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FAP-1-Mediated Activation of NF-κB Induces Resistance of Head and Neck Cancer to Fas-Induced Apoptosis

Eva Wieckowski,[1,2] Yoshinari Atarashi,[1] Joanna Stanson,[1] Taka-Aki Sato,[3] and Theresa L. Whiteside[1,2]*

[1]University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213
[2]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213
[3]Department of Pathology, Columbia University, New York, New York 10032

Abstract Molecular mechanisms responsible for tumor resistance to apoptosis often involve the Fas/FasL pathway. While squamous cell carcinomas of the head and neck (SCCHN) express both Fas and FasL, their resistance to self-induced apoptosis or apoptosis mediated by Fas agonistic antibody (CH-11Ab) was independent of the level of Fas surface expression or the presence of soluble Fas in supernatants of primary or metastatic SCCHN cell lines. By in vitro immunoselection, using PCI-15A cell line treated with successive cycles of CH-11 Ab, Fas-resistant sublines with the parental genotype were selected. Such sublines failed to cleave caspase-8 upon Fas engagement and were resistant to CH-11 Ab, although they remained sensitive to VP-16 or staurosporin. In the presence of cycloheximide, the selected SCCHN sublines become susceptible to CH-11 Ab, and showed cleavage of caspase-8, suggesting that apoptosis resistance was mediated by an inhibitory protein(s) acting upstream of caspase-8. Overexpression of Fas-associated phosphatase 1 (FAP-1), but not cellular FLICE-inhibitory protein (cFLIP) in SCCHN sublines was documented by Western blots and RT-PCR analyses. The FAP-1$^+$ selected sublines also downregulated cell surface Fas. A high phosphorylation level of IκBκ, NFκB activation and upregulation of Bcl-2 expression were observed in the FAP-1$^+$ sublines. Treatment with the phosphatase inhibitor, orthovanadate, or silencing of FAP-1 with siRNA abolished their resistance to apoptosis, suggesting that FAP-1 phosphatase activity could be responsible for NF-κB activation and resistance of SCCHN cells to Fas-mediated apoptosis. J. Cell. Biochem. 100: 16–28, 2007.

Key words: apoptosis; FAP-1; head and neck cancer; Fas resistance; immune escape

In mammalian cells, death receptors of the TNF receptor superfamily, such as CD95/Fas, mediate apoptosis upon interaction with their natural ligands or agonistic antibodies [Ochm et al., 1992; Peter and Krammer, 1998, 2003]. Fas engagement leads to recruitment of the FAS-associated death domain (FADD) and caspase-8 to the receptor, forming a death signal-inducing complex (DISC) [Chinnaiyan et al., 1996; Peter and Krammer, 2003]. Activation of caspase-8 initiates apoptosis by cleavage of downstream effector caspases [Medema et al., 1997; Thornberry and Lazebnik, 1998]. To avoid cell death, signals mediating death receptors must be tightly controlled [Peter and Krammer, 2003]. The Fas/FasL pathway can be inhibited at different checkpoints by a variety of mechanisms. For example, at the receptor level, it can be blocked by receptor mutation or downregulation of its expression, activation of decoy receptors or by binding of soluble ligands [Suda et al., 1997]. During signal transduction, inhibitory molecules, including c-FLIP regulate the pathway activity [Tschopp et al., 1998]. c-FLIP, which structurally resembles caspase-8 but lacks proteolytic activity, is recruited to the DISC through the adaptor molecule FADD, and it prevents caspase-8 recruitment into the complex and thus its activation [Tschopp et al., 1998; Scaffidi et al., 1999]. In the effector phase,

Abbreviations used: SCCHN, squamous cell carcinoma of head and neck; FAP-1, Fas-associated phosphatase 1; cFLIP, cellular FLICE-inhibitory protein; siRNA, small interfering RNA; FADD, Fas-associated death domain; DISC, death signal-inducing complex; PTP, protein tyrosine phosphatase; SOV, sodium orthovanadate; CHX, cycloheximide.

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*Correspondence to: Theresa L. Whiteside, PhD, Hillman Cancer Center, Suite L 1.27, 5117 Centre Avenue, Pittsburgh, PA 15213. E-mail: whitesidetl@upmc.edu

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inhibitors of apoptotic proteins, IAPs play a key role in regulation of the Fas/FasL pathway by interfering with activation of the effector caspases-3 and -7 [Deveraux and Reed, 1999]. At the mitochondrial level, apoptosis can be inhibited by anti-apoptotic members of the Bcl-2 family, such Bcl-2 or Bcl-X_L, which prevent relocalization of cytochrome c from mitochondria into cytosol and the formation of apoptosome [Zou et al., 1999].

Induction of tumor cell death by triggering of death receptors is thought to be a prominent mechanism for tumor clearance in vivo, and a blockade of Fas-induced apoptosis has been implicated in tumorigenesis as well as resistance to therapy [French and Tschopp, 1999; Medema et al., 1999]. Squamous cell carcinoma of head and neck (SCCHN) is one of the most immunoresistant human tumors. Although SCCHN express Fas as well as FasL on the cell surface, they are often resistant to FasL-induced apoptosis [Ungefroren et al., 1998; Gastman et al., 1999], which may explain their capability to escape from immune surveillance. The molecular mechanisms of this escape process are still unknown. One of the possible mechanisms of Fas resistance of SCCHN might involve abnormalities in the Fas pathway signaling.

Fas-associated phosphatase 1 (FAP-1), is a 250-kDa protein tyrosine phosphatase (PTP) also known as PTP-BAS, PTPL1, PTP1E, and PTP-BL. It interacts with the intracellular domain of Fas [Maekawa et al., 1994; Saras et al., 1994; Sato et al., 1995] and is the only molecule known to associate with the negative regulatory domain of Fas represented by 15 C-terminal amino acids [Sato et al., 1995; Li et al., 1997; Saras et al., 1997; Yanagisawa et al., 1997]. By interacting with the cytoplasmic death domain of Fas receptors, FAP-1 acts as a negative switch in the Fas pathway. FAP-1 overexpression correlates with the resistance of some human malignant cells to Fas-mediated apoptosis [Arai et al., 1998; Lee et al., 1999b, 2001; Elnemr et al., 2001; Meinhold-Heerlein et al., 2001; Ungefroren et al., 2001].

