bFGF rescues 423-cells from serum starvation-induced apoptosis downstream of activated caspase-3

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Abstract Serum withdrawal rapidly induces apoptosis in rat 423-cells, while addition of bFGF results in cell survival. However, surviving cells initially display morphological changes characteristic for apoptotic cells and even process caspases. Active caspase-3 was detected at the single-cell level in those finally bFGF-rescued cells, while mitochondrial integrity was maintained. Generation of cleavage products of caspase targets was confirmed in surviving cells. Proteome analysis indicated multi-faceted survival activities of bFGF including upregulation of inhibitor-of-apoptosis and heat shock protein family members directly interfering with caspases. Our data suggest that the "point-of-no-return" in death-induced cells has to be moved downstream of activated caspase-3.

Keywords: Apoptosis; Survival; Serum withdrawal; Caspase; Basic fibroblast growth factor; Inhibitor-of-apoptosis protein

1. Introduction

The homeostasis of each tissue, and thus of an entire organism, is regulated by the balance between cell proliferation and apoptosis, a form of programmed cell death. This is characterised by morphological changes like cell shrinkage, membrane blebbing, detachment and finally fragmentation of cell content into vesicles. The progress of apoptosis is well concerted in a spaciotemporal manner by a cascade of death specific proteases, termed caspases [1]. In this cascade initiator caspases activate executioner caspases. To ensure proper performance of death signalling, a sophisticated caspase network exists [2]. Once, executioner caspases are activated, they cleave cellular target proteins, most of them with a discrete function in propagation of cell death processes [3]. Distinct safety steps to prevent aberrant caspase activation were implemented during evolution. Most directly, inhibitor-of-apoptosis proteins (IAP) block processing of caspases and inhibit also processed caspases [4]. Additionally, several members of the heat shock protein (hsp) family interfere with apoptotic pathways and prevent caspase cleavage [5]. Another important event is to preserve the integrity of intracellular membranes like mitochondria and the endoplasmatic reticulum by anti-apoptotic bcl-family members [6]. Some intermembrane space proteins can induce or enhance caspase activation, whereas others might act in a caspase-independent manner to promote apoptosis [7]. This fine-tuned cellular equilibrium between life and death is overbalanced not only upon triggering apoptosis, but also by the addition of survival factors, like basic fibroblast growth factor (bFGF). bFGF plays an important role in cell proliferation, differentiation, neoangiogenesis and survival in various tissues and cell systems, but also malignancies [8]. The abundance of this ubiquitary expressed growth factor contributes to resistance in chemotherapy and enhances survival of malignant cells [9–11].

We took advantage of a rat cell line, 423-cells, that undergo apoptosis upon serum withdrawal in a caspase-dependent manner within a few hours, while bFGF was described as potent survival factor for this cell culture model system [12]. Despite morphological alterations and even caspase-3 activation, bFGF-treated 423-cells escape from termination phase of apoptosis. The purpose of the present study was to determine how far apoptotic signalling proceeded under the influence of the survival factor bFGF and to characterise these survival effects by means of proteome analysis.

2. Materials and methods

2.1. Chemicals and antibodies

Antibodies directed against caspase-3, nPKC-δ and survivin were supplied by Santa Cruz, caspase-8 by NeoMarkers, vimentin and β-actin by Sigma, c-IAP-1 and PARP (C2-10) by R&D, and cleaved caspase-3-p17 by New England Biolabs. Peroxidase-secondary antibodies and Cy3-conjugated anti-rabbit antibody were purchased from Calbiochem. 4′-6-Diamidine-2-phenyl indole (DAPI), JC-1 and bFGF from Calbiochem, caspase dye, Di-L-aspartate-rhodamine 110 (D_2R) from Alexis and Complete® Protease Inhibitor Cocktail from Roche Diagnostics.

2.2. Cell culture

The non-transformed, G418-resistant embryonic rat cell line, 423, immortalised by Human Papilloma Virus type 11 DNA [13], was routinely cultivated in DMEM/10% FCS, penicillin/streptomycin (100 U/ml) and G418 (200 μg/ml). For induction of apoptosis, confluent cultures of 423-cells were washed twice with serum-free DMEM and further incubated in DMEM/0.1% FCS. bFGF (25 ng/ml) was added at the time point of serum withdrawal.

