Abstract. Background: Bleomycin induces apoptosis in alveolar epithelial cells. The expression of caveolin-1 and -2 in lung epithelial-derived A549 cells was analysed in terms of apoptosis after exposure to bleomycin. Materials and Methods: Apoptosis was investigated using flow cytometry, ELISA, immunohistochemistry and Western blot analysis. Caveolin-1 and -2 were determined at the protein level (Western blot). Intracellular caveolin-1 distribution was studied with immunofluorescence, as well as sucrose density gradient centrifugation. Results: Caveolin-1 and -2 were up-regulated 1 h after exposure to bleomycin and preceding the occurrence of caspase-8, and of caspase-3 and caspase-9 cleavage products. Sucrose density gradient centrifugation revealed that bleomycin exposure led to a partial translocation of caveolin-1 from caveolin-rich membrane fractions to non-raft fractions. Successful inhibition of bleomycin-induced apoptosis by the broad-spectrum caspase inhibitor zVAD-fmk did not influence the amount of caveolin-1 and -2. Conclusion: The early up-regulation of caveolin-1 and -2 following bleomycin exposure is a rather apoptosis-independent event related to other unknown mechanisms of bleomycin-mediated cell injury.

Caveolae are flask-shaped invaginations of the plasma membrane and are present in most tissue cells. Caveolins are a family of integral membrane proteins (caveolin-1, -2, and -3) that are important structural components of caveolae (1). Although individual caveolin members are differentially expressed in various cell types (2), lung tissue has abundant caveolin-1, and -2 in both endothelial and alveolar type I cells (3, 4). Caveolae and caveolin-1 play a key role in a number of cellular functions, including tumor suppressor function and apoptosis (1), cholesterol trafficking (5), modulation of signalling events (6) and involvement in potocytosis and transcytosis (7). Recent data also describe caveolae as a portal for the entry of a range of bacteria and viruses (8).

Caveolin-1 has also been implicated in cellular transformation and tumorigenesis. Caveolin-1 levels are down-regulated in a variety of carcinoma cell lines (1). Cells, including tumor cells, constantly face the decision of whether to survive and proliferate, or to undergo cellular senescence or programmed cell death (apoptosis). Therefore, identifying the pathways that are pro-apoptotic or anti-apoptotic has important implications for controlling tumor cell growth. Data on the role of caveolin-1 in apoptosis are still controversial. Overexpression of caveolin-1 in Rat-1 fibroblasts facilitated ceramide-induced cell death through a PI3-kinase-dependent mechanism (9). Liu et al. (10) observed that caveolin-1 expression sensitized NIH-3T3 fibroblasts and T24 bladder carcinoma cells to apoptotic stimulation initiated by staurosporine. In contrast, caveolin-1 overexpression in Rat1A cells and human prostate cancer cells (LNCaP), or caveolin-1 upregulation in androgen-insensitive LNCaP clones rendered these cells more resistant to apoptosis (11).

To address the potential role of caveolin-1 and -2 in apoptosis, we studied their behaviour in A549 lung cancer cells after treatment with the apoptotic stimulus bleomycin. Bleomycin-induced apoptosis of epithelial and non-epithelial cells plays an important role during pulmonary fibrogenesis (12). We used several assays to directly assess apoptosis and their relationship to the expression of caveolin-1 and -2 in bleomycin-treated A549 cells.

Materials and Methods

Cell line, reagents. The A549 cell line (human lung adenocarcinoma cells) was from ATCC. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from PAA Laboratories GmbH, Colbe, Germany. Fetal calf serum (FCS) was obtained from HyClone, Perbio
Science Deutschland GmbH, Bonn, Germany. L-Glutamine and Trypsin/EDTA were purchased from BIOCHROM AG seromed®, Berlin, Germany. BLEO-cell® was obtained from cell pharm GmbH, Hannover, Germany. Z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) was from ALEXIS® Biochemicals, Lausen, Switzerland.