In this study, the mechanisms responsible for FAP-1-mediated resistance of tumor cells to apoptosis were investigated using as a model the SCCHN sublines selected by exposure to CH-11 Ab. This model is relevant to events in vivo, where the tumor is constantly subjected to signals generated in the microenvironment and resulting in selection of immunoresistant tumor variants. Indeed, in the FAP-1^+ sublines elevated NFκB activity as well as Bcl-2 overexpression were consistent with better tumor survival. Silencing of FAP-1 expression or inhibition of phosphatase activity abolished tumor cell resistance to apoptosis. Thus FAP-1 activity is responsible for resistance to Fas-mediated apoptosis, which characterizes most of head and neck tumors.

MATERIALS AND METHODS

Cell Lines

Human SCCHN cell lines were established and maintained in our laboratory [Heo et al., 1989]. The lines PCI-13, PCI-15A, PCI-22A, PCI-37A, PCI-4A, and PCI-6A were established from primary tumors and PCI-15B, PCI-22B, PCI-37B, PCI-4B, and PCI-6B from lymph node metastasis in the same patients. The cell lines were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies Inc., Grand Island, NY). Jurkat cells were obtained from the ATCC (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS, antibiotics, and 25 mM Hepes buffer (Life Technologies).

Antibodies and Reagents

Cycloheximide (CHX), sodium orthovanadate (SOV), sulfasalazine, MG132, VP16, staurosporine, and anti-βactin mAb (clone AC-15) were purchased from Sigma (St. Louis, MO). Rabbit anti-caspases-3 and -7 Abs were from BD-PharMingen; rabbit anti-caspase-8 polyclonal Ab was from StressGen (Victoria, BC). Two different polyclonal rabbit anti-FAP-1 Abs were used, one purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the other provided by Dr. Taka-Aki Sato (23). The Ab to phosphorylated IκBα was from Cell Signaling (Beverly, MA), to cellular FLICE-inhibitory protein (cFLIP) from Alexis Biochemicals (San Diego, CA) and CH-11 Ab was from Upstate Biotechnology (Lake Placid, NY). IL-2 was purchased from PeproTech (Rocky Hill, NJ) and recombinant human TRAIL from R&D Systems (Minneapolis, MN).

Flow Cytometry

Fas (CD95) expression on the tumor cell surface was evaluated by flow cytometry after

detaching tumor cells with cell-dissociation solution (Sigma). Cells stained with FITC-labeled anti-CD95 mAb (30 min at 4°C) were fixed in 1% paraformaldehyde and analyzed using a four-color cytometer (EPICS XL, Beckman Coulter, Miami, FL). FITC-conjugated mouse IgG1 served as isotype control. Mean fluorescence intensity (MFI) was calculated using Expo 32v1.2 analysis software (Beckman Coulter). To evaluate Bax, Bcl-2, and Bcl-X_L expression, PCI-15A cells were permeabilized with 0.1% saponin in PBS for 30 min and then stained with FITC-conjugated mAb. To quantitate the expression level of each protein, molecular equivalents of soluble fluorochrome (MESF) units were determined, using a standard curve generated with four standard beads of known fluorescence intensity and unlabeled beads (Bangs Laboratories, Fishers, IN). MFI of each sample was transformed into MESF units, using the calibration curve calculated by the Quick Cal program (Bangs Laboratories).

Sensitivity of SCCHN to Fas-Mediated Apoptosis

Susceptibility of tumor cells to Fas-mediated apoptosis was measured in JAM assays [Matzinger, 1991]. Briefly, cells were incubated overnight with 5 μCi/ml methyl (^{3}H)-thymidine (NEN: 147.9 Gbq/mmol), washed and plated (25,000/well) in 96-well flat-bottom plates +/− CH-11Ab (200 ng/ml). Some cell lines were also pretreated with CHX (10 μg/ml), SOV (50–150 μM), IL-2 (100 U/ml), sulfasalazine (0.5 mM), or MG132 (20 μM). Cells were disrupted by three cycles of freezing (−20°C) and thawing (RT) and harvested onto fiberglass filters. The radioactivity was counted in an LKB Betaplate counter (Amersham Biosciences, Piscataway, NJ). The percentage of DNA fragmentation was determined by the formula $(C-E)/C \times 100$, where E (experimental) = cpm of cells incubated with an apoptosis inducer and C (control) = cpm of cells incubated with medium.

Selection of Fas-Resistant Tumor Cells

PCI-15A cell line was cultured in the presence of CH-11 Ab (200 ng/ml) for 7 days. Dead cells were removed and the remaining cells was again incubated with CH-11 Ab for 7 days, and dead cells were discarded. The cycle was repeated 8–10 times. Following this selection, surviving cells were treated with 500 ng/ml of CH-11 Ab or IgM isotype control, 40 μM VP16 or 0.5 μM staurosporin and tested for resistance to Fas-mediated apoptosis in JAM assays.

Western Blots

For Western blots cells were lysed in 0.5% Nonidet P-40, 10 mM HEPES, (pH 7.4), 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The proteins present in cell extracts were resolved by SDS–PAGE and transferred to polyvinylidene difluoride membranes as described [Wieckowski et al., 2002]. Following probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL).

siRNA for FAP-1

To silence the expression of FAP-1, small interfering RNA (siRNA) was used from the siGENOME SMARTpool reagent for PTPN13 (FAP-1) (Dharmacon, Lafayette, CO). Non-targeting siRNA pool was used as a negative control, and siRNA for lamin as a positive control for transfection efficiency. The manufacturer's protocol was followed. Silencing of FAP-1 expression was evaluated 48 and 72 h after transfection by Western blotting, using two different anti-FAP-1 Abs. Silencing of lamin expression was evaluated with anti-lamin A/C antibody from Santa Cruz.