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Abbreviations: bFGF, basic fibroblast growth factor; IAP, inhibitor-of-apoptosis protein; IF, intermediate filament; IL, interleukine; hsp, heat shock protein; NSCLC, non-small cell lung cancer; ROS, reactive oxygen species; TNFR, tumour necrosis factor receptor

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2.3. Western blotting

For total protein lysates, cells were harvested by scraping, washed twice in ice cold PBS and lysed in RIPAII (500 mM NaCl; 50 mM Tris, pH 7.4; 0.1% SDS; 1% NP-40; 0.5% Na-DOC; 0.05% NaN_3 and complete protease inhibitor mix) for 30 min on ice. Lysates were sonificated, centrifuged at 12 000×*g*. Supernatants were used as total cell lysates. SDS–PAGE and Immuno Blot procedure was performed as described in [14].

2.4. Immunofluorescence and FACS analysis

423-cells were plated on coverslips and cultivated in the appropriate media for at least 48 h before fixation. Serum withdrawal in the presence or absence of bFGF was performed for 4 h. Cells were fixed and stained with anti-cleaved caspase-3/anti-rabbit-Cy3 and DAPI as described in [14].

423-cells were seeded in 24-well plates and apoptosis was induced up to 6 h. Twenty minutes before harvesting floating and attached cells together, D_2R was added to the medium [15]. For determination of mitochondrial integrity, floating and attached cells were combined in DMEM, 2.5% FCS and stained with 5 μM JC-1 for 10 min in the dark [16,17]. Samples were analysed in a Becton Dickinson FACSCalibur system using CELLQuest software.

2.5. Metabolic labelling and two-dimensional electrophoresis

Labelling, fractionation, isoelectric focusing, SDS–PAGE, autoradiography and silver staining were performed as previously described [18]. Briefly, confluent 423-cells were washed in serum-free medium and further incubated in methionine-free medium containing 10% FCS (control cells), 0.1% FCS (apoptotic cells) and 0.1% FCS plus bFGF (surviving or rescued cells), respectively. Labelling occurred by addition of 0.8 mCi/10^7 cells ^{35}S-protein labelling mix (NEG-072, Perkin-Elmer Life Sciences) at the time point of medium change. After a 4 h labelling time, cells were collected by centrifugation, fractionated and 100 μg protein were separated and comparative gel analysis was performed using the 2-D Analyzer™ V 6.1 software package. Identification of protein spots by mass spectrometry fingerprinting was performed as described in [18].

3. Results

3.1. Serum withdrawal in the absence and presence of bFGF

We compared 423-cells induced to undergo apoptosis with the bFGF-treated, finally surviving cells. Monitoring the morphological changes after serum withdrawal by light microscopy (Fig. 1A); we found that 423-cells rounded up rapidly, irrespective of bFGF addition. At 2 h after serum deprivation, bFGF-treated cells were undistinguishable from those committed for apoptosis. However, the apoptotic cell population already displayed some DNA condensation, while bFGF-treated cells did not (Fig. 1A, white arrows). Blebbing and massive cell detachment started after 3 h and preceded further in the apoptotic 423-cells, but remained undetectable in the bFGF-treated cells. Furthermore, the bFGF-rescued cells, still maintained in low serum conditions, reattached between the forth and fifth hour and finally completed restructuring of the confluent layer without any signs of apoptosis 12 h after.

As the morphology of 423-cells was apparently destroyed within minutes after serum withdrawal, we looked for a marker for cytoskeletal disruption. The intermediate filament (IF) protein vimentin is a caspase target [19], like other cytoskeletal proteins as cytokeratins [20] and myosin heavy chain [18]. In contrast to monomeric vimentin, the filamentous form is highly insoluble in commonly used lysis buffers [21], and may