Cell culture. A549 cells were cultured in DMEM supplemented with 5% FCS, L-Glutamine and sodium pyruvate. They were grown at 37°C in a 5.5% CO2 atmosphere. Cells were seeded at a density of 1-2x10⁴ cells/ml and passaged continuously. Subconfluent cells were treated with bleomycin in culture medium at a concentration of 50 mU/ml. For inhibition experiments, bleomycin (BLM) and the broad-spectrum caspase inhibitor zVAD-fmk (40 μM) were added to the culture medium at the same time.

Immunohistochemistry. Clotting, paraffin embedding, sectioning and immunostaining of cells used here has been described elsewhere (13). The following primary antibodies were used: (i) polyclonal rabbit anti-cleaved caspase-3 (Asp175; Cell Signaling Technology, distributed by New England Biolabs GmbH, Frankfurt a.M., Germany); dilution 1:50; (ii) polyclonal rabbit anti-cleavage product of vimentin V1, (14); dilution 1:8000.

Briefly, the 5-μm thick paraffin sections cut from formalin-fixed material were deparaffinized and pretreated with microwaves, washed in phosphated buffered saline (PBS) and then incubated with normal horse serum followed by incubation with the primary antibody. All steps were performed at room temperature (RT). For immunodetection a commercially available ABC-technique (mouse or rabbit kit; for detection of the mouse or rabbit antibodies, respectively; Vectastain Elite Kit, Serva, Heidelberg, Germany) was used according to the manufacturer’s instructions. Visualization of peroxidase localization was performed using 3,3′-diaminobenzidine as substrate. As negative controls the primary antibodies were replaced with PBS. Immunostaining was examined with a BX60 fluorescent microscope (Olympus, Hamburg, Germany).

Quantitative determination of immunoreactivities. Cells were considered active caspase-3- and V1-positive when brown cytoplasmic staining could be detected. The total cell number (haematoxylin counterstained) and the immunopositive cell number were counted randomly for 5-10 high-power fields (area 0.04 mm²) per slide using a microscope (Nikon Optiphot-2, Nikon GmbH, Düsseldorf, Germany). The labelling index was calculated as the percentage of positive cells of the total cells per area.

Western blot analysis. After different times, bleomycin-treated and control cells were rinsed with PBS without Mg²⁺ and Ca²⁺, incubated with Trypsin/EDTA for 5 min at 37°C, 5.5% CO₂. Trypsin was stopped using culture medium. Cells were pelleted by centrifugation at 220 xg for 5 min at RT. Pellets were then washed with PBS twice. The pellets were resuspended in lysis buffer containing 20 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1% protease inhibitors. After incubation for 30 min on ice, cell lysates were centrifuged at 13000 rpm for 20 min at 4°C. Total protein of the lysate supernatant was determined using BCA Protein Assay Reagent Kit (Pierce, Rockford, Ireland) using 50 μg of total protein of each sample redissolved in 6 x SDS sample buffer (300 mM Tris-HCl, pH 6.8; 30% (w/v) glycerol; 10% (w/v) SDS; 0.1% bromphenol blue; 100 mM DTT). After boiling the samples for 5 min at 95°C they were loaded on a 12% SDS-polyacrylamide gel. The separated proteins were transferred to a 0.45 μm PVDF-membrane (Immobilon-P™, Millipore; Carl Roth

