21.1 (16.39; 25.82)	20.9 (16.62; 25.15)	17.7 (13.81; 21.59)
BLM + Z-VAD-fmk	0.8 (0.5; 1.16)	5.9 (2.52; 9.31)	0.2 (0.15; 0.25)
BLM + ZB4-antibody	17.2 (15.7; 18.69)	18.7 (17.49; 19.95)	14.7 (13.41; 16.03)

proportion of apoptotic L132 cells induced by BLM, flow cytometry was employed using anti-CD44s antibody and anti-cleaved-caspase-3 antibody, respectively. The data are summarized in Table I and the results of one representative FACS analysis are shown in Figure 4. In controls (Fig. 4A) about 3% of the cells did not express CD44s and about 1% of the cells were apoptotic. In contrast, after incubation with BLM for 24 h (Fig. 4C), both, the number of apoptotic cells and the number of CD44-negative cells significantly increased to about 21% (cleaved caspase-3-positive cells: $P < 0.001$; CD44s-negative cells: $P < 0.0005$). The determination of the percentage of apoptotic/CD-44s-negative cells (Table I: about 18%) proved the result of the microscopic studies described above that there is a coincidence of caspase-3-activation and loss of surface CD44s (Fig. 4C; lower right part of the panel).

To investigate a possible relationship of BLM-effects on the CD44 release and on the activation of the apoptotic cascade, the broad range caspase-inhibitor Z-VAD-fmk was employed (Fig. 4E). Incubation of L132 cells with Z-VAD-fmk completely prevented the BLM-induced activation of caspase-3 ($P < 0.001$) and inhibited the loss of CD44s. The proportion of CD44s-negative cells was reduced from 21% to about 6% by Z-VAD-fmk (Table I) ($P < 0.0005$).

To test whether BLM-induced apoptosis and the decrease of CD44s surface expression by L132 cells was mediated via the CD95-receptor pathway of apoptosis initiation, the antagonistic anti-CD95 antibody ZB4 was added to the cell culture medium during exposition to BLM (Table I and Fig. 4F). ZB4 antibody did not exert any effect neither on BLM-induced apoptosis nor on the loss of CD44s in L132 cells. There was no significant change of the percentage of apoptotic/CD44s-negative cells from 17.7% after BLM-treatment to 14.7% in cultures exposed to BLM + ZB4-antibody (Table I, Fig. 4C,F, $P = 0.501$).

Kinetics of BLM-Induced Activation of Caspase-3 and of CD44 Release by L132 Cells

The time courses of initiation of apoptosis represented by activation of caspase-3 and of the release of CD44 were investigated. Soluble CD44 was determined in culture supernatants at given times after BLM-treatment of the cells (Fig. 5A). Cleaved caspase-3 was detected by immunoblot analysis and quantified by densitometric evaluation of the 19 kDa band normalized to γ-tubulin (ratio CC3/γ-tubulin, Fig. 5B–D). The densitometric evaluation of immunoblots resulted in a very low value of this ratio (0.0081) for the controls, representing the absence of caspase-3 activation in untreated cells (Fig. 5C). After 2 h of BLM-treatment of the cells, the ratio CC3/γ-tubulin was elevated to about the 22-fold of the control level, followed by a 61-fold increase at 3 h. At these time points, the concentration of soluble CD44 in the medium was raised to the 1.3-fold and 2.2-fold of control values, respectively (Fig. 5A). The activation of caspase-3 showed a maximum at about 6–8 h after BLM-treatment, whereas the concentration of soluble CD44 did not reach a maximum in this time course. Statistical evaluation of both series of data revealed the first contrast showing a significant difference for soluble CD44 at 3 h against the average of the values at 0 and 2 h. For activated caspase-3, the first significant difference was determined at 4.5 h versus the average of the values at 0, 2, and 3 h.

Cytochrome C Release by L132 Cells

Measurement of cytochrome C revealed a strong release after 2 and 4 h of BLM exposure (Fig. 6). The concentration of cytochrome C in cytosolic fractions of L132 cells was increased to