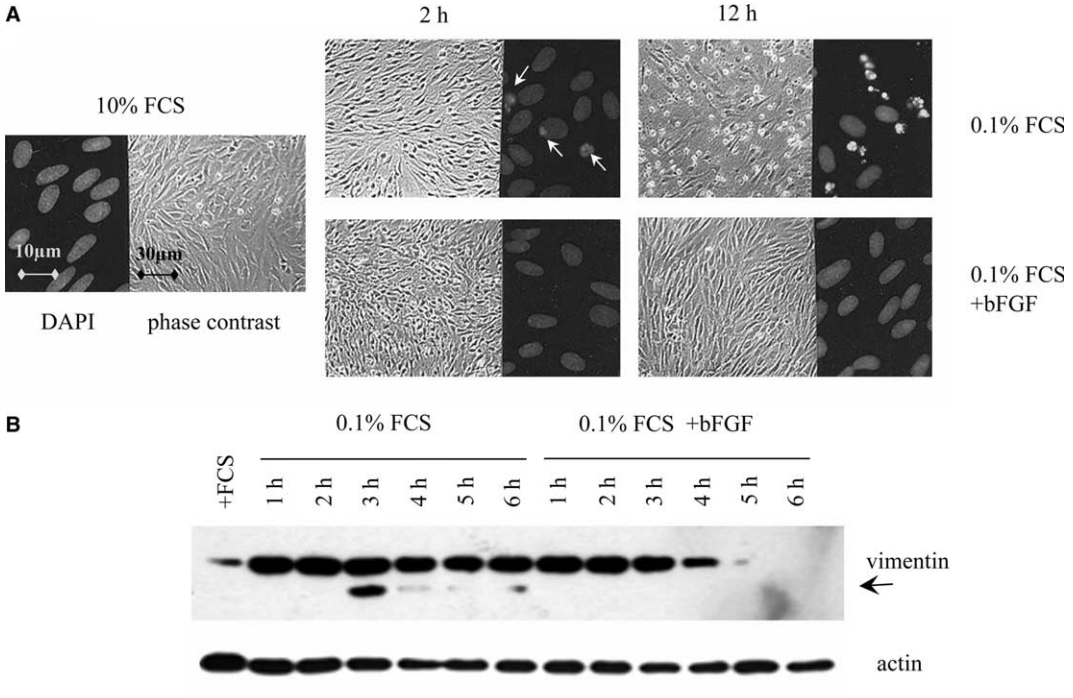

Fig. 1. Serum withdrawal in the presence or absence of bFGF. (A) bFGF-treated and untreated 423-cells were imaged by phase contrast (left panel) and by DAPI staining (right panel) during a time course. Morphological alterations, spherical cells, occurred during serum starvation-induced apoptosis and bFGF-mediated rescue at 2 h. At 12 h bFGF-treated 423-cells recovered (lower panel), while a mixture of remaining cells in the layer and apoptotic bodies were detected in untreated cells (upper panel). Condensed nuclei are marked by white arrows. (B) Intermediate filament protein vimentin as indicator for cytoskeletal breakdown during bFGF-mediated rescue. Detection of soluble vimentin in serum-starved 423-cells in the presence or absence of bFGF during a time course was performed by Western analysis. Disappearance of vimentin in bFGF-treated cells correlated with remodelling of morphology. Caspase-specific vimentin fragment is marked by an arrow. Actin was used as loading control.

hence remain hardly detectable by Western analysis. We analysed vimentin in 423-cells after serum withdrawal in a time course in the absence or presence of bFGF, respectively (Fig. 1B). In untreated attached cells only low level of vimentin was detectable, but the amount increased upon serum withdrawal, irrespective of bFGF supplement, correlating with shrunken morphology of the cells. Under apoptotic condition, vimentin cleavage occurred within 3 h, whereas in bFGF-treated cultures no cleavage was observed. Three hours after bFGF treatment, when cells started to reattach, detectable vimentin levels started to decrease rapidly, indicative of rebuilding of the filamentous network. These morphological and biochemical observations suggested that early apoptotic events upon serum withdrawal proceeded regardless of the presence of a potent survival factor.

3.2. Caspases processing despite the presence of bFGF

To investigate how far apoptosis proceeds in the presence of bFGF, we performed a time course of caspases processing (Fig. 2). Intriguingly, caspase-8 and subsequently caspase-3 were processed upon serum withdrawal in the absence but also in the presence of bFGF. In bFGF-treated cells, caspase-3 processing was weaker and the cleaved fragment became undetectable within 5 h. In order to determine proteolytic activity of the processed caspases, we investigated the cleavage of a cytoplasmic and a nuclear caspase-3 target, nPKC-δ and PARP. Indeed, cleavage fragments of both caspase targets were detected in the surviving cell population treated with bFGF. These data suggested that caspase processing and activation was actually triggered upon serum withdrawal in the presence of a potent survival factor eventually resulting in cell survival.