Figure 1. Caspase-cleaved CK18 (CK18-Asp396-NE) was measured after 48 h in untreated (control) and bleomycin-treated A549 cells (total and adherent cells) using the M30-ELISA. Results are shown as means in the presence or absence of the broad-spectrum caspase inhibitor zVAD-fmk; BLM: bleomycin, bars: ±SD.
Figure 2. Detection of apoptosis in A549 cells. After exposure to bleomycin (BLM) at 50 mU/ml for 48 h cells were fixed in formalin and paraffin embedded (see Materials and Methods). Control: untreated cells; BLM+zVAD-fmk: 40 μM broad-spectrum caspase inhibitor and 50 mU/ml BLM. Immunohistochemical data are representative of three independent experiments. Magnification x300; V1: caspase-9 cleavage product of vimentin, V1; CC3: active caspase-3 labelling index for A549 cells. Data are shown as percentages compared to all cells as mean±SD.
GmbH & Co., Karlsruhe, Germany). After blocking the membrane in TBS-T (137 mM NaCl, 2.7 mM KCl, 20 mM Tris-HCl, 0.2% Tween20; pH 7.4) containing 5% (w/v) non-fat dry milk for 1 h at RT, or overnight at 4°C, it was incubated with monoclonal mouse anti-caveolin-1 (clone 2297; BD Biosciences, Pharmingen, US; dilution 1:1000), monoclonal mouse anti-caveolin-2 (clone 65; BD Biosciences; dilution 1:500), monoclonal mouse anti-γ-tubulin (clone GTU-88; Sigma-Aldrich Chemie GmbH, Munich, Germany; dilution 1:1000), for 2 h at RT or overnight at 4°C. The membrane was washed three times for 10 min. Incubation with secondary HRP conjugated antibodies (ECL anti-mouse IgG, Amersham Biosciences, Buckinghamshire, UK; dilution 1:2000) for 1 h at RT followed before washing again (three times for 10 min). Chemiluminescent signal was generated using ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Uppsala, Sweden) and detected with an Image Reader LAS3000 (Fujifilm, Tokyo, Japan).

**Measurement of apoptosis using ELISA.** Cells were seeded at a concentration of 1.5-2x10^4 cells/ml. Subconfluent cells were treated with bleomycin, bleomycin and zVAD-fmk. After 48 h, the M30-Apoptosense® ELISA (Peviva, Axxora Deutschland GmbH, Grünberg, Germany) was performed according to the protocol of the supplier. Absorption was determined using a TECAN Sunrise reader (Crailsheim, Germany).

**Flow cytometry with antibodies against intracellular antigens (V1, caveolin-1).** Cells were rinsed with PBS without Mg^2+ and Ca^2+ and collected by using Trypsin/EDTA as described above. Afterwards they were resuspended and fixed in PBS containing 4% formaldehyde (1:1) for 20 min. Centrifugation at 1200 rpm for 4 min at 4°C followed. Cells were permeabilized in PBS containing 0.5% saponin for 30 min, then centrifuged again under the same conditions. Cells were blocked in 0.5% saponin and 0.5% BSA in PBS for 30 min, then incubated with rabbit anti-caveolin-1 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany; dilution 1:20) or V1-antibody (dilution 1:50) for 1 h. Afterwards, cells were resuspended in PBS containing 0.5% saponin and 0.5% BSA. After centrifugation at 1200 rpm for 5 min cells were resuspended and incubated with FITC-coupled polyclonal goat anti-rabbit IgG (Dianova; dilution 1:100) in PBS containing 0.5% saponin for 1 h in the dark. After incubation, cells were centrifuged again, resuspended in PBS and analysed using flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany). For each analysis, 10000 events were recorded.

**Measurement of caspase-8 using flow cytometry.** The medium of subconfluent treated and untreated cells was not changed but supplemented with 2 μM Z-IETD-Rhodamine110 (Molecular Probes Europe BV, Leiden, The Netherlands). After 1 h incubation, all cells were detached as described above, resuspended in PBS and analysed with flow cytometry. For each analysis, 10000 events were recorded.