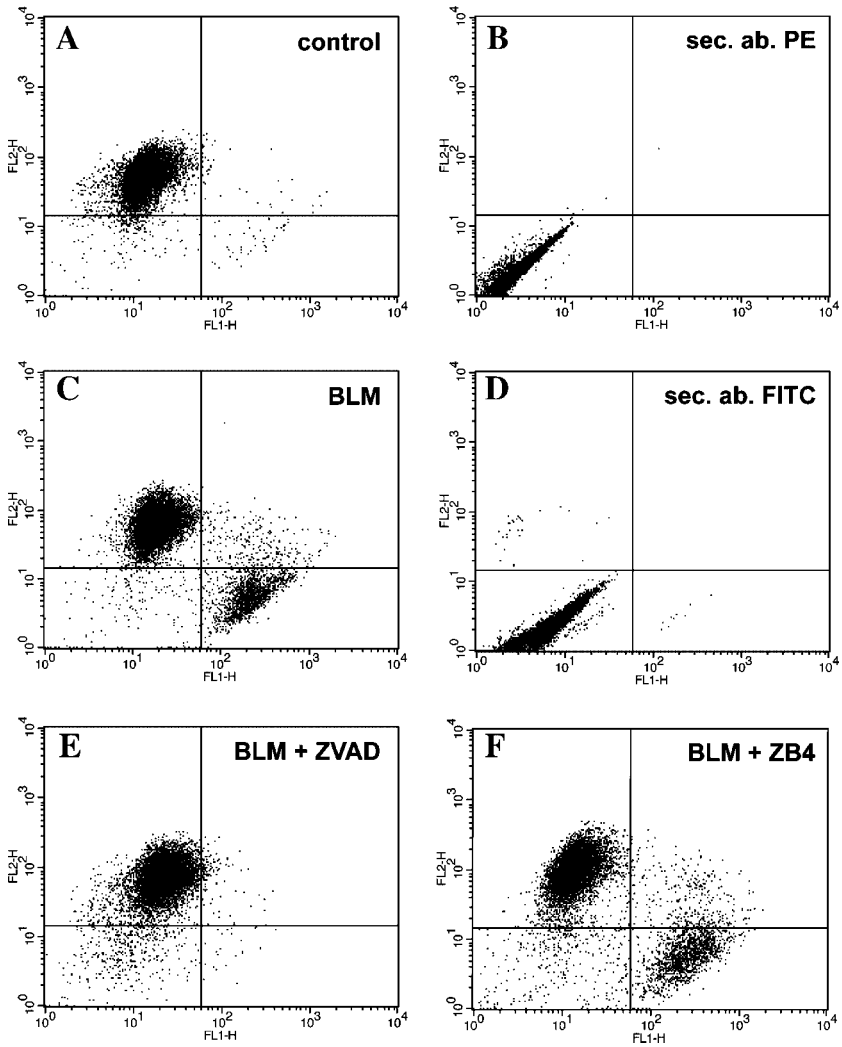

Fig. 4. Determination of CD44s expression and of the apoptosis marker active caspase-3 in L132 cells in response to BLM-treatment by flow cytometry. The cells were cultured in the absence (**A**) or presence (**C**, **E**, **F**) of 100 mU BLM/ml. Suppression of the BLM-effect was tested by preincubation of the cells with the broad range caspase inhibitor Z-VAD-fmk (E) or with the antagonistic anti-CD95 antibody ZB4 (F). For assessment of cell surface CD44s expression, cells were stained with monoclonal mouse antibody against human CD44s combined with phycoerythrine (PE)-conjugate of a secondary antibody (y-axis). Activation of caspase-3 was detected using polyclonal anti-active caspase-3 antibody combined with a FITC-coupled anti-rabbit IgG (x-axis). Cells were analyzed at a FACSCalibur flow cytometer. **B**, **D**: They show the histograms of control experiments using the secondary antibody-conjugates for determination of fluorescence thresholds. The histograms show the results of one representative experiment.

about 40-fold upon treatment of the cells with BLM.

ROS Measurements

As proof of generation of ROS upon treatment of L132 cells with BLM, the conversion of the redox-sensitive agent DCFH-DA to the highly fluorescent dye DCF was determined. Figure 7 shows the results of one representative FACS analysis experiment. Exposition of the cells to H_2O_2 used as positive control for the test led to a strong shift of cellular fluorescence. For treatment with BLM, the shift was less but clearly detectable and indicates the generation of ROS.

DISCUSSION

In the present study, we demonstrated apoptosis in human lung epithelial cells and a concomitant decrease of surface expression of CD44s by these cells accompanied by a release into the culture medium induced by in vitro exposition to the pulmotoxic agent BLM. We