3.3. Caspases are uniformly activated upon serum withdrawal regardless of bFGF treatment

The question arose, whether the detected caspase cleavage products might derive from a small minority of dying cells within the bulk of surviving bFGF-treated 423-cells. Therefore, we analysed caspase activity at single cell level by fluorescence detection of the Rhodamine-linked caspase substrate dye D_2R [15] in 423-cells in the presence and absence of bFGF upon serum withdrawal. Under apoptotic and surviving conditions, similar amounts of cells processed this caspase-specific dye within the first 2 h (Fig. 3A). Elevated caspase activity in bFGF-treated cells (Fig. 3A, open circles) may reflect contribution of maybe apoptosis-independent caspases, other than caspase-8 and -3 (compare to Fig. 2). After 4 h the percentage of bFGF-treated, serum starved cells with caspase activity decreased, while the fraction of serum starved cells with caspase activity (Fig. 3A, filled circles) remained constant. As a second approach, we performed immunofluorescence in untreated, apoptotic and surviving 423-cells with an antibody specific for cleaved caspase-3 (Fig. 3B). Importantly, cleaved caspase-3 was detectable in similar amounts in apoptotic and surviving cells, but not in untreated controls. Taken together, the apoptotic machinery was found switched on upon serum

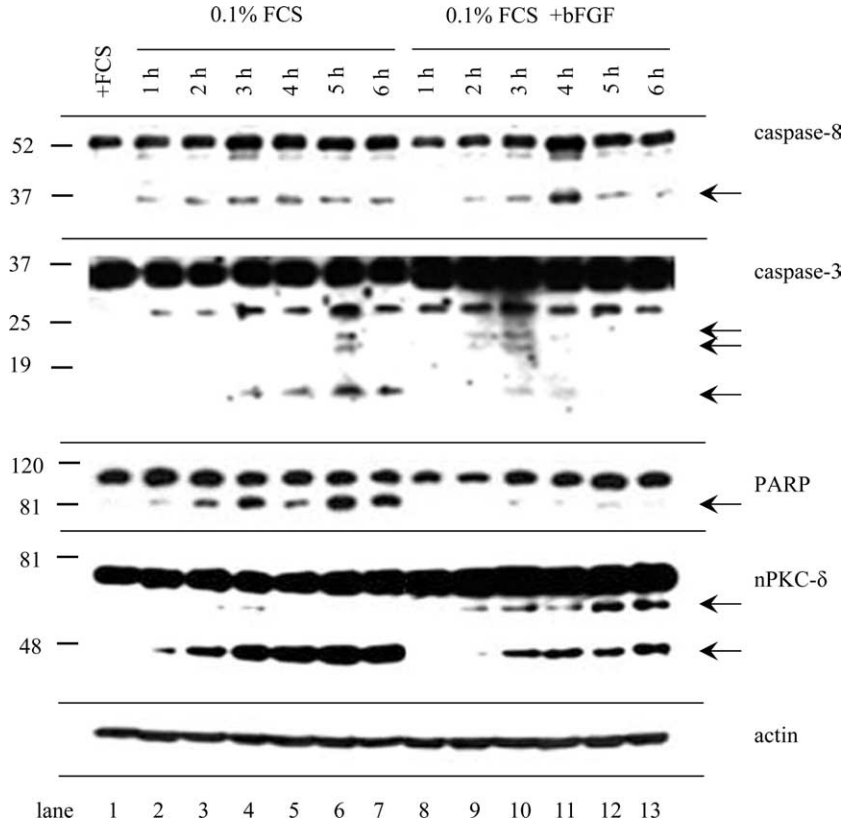

Fig. 2. Activation of caspases in serum-starved and bFGF-treated 423-cells during a time course. Processing of caspase-8 and subsequently that of caspase-3 was evidenced by detection of characteristic cleavage products, on both conditions. PARP and nPKCδ, as caspase-3 targets, were cleaved in the presence and absence of bFGF. Cleavage products are marked by arrows. Actin was used as loading control.