NFκB Activation Assay

Translocation of NFκB to the nucleus was measured in parental and selected PCI-15A cells at a single-cell level. Cells (1×10^4/well) were plated in flat-bottom 96-well plates (Becton Dickinson) pretreated with carbonate buffer (pH 9.6) and adhered overnight. They were then stimulated with TNFα (10 ng/ml) for 15 min, fixed with 1% (w/v) paraformaldehyde in PBS for 20 min, washed and permeabilized with 0.1% Triton X-100 in PBS for 90 s. The cells were incubated with anti-NFκB (p65) mAb for 1 h, washed, re-treated with the detergent for 10 min, and incubated in the presence of goat anti-mouse IgG conjugated with Alexa 488 fluorochrome (Cellomics Inc., Pittsburgh, PA) and 1 μg/ml solution of Hoechst 33342 (Molecular Probes Inc., Eugene, OR) for 1 h. Following incubation in the buffered detergent for 15 min, the cells were washed and stored at 4°C for

analysis in an ArrayScan HCS imaging cytometer (Cellomics Inc.), equipped with emission and excitation filters (XF93, Omega Optical, Brattleboro, VT) for selectively imaging fluorescent signals emitted by Hoechst 33342, Alexa 488, and Alexa 666. Data were analyzed with ArrayScan II Data 8 Acquisition and Data Viewer version 3.0 (Cellomics Inc.), Quattro Pro version 10.0.0 (Corel Corporation, Ottawa, Ontario, Canada), and MS Excel 2002 (Microsoft Corporation, Redmond, WA).

ELISA for Soluble Fas (sFas)

Tumor cells (2×10^6 cells) were seeded in 100 mm^2 dishes (Corning Inc., Acton, MA) in 5 ml of a complete medium. After 24 h incubation, supernatants were collected, centrifuged once at 1,500 rpm and tested for the presence of sFas by ELISA (BD/Pharmingen). Supernatants of PBMC which were activated by PMA (2 ng/ml) and ionomycin (1 μM) and cultured for 24 h were used as positive controls for sFas. The sensitivity of the assay was 0.02 ng/ml.

Statistical Analysis

Results were analyzed using a paired Student's t test. Values of $P < 0.05$ were considered to be significant.

RESULTS

Susceptibility of Human SCCHN Cells to Fas-Induced Apoptosis

Human SCCHN cell lines ($n = 11$) were incubated with CH-11 Ab for 24 h. JAM assays were performed to measure the percentage of cells with fragmented DNA in the population of the plated cells. Out of five A and B pairs (A, derived from primary tumors and B, from metastatic lesions), primary cells in three pairs (PCI-4, PCI-15, and PCI-22) were more resistant to CH-11 Ab-induced apoptosis ($P < 0.05$) than metastatic cells. In two pairs (PCI-37 and PCI-6), metastatic cells were more resistant. The susceptibility to Fas-mediated apoptosis widely varied among SCCHN cell lines and did not reflect their origin from primary versus lymph node-invading tumors (Table I).

Expression of Fas on the Surface of SCCHN Cells

Fas (CD95) expression on the cell surface in nine human SCCHN cell lines was measured by flow cytometry. The cell lines expressed various levels of surface Fas (Table I). There was no correlation between the origin of the cell lines (primary tumor vs. tumor metastatic to lymph nodes), their resistance to CH-11 Ab-induced apoptosis and the level of Fas expression.

Detection of Soluble Fas in Supernatants of SCCHN Cells

A possibility was considered that Fas was released in a soluble form (sFas) into cell supernatants during culture. sFas was detected by ELISA in culture supernatants of all the human SCCHN cell lines tested. The Fas-sensitive cell lines produced more sFas than the Fas-resistant cell lines (85 ± 46 pg/ml vs. 41 ± 24 pg/ml). However, no correlation was

TABLE I. Characteristics of SCCHN Cell Lines

Cell line	% DNA fragmentation after CH-11 Ab treatment[a]	Surface Fas expression[b]	sFas (pg/ml)[c]	cFLIP expression[d]
PCI-13	18 ± 1.7	60	36	46
PCI-15A	13 ± 3.1	70	60	114
PCI-15B	39 ± 2.9	40	53	98
PCI-22A	2 ± 1.7	30	40	na
PCI-22B	22 ± 4.3	50	64	na
PCI-37A	13 ± 1.7	na	na	na
PCI-37B	5 ± 1.2	na	na	na
PCI-4A	5 ± 1.0	45	7	192
PCI-4B	10 ± 0.7	80	26	139
PCI-6A	35 ± 1.4	40	137	147
PCI-6B	11 ± 1.2	60	76	65

na, not analyzed.
[a]% DNA fragmentation in 24 h JAM test performed as described in Materials and Methods. Data are mean±SD.
[b]CD95 expression as MFI.
[c]sFas concentrations were determined by ELISA and expressed per 2×10^6 tumor cells incubated for 24 h.
[d]cFLIP expression was analyzed by Western blot as described in Materials and Methods. The intensity of bands was measured with Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and expressed in arbitrary units.

observed between the ability of the cell lines to produce sFas and their resistance to CH11 Ab-induced apoptosis (Table I). The ability to produce sFas did not correlate with the origin of the cell lines from primary or metastatic tumor sites.

Expression of cFLIP by SCCHN Cells

cFLIP is often overexpressed by tumor cells resistant to apoptosis. Therefore, we tested SCCHN cell lines for cFLIP expression. Western blot analyses showed that all of the tested cell lines expressed cFLIP (Table I) and there was no correlation between the origin of the cell lines (primary tumor vs. tumor metastatic to lymph nodes), their resistance to CH-11 Ab-induced apoptosis and the level of Fas expression.

Generation of a Fas-Resistant SCCHN Cell Line

As the next approach to a more extensive analysis of mechanism of resistance to apoptosis in SCCHN cells, we proceeded to generate a completely Fas-resistant cell line. The PCI-15A cell line, previously found to be relatively resistant to Fas-mediated apoptosis, was repeatedly exposed to low concentrations of CH-11 mAb (200 ng/ml) for 8–10 treatment cycles. Using this selection procedure, a completely resistant cell line was obtained (Fig. 1A). The selection process was independently repeated three times, consistently yielding sublines with similar levels of resistance. The selected PCI-15A cell lines remained sensitive to TRAIL-induced apoptosis at 100 ng/ml, indicating that only Fas/FasL pathway was affected (data not shown).