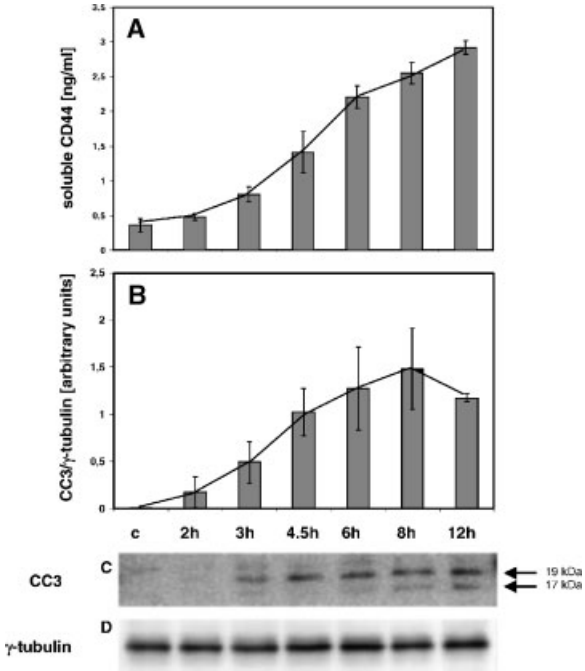

Fig. 5. Kinetics of CD44 release (**A**) and of activation of caspase-3 (**B**) in BLM-treated L132 cells. L132 cells were exposed to BLM and at given times the concentration of soluble CD44 in culture supernatants was determined by ELISA. At the same time points, cells were harvested and the relative amount of cleaved caspase-3 (ratio CC3/γ-tubulin) was determined by immunoblotting. **Panel B** shows the results of the densitometric evaluation of the 19 kDa band of CC3 in immunoblots and **panel C** shows one representative blot. **Panel D**: Immunoblot for γ-tubulin as an unregulated protein. Data in (A) and (B) represent the mean ± SD (n = 3).

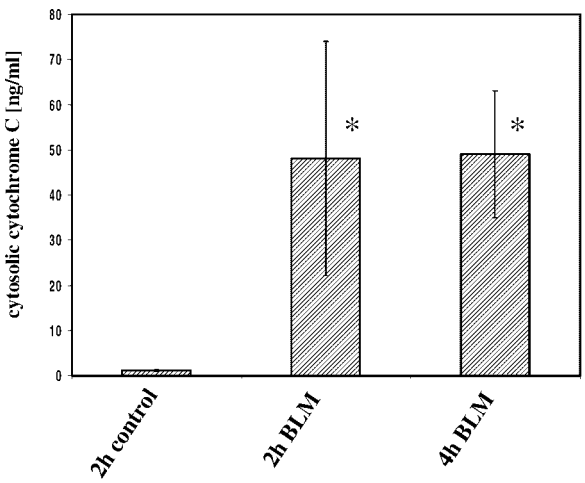

Fig. 6. Effect of BLM exposition of L132 cells on the mitochondrial release of cytochrome C. The cytosolic fraction of the cells was prepared and cytochrome C was determined by ELISA. Data represent the mean ± SD. (n = 3, * values are significantly different from untreated controls, $P < 0.05$).

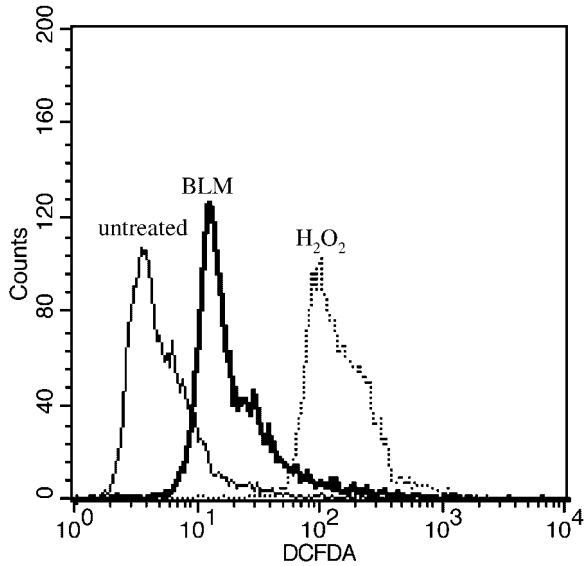

Fig. 7. Histogram of L132 cells loaded with DCFH-DA for determination of ROS. L132 cells were treated with BLM or H_2O_2, respectively, (see Material and Methods) and the generation of ROS was detected by conversion of the redox-sensitive dye DCFH-DA to the highly fluorescent DCF. Cells (10^4) each were evaluated by flow cytometry. The figure shows the results of one representative experiment (n = 3).

employed the caspase inhibitor Z-VAD-fmk for a suppression of apoptosis and studied if a presumed prevention of loss of CD44s surface expression and CD44 release was detectable. Our data clearly show that suppression of apoptosis abolished the BLM effect for L132 cells and reduced it statistically significant for A549 cells. The usage of the antagonistic CD95 antibody, however, did not exert any effect neither on BLM-induced apoptosis nor on the loss of CD44s by L132 cells.