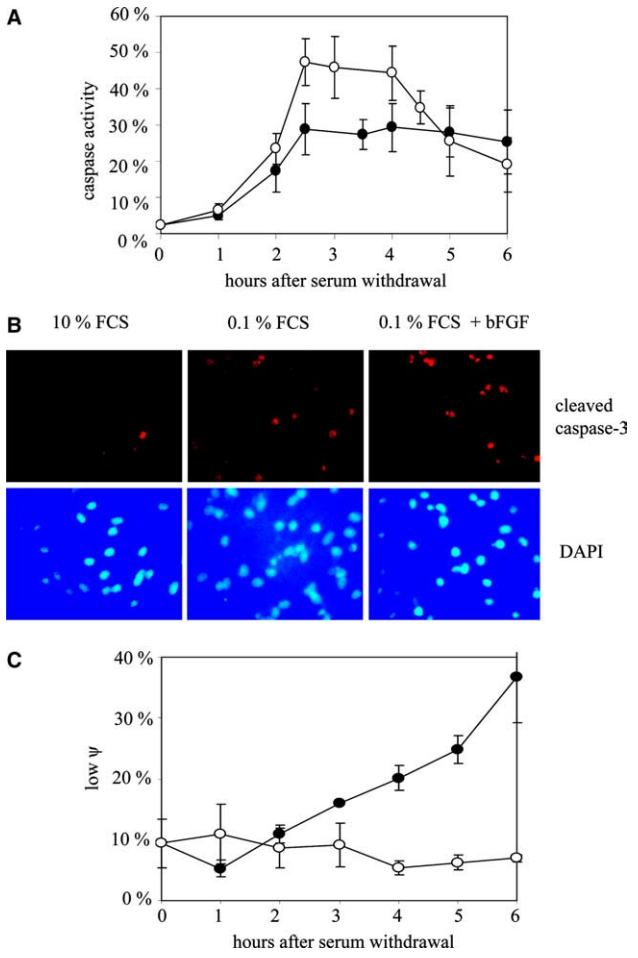

Fig. 3. Caspase activation, but maintenance of mitochondrial integrity in bFGF-rescued 423-cells. (A) Measurement of general caspase-activity by R_2D. Caspase activity, measured by Rhodamine-fluorescence, was determined by FACS analysis after serum starvation in the absence (filled circles) or presence (open circles) of bFGF in a time course up to 6 h. The bars represent S.D. from at least three independent experiments. (B) Evaluation of cleaved caspase-3. Immunofluorescence with cleaved caspase-3-specific antibody was performed 3 h after serum starvation in the presence or absence of bFGF and in 423-control cells. Nuclei were counterstained with DAPI. (C) Preservation of mitochondrial integrity by bFGF. Mitochondrial integrity, measuring a fluorescence shift of the mitochondrial dye JC-1, was determined by FACS analysis after serum starvation in the absence (filled circles) or presence (open circles) of bFGF in a time course up to 6 h. The bars represent S.D. from at least three independent experiments.

withdrawal regardless of bFGF treatment. Evidently, the anti-apoptotic effects of bFGF may act downstream of activated execution caspases.

3.4. bFGF preserves mitochondrial integrity

Mitochondria are an important part of the apoptotic machinery. Mitochondrial damage promotes apoptosis in two ways. On the one hand, it leads to the release of apoptogenic factors [7] and, on the other hand, it disrupts energy production of the cell. We examined changes in mitochondrial membrane potential by flow cytometric analysis of JC-1 complexes [17] during a time course after serum withdrawal in the absence and presence of bFGF. The percentage of 423-cells with low mitochondrial membrane potential increased from a basal level of 10% up to 35% after 6 h at apoptotic conditions (Fig. 3C, filled circles), while bFGF-treated 423-cells (Fig. 3C, open circles) maintained mitochondrial integrity.

3.5. Cleavage of a subset of proteins in bFGF-rescued 423-cells

Caspase-3 was found activated in surviving cells, but its proteolytic activity was apparently intercepted to circumvent irreversible commitment for apoptosis. Therefore, we screened for possible differences of caspase activity at apoptotic and surviving conditions. We compared the cytoplasmic fraction at a 4-h time point of control, apoptotic and bFGF-treated 423-cells by proteome analysis. As expected, several caspase-generated cleavage products of most notably cytoskeletal proteins including actin, fodrin, cytokeratin 19, myosin heavy chain and vimentin were detected in apoptotic 423 cells (Fig. 4C), but not in controls (Fig. 4A). Intriguingly, only a subset of cytoskeletal proteins including actin and vimentin were found partially degraded in bFGF-treated cells (Fig. 4E).

3.6. Induction of inhibitor-of-apoptosis proteins and heat shock proteins in death-induced, but bFGF-rescued 423-cells

To elucidate mechanisms involved in the anti-apoptotic capability of bFGF, we investigated the cytoplasmic protein expression profile of ^{35}S-radiolabelled cells at the same time point. ^{35}S-incorporation was found strongly decreased in apoptotic cells (Fig. 4D) compared to controls, and partially restored in bFGF-treated cells (Fig. 4E). Several proteins, including calreticulin, c-IAP-1, antiox2, grp78, hsp27 and SOD were significantly upregulated by bFGF (Fig. 4F compared to 4D, Table 1). Upregulation of cellular caspase inhibitor protein c-IAP-1 was verified by Western analysis. In addition, a second member of the IAP family, survivin, was found induced by bFGF (Fig. 5). Another family of anti-apoptotic proteins, hsps, were affected in different ways. Hsp70 expression was found upregulated and hsp27 phosphorylated at apoptotic conditions. Soluble cytoplasmic hsp90 became undetectable in bFGF-rescued cells, and was found translocated to the nuclear compartment (data not shown), comparable to translocation in fas-induced Jurkats [22]. These data suggest that bFGF may mediate cell survival by direct caspase inhibition via upregulation and activation of anti-apoptotic proteins.