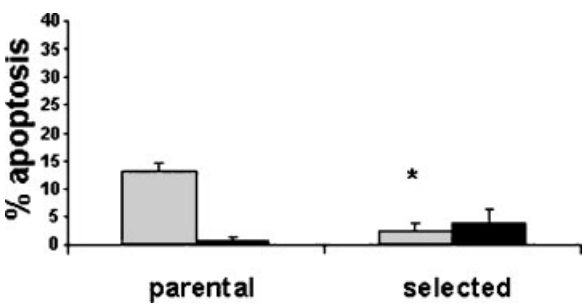

Fig. 1. CH-11 Ab selection of a cell line resistant to apoptosis. Susceptibility to CH-11 Ab-induced apoptosis in parental versus selected PCI-15A cells was measured in JAM assays, as described in Materials and Methods. The data are mean ± SD. Gray bars represent CH-11 Ab treatment; black bars, control IgM treatment. Five experiments were performed. The asterisk indicates significant ($P < 0.05$) difference in sensitivity to CH-11 Ab-induced apoptosis between the parental and selected cell lines.

Effects of Cycloheximide on Selected SCCHN Cell Line Resistance to Fas-Mediated Apoptosis

To determine whether treatment with a protein synthesis inhibitor, for example, CHX, renders resistant SCCHN cells sensitive to Fas-mediated apoptosis, PCI-15A selected sublines were incubated with CHX prior to incubation with CH-11 Ab. As shown in Figure 2A, the susceptibility to CH-11 Ab-mediated apoptosis was significantly increased ($P < 0.02$) after incubation with CHX. Incubation with CHX alone was not toxic to the cells.

The effect of CHX on caspase-8 activation in PCI-15A parental and selected cells was next evaluated by Western blotting. While caspase-8 cleavage was demonstrable in the parental PCI-15A cells after only 4 h of treatment with CH-11 Ab + CHX, caspase-8 prodomain was still visible in the selected cells after 18 h of treatment (Fig. 2B). Therefore, increased resistance of the selected cells to Fas-mediated apoptosis was, in part, explainable by lower levels of caspase-8 cleavage. The same results were obtained with caspases-3 and -7 (data not shown). Incubation with CH-11 Ab alone or CHX alone did not induce caspase-8 activation. These results indicated that CHX blocked synthesis of a protein inhibitor, which was upstream of caspase-8 and which mediated the resistance of SCCHN cell line to Fas-mediated apoptosis.

The expression of two inhibitors, FAP-1 and cFLIP, known to inhibit caspase-8 activation, were next evaluated in parental and selected PCI-15A cells. Western blot analysis (Fig. 2C) revealed that while the selected sublines expressed FAP-1, parental PCI-15A cells did not. The expression of cFLIP protein was unchanged in both cell lines. Since treatment with CHX significantly increased susceptibility of the selected Fas-resistant SCCHN cell line to CH-11 Ab-induced apoptosis, effects of CHX on the expression of FAP-1 and cFLIP proteins was investigated in time-course experiments followed by Western blots (Fig. 2D). FAP-1 expression in selected PCI-15A cells decreased after 6 h incubation with CHX. In contrast, cFLIP expression increased in the presence of CHX. This finding was reproducible, and it could be explained by the accumulation of cFLIP as a result of its decreased de-gradation in the presence of CHX. These results suggest that

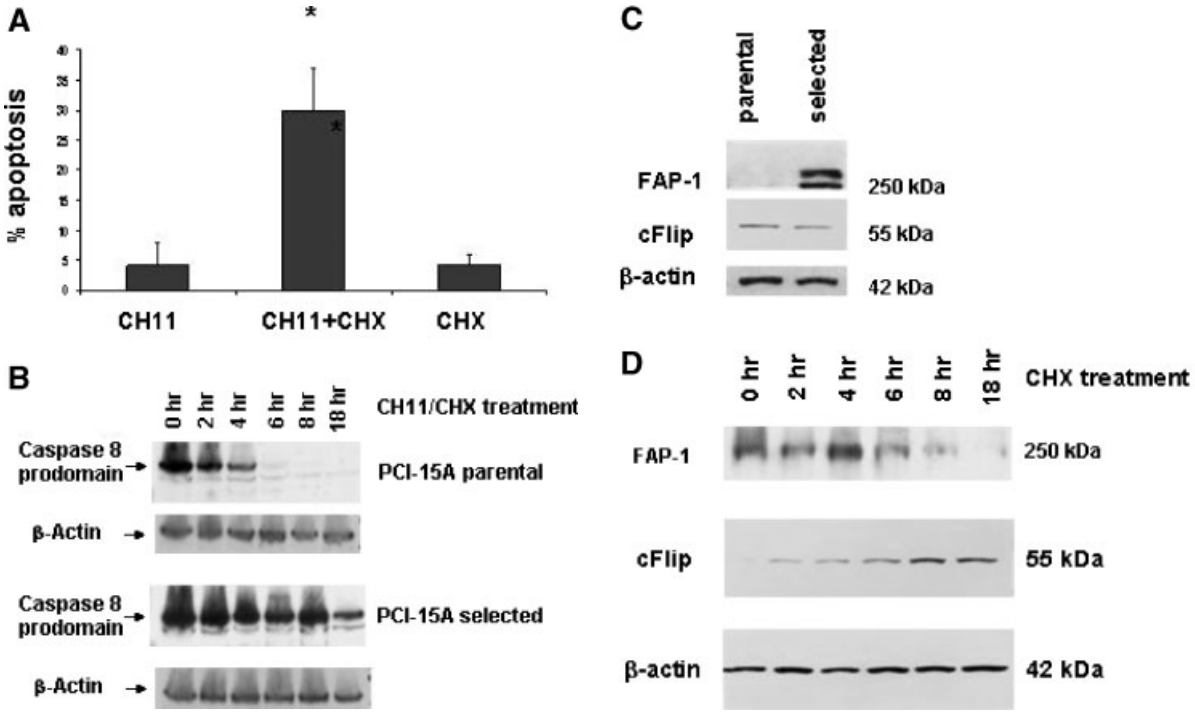

Fig. 2. Effect of cycloheximide (CHX) treatment on resistance of PCI-15A cells to Fas-mediated apoptosis. **A**: Treatment with CHX, as described in Materials and Methods, increased the susceptibility of PCI-15A selected cells to CH-11 Ab-induced apoptosis in JAM assays. The data are mean ± SD from triplicate wells. The asterisk indicates significant ($P < 0.05$) difference in sensitivities to CH-11 Ab versus CHX + CH-11 Ab-induced apoptosis. Results of a representative experiment of four performed are shown. **B**: The time course of CHX treatment of the parental and selected PCI-15A cell lines. Western blot analysis, as described in Materials and Methods, revealed that combined treatment with CHX and CH-11 Ab induces caspase-8 cleavage in both parental and CH-11 Ab-selected PCI-15A cells. However, caspase-8 cleavage in the selected cell line is substantially slower than that in the parental line. **C**: Expression of FAP-1 and cFLIP proteins in the parental and selected PCI-15A cells by Western blots. **D**: The time course of CHX effects on expression of FAP-1 and cFLIP in PCI-15A selected cells was measured in Western blots. β-actin was used as a loading control. A representative of three performed experiments is shown.