The induction of apoptosis in lung epithelial cells during BLM-induced lung injury is a common process [Kuwano et al., 1996]. In a study on apoptosis of epithelial cells and infiltrating lymphocytes in BLM-induced pulmonary fibrosis in mice, Kuwano et al. [1999] demonstrated an essential role for the Fas (CD95-receptor)—Fas ligand (FasL, CD95) pathway of initiation of apoptosis. Similarly, Hagimoto et al. [1997] reported on an upregulation of Fas mRNA in alveolar epithelial cells after BLM-administration to mice. The importance of the Fas/FasL-system for BLM-effects on apoptosis induction was also emphasized by a study by Muller et al. [1997] on HepG2 cells. The results of our present study clearly indicate, at least for L132 cells, that the BLM-induced

apoptosis is CD95 independent, but involves a mitochondrion-dependent pathway, which includes the release of cytochrome C. This was recently shown by Zhao et al. [2004] in BLM-exposed alveolar macrophages. In addition, our ROS measurement data suggest a role of reactive oxygen metabolites in BLM-induced apoptosis.

The primary role of CD44s is to promote the maintenance of tissue architecture during development and repair. In the lung, type II epithelial cells express CD44s predominantly at the basolateral site indicating a function as adhesion protein mediating contact to the underlying matrix [Kasper et al., 1995]. In epithelia, increased expression of CD44 in response to proliferative stimuli or to repair processes has been observed [Svee et al., 1996]. In a previous study on lung fibrosis, we showed that pneumocytes in fibrotic foci were devoid of CD44, particularly in advanced stages of disease. In contrast, hyperplastic alveolar epithelium strongly expressed CD44 [Kasper et al., 1996]. Therefore, under pathologic conditions both processes upregulation and loss of CD44 by epithelial lung cells occur.

The release or shedding of CD44 by cells is a constitutive process that is modulated by several factors [Ristamaki et al., 1997; Cichy and Pure, 2003]. Several data support the concept of a shedding of this adhesion protein by action of proteinases [Cichy et al., 2002; Lazaar et al., 2002] with the consequence of accumulation in serum or bronchoalveolar lavage under pathologic conditions connected with inflammation and matrix remodeling [Cichy and Pure, 2003]. The CD44 immunoreactivity in intracellular vesicles located near the cell surface of apoptotic cells observed in the present study might represent part of the CD44 molecule internalized after proteolytic cleavage.

It is assumed that soluble CD44 has the potential to compete with membrane bound CD44 in interactions with hyaluronan or other ligands with the consequence of a diminished capacity of cells to adhere to extracellular matrix components [Peterson et al., 2000].

The observation by Gunthert et al. [1996] of a functional link between the loss of CD44 and apoptosis raised the question whether apoptosis processes trigger CD44-loss or whether CD44-depletion and cell detachment are the reason for induction of apoptosis. The authors of the mentioned study hypothesized that CD95-induced apoptosis triggers CD44 shedding in colon carcinoma cells. In the present study, we compared the kinetics of activation of caspase-3 and of CD44 release by L132 cells upon BLM-treatment. These experiments demonstrated a strong increase of the amount of cleaved caspase-3 as early as 2 and 3 h after BLM-treatment accompanied by a moderate elevation of soluble CD44 in culture media. Statistical analysis of the data, however, did not show a significant difference between the start of apoptosis induction and of CD44 release. One reason for this fact might be the existence of great standard deviations of the values for caspase-3 activation. Furthermore, in each cell the temporal difference between caspase activation and CD44 release might be extremely short leading this approach to its limit to discriminate between the initiation of both processes. Much more stringent data are provided by the inhibition experiments using Z-VAD-fmk as a broad range caspase inhibitor. The results of these inhibition experiments clearly show that CD44s release depends on induction of apoptosis. These data confirm observations by Gunthert et al. [1996]. However, we demonstrated the independence of these processes from CD95-activation. We have to take in consideration that these observations might be limited to specific lines of epithelial lung cells. The molecular mechanisms connecting apoptosis processes with the release of CD44 have to be discovered.

From the data presented above, we suggest that soluble CD44 can be regarded as a potent modulator of inflammatory and repair processes during development of fibrosis. The release of CD44 by BLM-treated epithelial cells might reflect part of a protective mechanism in lung tissue for maintaining the integrity of the epithelium by competing with membrane bound CD44 as a target for proteinases and by serving as an organizer of extracellular matrix assembly in close proximity to the cells [Cichy et al., 2002; Cichy and Pure, 2003]. Furthermore, soluble CD44 might impede the migration of fibroblasts at sites of destruction of alveolar epithelium thus preventing fibrosis [Svee et al., 1996].

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