4. Discussion

Escape from apoptosis is not only a prerequisite for early stages of transformation processes, but also a major obstacle in cancer therapy. bFGF, a growth factor frequently expressed in normal tissues and overexpressed in carcinomas [23], can mediate strong survival benefit. In this study, we demonstrated that bFGF was capable to counteract late apoptotic events, including caspase-3 activity. Upon serum withdrawal, apoptosis was rapidly induced in rat 423-cells. While initial morphological alterations were found unaffected, bFGF prevented membrane blebbing and DNA fragmentation (Fig. 1A). Processing of initiator caspase-8 was similar in apoptotic and bFGF-rescued cells (Figs. 2 and 3A). Caspase-3 processing was reduced in bFGF-treated cells, but still resulted in the cleavage of target proteins. The unexpected finding of caspase-3 activation without subsequent cell death was already described for T lymphocytes stimulated with interleukine (IL)-2 or phytohaemaglutinin A [24–26], ras-overexpressing Ba/F3

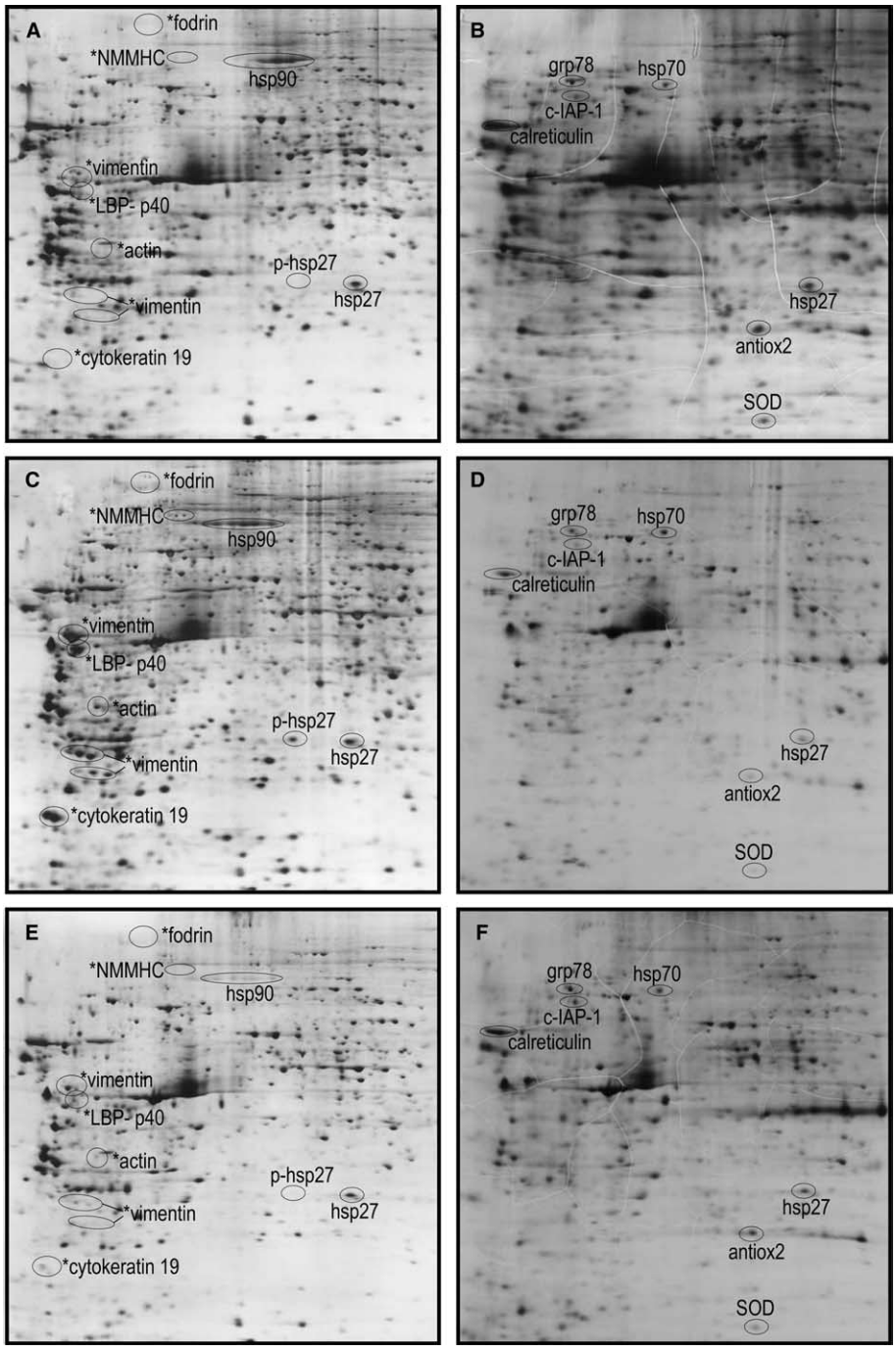

Fig. 4. Expression profiling of apoptosis-induced, but bFGF-rescued cells. Silver stained 2D-gels of cytoplasmic fractions of control, apoptotic (4 h) and bFGF-rescued (4 h) of ^{35}S-radiolabelled 423-cells (A, C and E) and the corresponding autoradiographs (B, D and F) reveal caspase activities and bFGF-survival effects, respectively. Cleaved and altered proteins are encircled and annotated.

upon IL-3 withdrawal [27] and non-small cell lung cancer (NSCLC) upon irradiation and etoposide treatment [28]. In NSCLC, only cytoplasmic targets were affected, while in hematopoetic cells cleavage of nuclear caspase targets, such as PARP and lamin B, also occurred. In surviving 423-cells, cytoplasmic and nuclear caspase targets were found cleaved (Figs. 2 and 4). We performed comparative proteome analysis of control, apoptotic and bFGF-rescued 423-cells to gain insight into the multi-faceted survival activities of bFGF. Taken together, the following events were observed:

1. Induction and upregulation of IAP and heat shock protein family members to counteract caspase activities.
2. Upregulation of chaperones to facilitate protein repair.
3. Upregulation of proteins involved in reactive oxygen species metabolism.
4. Preservation of mitochondrial integrity to maintain cellular energy supply.
5. Restoration of cellular morphology after initial crisis.

IAP [29] and hsp [4], most notably hsp70 [30], may act as emergency brake to inhibit further proteolytic caspase activity.

Table 1
Proteins affected during apoptosis and bFGF-mediated survival

Protein name	Accession No.	Apoptotic vs. control	bFGF vs. control	bFGF vs. apoptotic	Identification
Actin	P60711	Cleaved	Cleaved	–	W, MS
Calreticulin	P18418	Down	Down	Up	MS
Grp78	P06761	Down	Down	Up	MS
Heat shock protein hsp90-β (hsp90)	P34058	–	Transl.	Transl.	W
Heat shock protein β-1 (hsp27)	P42930	Phosh.	–	Up	W, MS
Heat shock 70 kDa protein 1/2 (hsp70)	Q07439	Up	Up	Up	W
Inhibitor-of-apoptosis protein 1 (c-IAP-1)	Q9ESE9	down	Up	Up	W
Keratin, type I cytokeratin 19	Q63279	Cleaved	Cleaved	–	MS
Laminin binding precurser (LBP-p40)	P38983	Cleaved	Cleaved	–	MS
Myosin heavy chain, non-muscle type A (NMMHC)	Q62812	Cleaved	–	–	MS
Peroxiredoxin 6 (antiox2)	O35244	Down	Down	Up	MS
Spectrin α chain (fodrin)	P16086	Cleaved	–	–	MS
Superoxide dismutase [Cu–Zn] (SOD)	P07632	Down	Down	Up	MS
Vimentin	P31000	Cleaved	Cleaved	–	W

List of altered proteins during apoptosis and bFGF-mediated survival in 423-cells. – , unaffected; Cleaved, caspase-cleavage products; Down, downregulated; Phosph., phosphorylated; Transl., translocated; Up, upregulated; MS, mass spectrometry fingerprinting; W, Western blot.