FAP-1 is more likely to be responsible for the resistance to Fas-mediated apoptosis in this cell line than is cFLIP.

Surface Fas Expression

The resistance of FAP-1 overexpressing cells to Fas-induced apoptosis could be explained by FAP-1 association with Fas in the cytoplasm, resulting in the inhibition of Fas export to the cell surface [Ivanov et al., 2003]. Indeed, as shown in Figure 3A, the parental Fas-sensitive, FAP-1 negative PCI-15A cells expressed three times more of Fas on their surface ($P < 0.01$) compared to selected Fas-resistant, FAP-1 positive PCI-15A cells.

FAP-1 Phosphatase Activity

FAP-1 phosphatase activity could account for the acquired resistance of the selected PCI-15A cells to Fas-mediated apoptosis. Therefore, the susceptibility of these cells to CH-11 Ab-induced apoptosis was measured after pre-incubation with phosphatase inhibitor, SOV. This treatment resulted in a dose dependent sensitization, and the selected PCI-15A cells became as sensitive to CH-11 Ab-induced apoptosis as the parental PCI-15A cells (Fig. 3B). The same treatment did not have any effect on the susceptibility to apoptosis of the parental PCI-15A cells, which do not express FAP-1.

Inhibition of FAP-1 Expression by siRNA

To provide a proof-of-principle that expression of FAP-1 by the selected PCI-15A cells is responsible for their resistance to CH11 Ab-induced apoptosis, we used siRNA to silence expression of this protein. FAP-1 protein expression was eliminated in the selected PCI-15A cells 48 and 72 h after transient siRNA transfection (Fig. 4A), and these cells became sensitive to CH-11 Ab-induced apoptosis to the same level as the parental PCI-15A cells

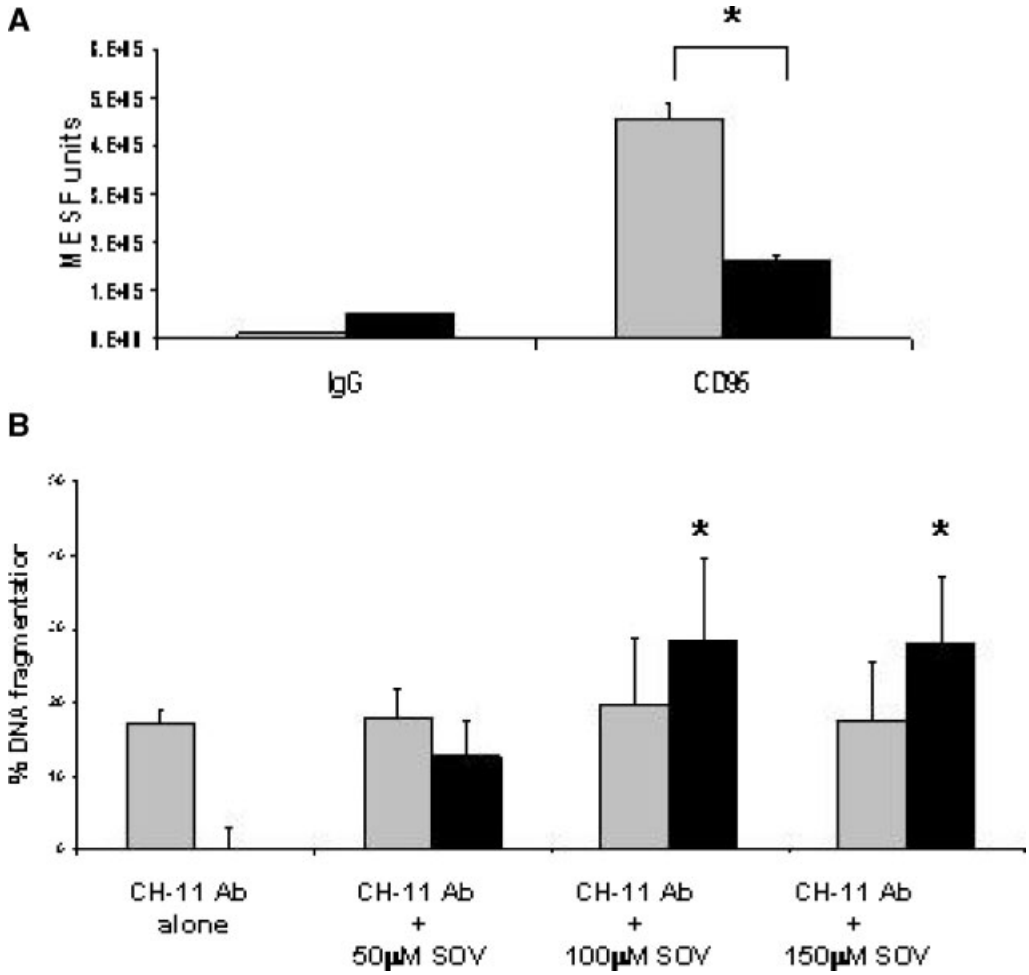

Fig. 3. **A**: Flow cytometry analysis for Fas expression by PCI-15A cell lines. The selected PCI-15A cells (black bars) express significantly less CD95 on their surface compared to parental (gray bars) PCI-15A cells. The asterisk indicates $P = 0.01$. **B**: Inhibition of phosphatase activity by sodium orthovanadate (SOV) sensitizes the selected PCI-15A cells (black bars) to CH-11 Ab-induced apoptosis but does not change the sensitivity of the parental PCI-15A cells (gray bars) as measured in JAM assays. A representative experiment of three performed is shown. The data are mean ± SD. Asterisks indicate significant differences ($P < 0.01$) between sensitivity to apoptosis of untreated and SOV-treated cells.