**Purification of caveolin-enriched membrane fractions.** Caveolin-enriched membrane fractions were purified using a detergent-free method as already described by Song et al. (16). All steps were performed at 4°C, each buffer contained protease inhibitors (Complete, EDTA-free; Roche, Penzberg, Germany). Subconfluent A549 cells were treated with 50 μM/ml BLM. After 48 h, treated and untreated cells were washed twice with PBS and scraped into 2 ml of 100 mM sodium carbonate (pH 11.0). The sample was homogenized using a tight-fitting Dounce homogenizer (15 strokes) and a sonicator (3 x 20 s bursts; UP100H, Dr. Hielser GmbH, Germany). Homogenates were mixed with 2 ml of 90% (w/v) sucrose in MBS (25 mM Mes, pH 6.5, 150 mM NaCl) and placed at the bottom of a 12 ml ultracentrifuge tube. A 5-35% (w/v) discontinuous sucrose gradient was formed above this with a 4 ml layer of 35% sucrose and followed by a 4 ml layer of 5% sucrose (both in MBS containing 250 mM sodium carbonate). Afterwards, samples were centrifuged at 39000 rpm for 18-20 h in a Beckman SW40 rotor (Beckman Coulter GmbH, Unterschleissheim-Lohhof, Germany). Thirteen x 1 ml gradient fractions were collected from the top of the gradient and subjected to Western blot analysis, performed as described above. Finally, PVDF-membranes were probed with monoclonal mouse anti-caveolin-1 (clone 2297; BD Biosciences, dilution 1:1000) and monoclonal mouse anti-flotillin-2/ETA (clone 29; BD Biosciences, dilution 1:1000).

**Results**

**Bleomycin-induced apoptosis in A549 cells.** Bleomycin, a potent anticancer drug, induces DNA cleavage by provoking changes in chromosome morphology and is a commonly used apoptotic inducer. To assess a potential role for caveolin-1 in promoting apoptosis, we first developed an apoptotic model system employing bleomycin-treated A549 cells. Exposure of A549 cells to bleomycin for 48 h caused apoptosis detectable by quantitative measurement of the apoptosis-associated CK18Asp396 ("M30") neoepitope (ELISA assay), and immunolabelling of the active form of caspase-3, and caspase-9 cleavage product of vimentin V1. The ELISA assay was used to quantify the levels of caspase-cleaved CK18-Asp396-NE fragments in the total cell population (adherent and detached cells) with and without bleomycin treatment (Figure 1). A parallel experiment comprised the assessment of adherent cells with and without bleomycin (Figure 1). A high content of CK18-Asp396-NE fragments (586 U/L) was detected in the total cell population of bleomycin-treated cells in comparison with a quite low content (235 U/L) in adherent cells. No difference was detected in the content of CK18-Asp396-NE fragments between the untreated total cell population (75 U/L) and untreated adherent cells (79 U/L). Bleomycin-induced apoptosis of A549 cells was inhibited by the broad-spectrum caspase inhibitor zVAD-fmk (40 μM), confirming the specificity of the assay for apoptosis (Figure 1).

**Immunohistochemistry using apoptosis markers for active caspase-3 and specific caspase-9 cleavage products of vimentin confirmed the ELISA data (Figure 2).** Under conditions of cell culture many epithelial cells, including A549 coexpress vimentin (17). The anti-V1 antibody detects the 21 and 31-kDa specific cleavage products of vimentin confirmed that caspase-9 in cells at an early stage of apoptosis to a similar extent as the determination of the active caspase-3 immunoreactivity (Figure 2). Figure 2 also
quantifies the increase in the cellular fraction of labelling with active caspase-3, which increased from 0.4% to 23.0% in the total cell population and from 0.4% to 3.2% in the adherent cells, and in cellular fraction labelling with V1, which increased from 0.4% to 14.2% and from 0.4% to 5.6%, respectively. Inhibition experiments using the broad-spectrum caspase inhibitor zVAD-fmk (40 ÌM) confirmed the specificity of both assays for apoptosis by inhibiting the labelling to approximately that of the control (Figure 2).

Effect of bleomycin on caveolin-1 and -2 expression in A549 cells. Bleomycin treatment of A549 cells was performed to investigate the expression of caveolin-1 and caveolin-2 under conditions known to induce epithelial injury in the lung (18). In comparison with controls a clearly increased expression of caveolin-1 and caveolin-2 protein levels after 48 h was detected (Figure 3). In order to ensure comparable amounts of protein γ-tubulin was also determined.