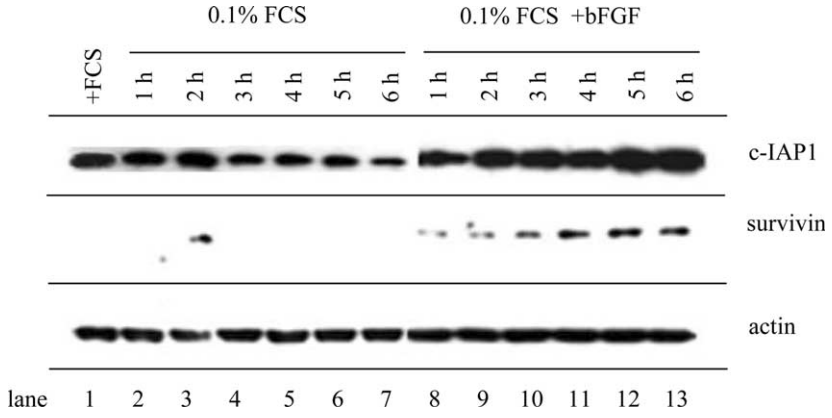

Fig. 5. bFGF induces IAP expression. Total protein lysates were prepared from control, serum-starved and bFGF-treated, serum-starved 423-cells during a time course. Induction of IAPs proceed after bFGF treatment, solely. bFGF-mediated c-IAP-1 upregulation and induction of survivin expression indicated regression of caspase activation. Actin was used as loading control.

We found two IAPs, c-IAP-1 and survivin, upregulated in bFGF-rescued 423-cells. c-IAP-1 was described as tumour necrosis factor receptor (TNFR2)-associated protein participating in TNFR signalling complexes and interfering with caspase-8 activation [31,32]. In addition, it was found to directly inhibit the executioner caspases, caspase-3 and -7 [33]. Recently, we showed that the strict cell-cycle-regulated expression of survivin can be deregulated by oncogenic c-H-ras [14]. Likewise, during bFGF-mediated survival, survivin was apparently upregulated independent from re-entry into cell cycle. We found no changes in cell cycle distribution during the first hours upon serum withdrawal and bFGF addition (data not shown). Intriguingly, hsp70 was also strongly upregulated under apoptotic conditions, suggesting a general stress response upon serum withdrawal not sufficient for cell survival.

In bFGF-rescued cells, additional members of the hsp family were increasingly translated, indicative of an enhanced requirement for protein repair [4]. hsp27, a member of the small hsp family, exerts its chaperone activity solely as oligomer by preventing aggregation of misfolded proteins [34]. Upon various stress stimuli, hsp27 becomes phosphorylated, resulting in the collapse of this chaperone complex, whereby it can no longer exert anti-apoptotic properties [35]. We observed hsp27 phosphorylation upon apoptosis induction, but not in bFGF-rescued 423-cells. Therefore, circumvention of hsp27 phosphorylation and subsequent preservation of its anti-apoptotic chaperone function may contribute to bFGF-mediated survival.

Indicative of further repair activity, we found a series of detoxification enzymes upregulated in bFGF-rescued cells. Free radical-derived reactive oxygen species (ROS) are formed by incomplete reduction of molecular oxygen and are constantly generated in most living tissue and cells in culture. ROS can potentially damage lipids, proteins and DNA, but also lead to reversible oxidation of proteins [36]. Upon growth factor withdrawal, ROS are produced apparently to enhance cell death, for example in PC-12 and mouse proximal tubular cells [37,38]. Therefore, upregulation of antiox2 and SOD, mediating ROS metabolism, by bFGF (Fig. 4F) may counteract apoptotic ROS production and signalling. Intriguingly, bFGF-mediated upregulation of antiox2 and SOD was only observed at serum-deprived, but not at full serum conditions (data not shown).

Mitochondrial damage enhances apoptosis by the release of apoptogenic factors [7], disruption of energy production [39] and subsequent production of ROS [40]. Preservation of mi-

tochondrial integrity mediated by bFGF (Fig. 3) seems to be of higher priority for successful cell survival than the complete inhibition of caspase activity.

Intact cellular structures are prerequisite for the function, proliferation, differentiation and survival of cells [41]. Breakdown of the structural integrity is a common feature of apoptotic cells. Intriguingly, a transient breakdown of cell architecture was observed in 423-cells struggling with death (Fig. 1). Consequent to our observations, the "point-of-no-return" in death-induced cells has to be moved downstream of activated caspase-3 into the execution phase of apoptosis. Our data suggest multi-faceted activities of bFGF in order to finally restore cellular integrity and enable survival.

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