(Fig. 4B). As a control, the selected PCI-15A cells were transfected with non-coding oligonucleotide sequences, and these cells still expressed FAP-1 protein after transfection and remained resistant to apoptosis (Fig. 4A,B). We also investigated the effect of FAP-1 siRNA on surface expression of Fas. As shown in Figure 4C, the surface Fas expression in selected PCI-15A cells increased significantly after FAP-1 siRNA. At the same time, Fas surface expression remained unchanged in selected PCI-15A cells subjected to siRNA with non-coding oligonucleotides. This finding further supports the hypothesis that resistance of the selected PCI-15A cells to CH-11 Ab-induced apoptosis was caused by FAP-1 overexpression and inhibition of Fas surface expression.

NFκB Activation in Fas-Resistant PCI-15A Cells

Interaction of FAP-1 with the NFκB inhibitor, IκBα, might determine its biologic activity. Initially, IκBα is dephosphorylated at Tyr42 by FAP-1. This facilitates its subsequent phosphorylation at serines 32 and 36, and the release of the p65 subunit, which then translocates to the nucleus [Nakai et al., 2000]. Therefore, we examined levels of IκBα phosphorylation in the selected PCI-15A cells. These cells showed much higher phosphorylation levels of IκBα than parental PCI-15A cells (Fig. 5A). When NFκB activity was measured in the parental

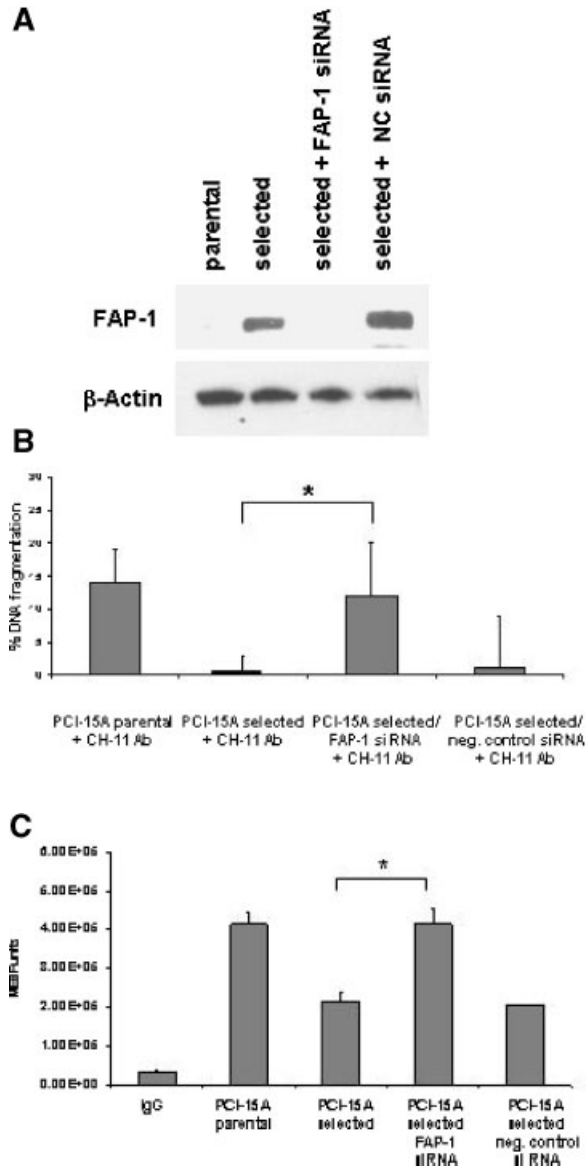

Fig. 4. Blocking of FAP-1 expression by siRNA sensitizes the selected PCI-15A cells to CH-11 Ab-induced apoptosis. **A**: Expression of FAP-1 was blocked by siRNA, as described in Materials and Methods. FAP-1 expression was measured in Western blots 48 h after transfection of the selected PCI-15A cells. A representative of five experiments performed is shown. **B**: Selected PCI-15A cells after FAP-1 siRNA treatment are as sensitive to CH-11 Ab-induced apoptosis as parental PCI-15A cells. Apoptosis was measured in JAM assays as described in Materials and Methods. A representative experiment of three performed is shown. The data are mean ± SD. The asterisk indicates a significant ($P < 0.01$) difference between sensitivity to CH-11 Ab-induced apoptosis of selected PCI-15A cells before and after siRNA treatment. **C**: Surface Fas (CD95) expression was restored in selected PCI-15A cells after FAP-1 siRNA as measured by flow cytometry. The asterisk indicates significant ($P = 0.01$) difference in Fas expression in parental PCI-15A cells before and 48 h after transfection.

and selected PCI-15A cells after a brief stimulation with TNFα, p65 translocation to the nucleus was increased to a significantly higher level in the selected (Fas-resistant) PCI-15A cells compared to the parental cells (Fig. 5B). Furthermore, this translocation, once induced, could not be inhibited with SN50, a cell-permeable NFκB peptide inhibitor. The basal NFκB