Caveolin-1 and -2 were equally up-regulated in apoptotic and non-apoptotic cells as revealed by a comparison of the number of total and adherent cells, the latter represented about 95% non-apoptotic cells. The remaining 5% of apoptotic cells did not account for the strong increase in caveolin-1 and -2 expression. Effects of broad-spectrum caspase inhibition on caveolin-1 and -2 expression. To test whether caspase activation modulated caveolin-1 and -2 expression, we also investigated the expression of caveolin-1 and -2 in bleomycin-treated A549 cells after inhibition of apoptosis with the broad-spectrum caspase inhibitor zVAD-fmk (Figure 3). Despite the complete inhibition of apoptosis by zVAD-fmk in the apoptosis ELISA (compare Figure 1), Western blot analysis revealed no changes in the amount of caveolin-1 and caveolin-2 protein expression in total and adherent A549 cells after inhibition experiments with zVAD-fmk (Figure 3).

Time-course of caveolin-1 expression, caspase-8 activation and of caspase-9 cleavage products during bleomycin exposure. Caspase-9 is the initiator caspase of the mitochondrial apoptotic cascade that is activated by Apaf-1 in the presence of cytochrome c and dATP (19). Therefore, we measured the kinetics of activation of caspases by FACS-analysis of total and adherent cells using caspase-8 and the caspase-9 cleavage products of vimentin as markers. In addition to the measurement of caspase-8 and the cleavage products of caspase-9, we estimated the expression of caveolin-1. As shown in Figure 4A for the total cell population, an increase

![Figure 3. Up-regulation of caveolin-1 and -2 in bleomycin treated A549 cells after 48 h. The amounts of caveolin-1 and -2 in untreated, bleomycin-treated and bleomycin-treated cells in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (total and adherent cells) were determined using SDS-PAGE and immunoblot analysis, with anti-γ-tubulin as a control for equal protein loading. Representative data from three separate experiments are shown.](image-url)
of caveolin-1 expression occurred 1 h after treatment with bleomycin which continued to rise after 3, 12 and 24 h. In contrast, activation of caspase-8 and caspase-9 cleavage products was detectable after 12 h and 24 h only. FACS-analysis of the adherent cell population revealed similar data (not shown).

For confirmation of the FACS-analysis data, the amount of caveolin-1 and the cleavage products of caspase-9 in A549 cells in response to bleomycin-treatment were determined using Western blotting. Figure 4B shows the levels of caveolin-1 and caspase-9 cleavage products in A549 cells exposed to bleomycin for the indicated periods in comparison
to untreated controls. The level of caveolin-1 increased after 3 h of bleomycin-treatment in comparison to untreated control cells. For the 21 kDa caspase-9 cleavage product, a slight increase of the amount of the protein was detected after 12 h. At 24 h after bleomycin administration, the presence of the 21 kDa fragment was clearly demonstrated.

Caveolin-1 expression in lipid rafts after bleomycin-treatment. Bleomycin treatment of lung epithelial cells has been shown to be associated with changes in the intracellular distribution of caveolin-1 (20). To address the presence of caveolin-1 in lipid rafts in control and bleomycin-treated A549 cells, we isolated CLM (caveolin-rich light membrane) fractions and assessed the distribution of caveolin-1 in these cells. Following density-gradient centrifugation of untreated and bleomycin-treated A549 cell homogenates extracted in sodium carbonate, caveolin-1 was enriched in fraction(s) 4-6 corresponding to a region of the gradient situated at the interface of the 5% and 35% (w/v) (Figure 5A). These fractions also contained the raft marker flotillin-2 (not shown). In addition, a translocation of caveolin-1 to (flotillin-2 negative) non-raft fractions 12 and 13 was detected in bleomycin-treated A549 cells.

The subcellular localization of caveolin-1 was also determined by immunofluorescence (Figure 5B, C). In untreated cells, caveolin-1 was targeted to the plasma membrane. In bleomycin-treated cells caveolin-1 was more retained intracellularly in the perinuclear compartment. The content of caveolin-1 in the plasma membrane was reduced in comparison to untreated cells.

Discussion

The majority of the in vitro data about apoptosis has been generated from experiments using the human alveolar epithelial-derived tumor cell line A549. A significant amount
of evidence has been accumulated during the last few years showing that the mitochondrial as well as the death receptor pathway play a role in apoptosis of A549 cells (21, 22).

Bleomycin is a chemotherapeutic agent that is used in the treatment of a variety of tumors. As a side-effect bleomycin induces lung injury and triggers apoptosis of alveolar epithelial cells (21). Bleomycin acts through its cytotoxicity by the induction of free radical production. These radicals cause DNA breaks leading ultimately to cell death (reviewed in (12)).

In this study, we have demonstrated (i) a moderate degree of apoptosis after treatment of A549 cells with bleomycin; (ii) a strong increase in the protein levels of caveolin-1 and -2 as well as an increase in cytoplasmic caveolin-1, and (iii) a partial translocation of caveolin-1 from the CLM fractions to non-raft fractions. As a second step towards the elucidation of the role of caveolin-1 in apoptosis, we compared the time course of caveolin-1 expression with the detection of active caspase-8 as well as the detection of early substrates of caspase-9. We found that the increase in caveolin-1 expression preceded the detection of apoptosis using FACS-analysis of A549 cells containing caspase-8 or caspase-9 cleavage products of vimentin. Furthermore, we observed that an almost complete inhibition of apoptosis in bleomycin-treated A549 cells did not reduce the total amount of caveolin-1 and -2.

From the discrepancy between the low degree of apoptosis after bleomycin exposure and the strong increase of caveolin-1 in the majority of cells, we suggest that caveolin-1 and -2 are not directly involved in apoptosis. There is also earlier evidence that treatment of A549 with paclitaxel and other chemotherapeutic agents induces an up-regulation of caveolin-1 (23). The increase of caveolin-1 in paclitaxel-treated A549 cells has been discussed in terms of terminal differentiation (23). Gargalovic et al. (24) described an increase in caveolin-1 expression independent of protein synthesis, caspase activation and nuclear DNA fragmentation, and proposed a role for caveolin-1 during phosphatidyserine (PS) externalization.

In our study, like the results of to Gargalovic et al. (24), the caveolin-1 and -2 expression was not reduced after the use of the broad-spectrum caspase-inhibitor zVAD-fmk. Taking into account that the increased caveolin-1 and -2 expression in the present study is more or less restricted to the population of non-apoptotic (adherent) A549 cells, the data again support an apoptosis-independent function of caveolin-1 and -2. However, a direct effect of caveolin-1 and -2 on the initiation of apoptosis in the early phase upstream to the activation of caspases cannot be ruled out.

Recent data propose a gatekeeper function of caveolin-1 in cellular senescence (25). Bleomycin induces cellular senescence in A549 cells (26). We have shown that under our experimental conditions about 75% of adherent cells were senescent (unpublished data). Bleomycin targets the cells during defined phases of the cell-cycle and induces G2/M arrest (27). Since caveolin-1 expression influences the cell-cycle (28), investigations of senescence in the context of our detected increase of caveolin-1 and -2 will allow definite conclusions about the role of caveolins in the bleomycin-exposed A549 lung cancer cells.

In this study it was not possible to address the intracellular, non-raft distribution of caveolin-1 in any cellular function or dysfunction of the A549 cells. One possible explanation is that bleomycin-induced senescence is associated with a redistribution of caveolin and caveolae in the cell as was shown for primary senescent fibroblasts in humans (29).

**Conclusion**

Our findings demonstrate that bleomycin induced quantitative and qualitative changes in the expression of caveolin-1 and -2 and that caspase activation during bleomycin-induced apoptosis did not modulate the caveolin expression.

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**References**


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