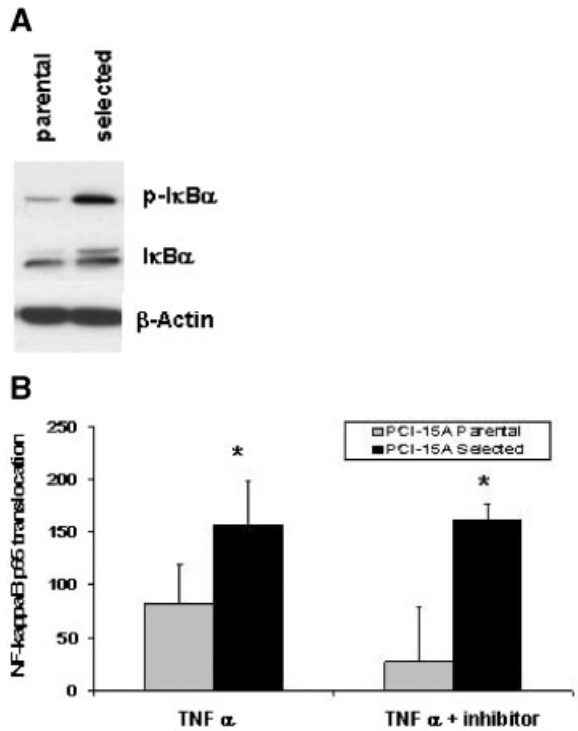

Fig. 5. The selected PCI-15A cells overexpressing FAP-1 exhibit higher NFκB activation than parental PCI-15A cells. **A**: Phosphorylation of IκBα in the selected PCI-15A cells was measured in Western blots as described in Materials and Methods. A representative Western blot of three performed is shown. **B**: Translocation of NFκB p65 to the nucleus in the parental (gray bars) and selected (black bars) PCI-15A cells. p65 translocation was measured after 15 min activation with TNFα as described in Materials and Methods. A representative experiment of three performed is shown. Asterisks indicate significant ($P < 0.05$) differences between p65 translocation in the parental versus selected PCI-15A cells. **C**: Intracellular expression of Bax, Bcl-2, and Bcl-X_L in the parental (gray bars) and selected (black bars) PCI-15A cells was measured by flow cytometry as described in Materials and Methods. Results are in MESF units ± SD. A representative experiment of three performed is shown. The asterisk indicates a significant ($P < 0.05$) difference in Bcl-2 expression between parental and selected PCI-15A cells. **D**: NFκB inhibitors sulfasalazine and MG 132 sensitize the parental (gray bars) and selected (black bars) PCI-15A cells to CH-11 Ab-induced apoptosis in JAM assays. A representative experiment of three performed is shown. Asterisks indicate significant ($P < 0.01$) differences in the sensitivity to CH-11 Ab-induced apoptosis between inhibitor treated versus non-treated PCI-15A cells.

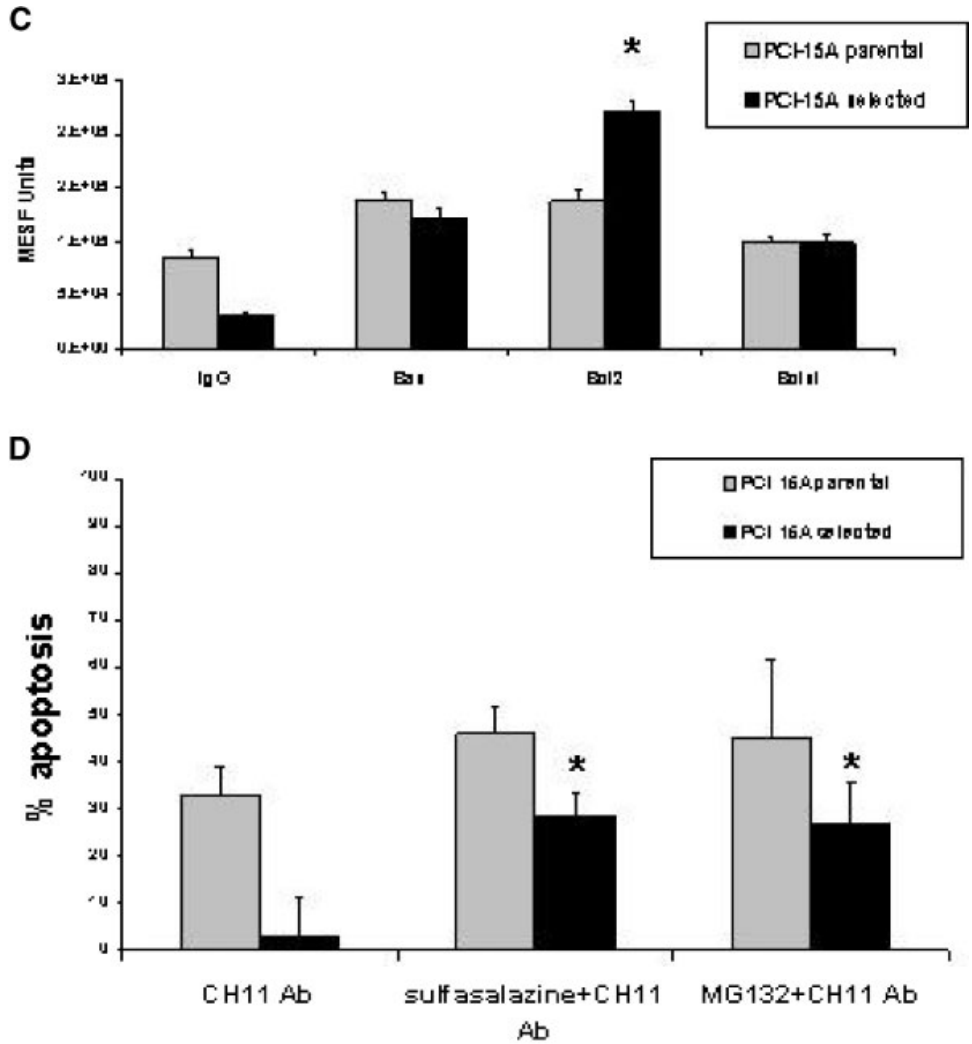

Fig. 5. (*Continued*)

activity in parental and selected PCI-15A cell was the same (data not shown).

Because NFκB activation leads to transcriptional upregulation of anti-apoptotic proteins, the parental and selected PCI-15A cells were tested for Bax, Bcl-2, and Bcl-X_L expression. Levels of Bcl-2 were significantly higher in the selected than parental cells, while Bcl-X_L and pro-apoptotic Bax expression was comparable (Fig. 5C). When the parental and selected PCI-15A cells were treated with sulfasalazine, an NFκB inhibitor, or with a proteasome inhibitor, MG132, NFκB activation was inhibited (data not shown). As expected, these treatments sensitized the parental and selected cells to CH-11 Ab-induced apoptosis (Fig. 5D), although the selected PCI-15A cells consistently remained less sensitive. Neither sulfasalazine nor MG132 treatment caused changes in the FAP-1 expression in these cells (data not shown). These results indicate that FAP-1 overexpression and its activity in the selected PCI-15A cells renders them resistant to Fas-mediated apoptosis, and that this resistance is mediated by activation of the NFκB pathway and upregulation of Bcl-2 synthesis.

DISCUSSION

The resistance of SCCHN to FasL-induced apoptosis may explain their capability to escape from immune surveillance. However, the molecular mechanisms of this escape process are still unknown. It has been suggested that Fas resistance of tumor cells is associated with defects in Fas signaling. Various inhibitors of

Fas-mediated apoptosis that have been described to date can be classified into soluble factors, inhibitors of DISC formation, inhibitors of effector caspases, and Bcl-2 family proteins [Cheng et al., 1994; Ungefroren et al., 1998; French and Tschopp, 1999; Gastman et al., 1999; Medema et al., 1999]. In this study, we focused on the inhibitors of DISC formation, using an experimental model of the paired SCCHN cell lines established from primary tumors and from metastatic lymph nodes, and SCCHN sublines selected to be Fas-resistant by prolonged treatment with CH-11 Ab. This model probably recapitulates in vivo selection taking place in the tumor subjected to signals generated in the microenvironment and targeting Fas on the cell surface.

Tumor cells, including SCCHN, secrete sFas. sFas is one of the alternatively spliced variants of Fas which does not have the transmembrane domain and thus is readily secreted [Cheng et al., 1994]. Serum levels of sFas are reported to be higher in cancer patients, including those with SCCHN, than in healthy controls [Knipping et al., 1995; Alecu et al., 2002; Furuya et al., 2003; Pignataro et al., 2003]. Although sFas was detected in culture supernatants of SCCHN cells, its levels did not correlate with the susceptibility of these cells to Fas-induced apoptosis. However, it is possible that the level of sFas produced by SCCHN cell lines was not sufficient to inhibit Fas-mediated apoptosis.

cFLIP inhibits DISC formation and inactivates caspase-8 [Tschopp et al., 1998]. The long form of cFLIP ($cFLIP_L$) contains two death effector domains (DEDs) and a caspase-8-like domain. In contrast, the short form of cFLIP ($cFLIP_S$) comprises only two DEDs. Both forms of cFLIP interfere with caspase-8 recruitment to DISC, and $cFLIP_L$ has more potent inhibitory activity [Tschopp et al., 1998]. cFLIP also inhibits apoptotic signals mediated by TNF-R1 and TRAIL-R [Griffith et al., 1998]. While many tumor cell lines express cFLIP, relatively high concentrations of cFLIP are needed to block apoptosis [Scaffidi et al., 1999]. All tested human SCCHN cell lines expressed cFLIP. However, no correlation could be established between the level of cFLIP expression and resistance of tumor cells to Fas-mediated apoptosis. Also, in the selected Fas-resistant PCI-15A sublines, cFLIP expression was comparable to that in the parental Fas-sensitive cells. Thus, this inhibitory molecule is probably not involved in downregulating Fas signaling in SCCHN.

FAP-1, a PTP [Sato et al., 1995], is known to bind to the carboxyl terminal 15-amino acid domain of Fas, called a negative regulatory domain, through its third or fifth PDZ domain [Yanagisawa et al., 1997]. This binding inhibits Fas-mediated signal transduction. Moreover, an association of FAP-1 with Fas in the cytoplasm attenuates export of Fas to the cell surface, rendering these cells resistant to Fas-mediated apoptosis [Ivanov et al., 2003]. Elevated levels of FAP-1 expression have been reported in colon, pancreatic [Ungefroren et al., 1998, 2001], hepatocellular [Lee et al., 1999a], hematological [Mundle et al., 1999], and ovarian cancers [Meinhold-Heerlein et al., 2001], and its expression was found to be highest in tissues and cell lines that were relatively resistant to Fas-mediated death. However, the only direct proof that FAP-1 is responsible for tumor cells resistance to Fas-mediated apoptosis comes from an experimental model, where the stable transfection of the Fas-sensitive FAP-1 negative pancreatic cancer cell line Capan-1 with a FAP-1 cDNA-induced strong resistance to Fas-induced apoptosis [Ungefroren et al., 2001]. Selection with CH-11 Ab of stably resistant PCI-15A cell lines provided us with another model for investigating the role of FAP-1 in the acquired resistance to Fas-mediated apoptosis. In the selected PCI-15A cells, FAP-1 protein was overexpressed, in contrast to its absence in the Fas-sensitive parental cells. While, the $FAP\text{-}1^+$ selected cell lines were resistant to CH-11 Ab, they remained sensitive to TRAIL, VP-16, or staurosporine treatments, an indication of selective resistance to Fas-mediated apoptosis. Inhibition of FAP-1 expression by siRNA eliminated the resistance of the selected PCI-15A cells to apoptosis. Blocking phosphatase activity by treatment with SOV also decreased their resistance to Fas-mediated apoptosis. Both treatments sensitized selected PCI-15A cells to CH-11 Ab-induced apoptosis, but had no effect on apoptosis sensitivity of the parental PCI-15A cells, which did not express FAP-1.

The molecular mechanism responsible for FAP-1-mediated resistance of tumor cells to Fas-mediated apoptosis could be regulated at the level of FAP-1 and Fas expression by the parental and selected PCI-15A cell lines. Upregulation of FAP-1 was previously reported to

cause downregulation of the surface Fas expression and thus resistance of the cells to Fas-mediated apoptosis [Ivanov et al., 2003]. Interestingly, the selected Fas-resistant PCI-15A cells expressed three times less of surface Fas compared to the Fas-sensitive parental PCI-15A cells. Incubation with IL-2 (100 IU/ml) for up to 48 h did not sensitize SCCHN cells to CH-11 Ab-induced apoptosis nor did it change expression levels of FAP-1 or Fas in these cells (data not shown), as also reported by Song for colon cancer cells [Song et al., 2000]. On the other hand, pre-incubation with sulfasalazine or MG132 [Fulda et al., 2000; Hermisson and Weller, 2003; Muerkoster et al., 2003], which are NFκB inhibitors, increased sensitivity of the selected PCI-15A cells to CH-11 Ab-induced apoptosis. Sulfasalazine is an anti-inflammatory drug, commonly used for therapy of rheumatoid arthritis and ulcerative colitis, and MG132 is a proteasome inhibitor. Their target of action is IκBα, an inhibitor of NFκB [Karin, 1999], and the blockade of NFκB activity by these drugs is generally believed to downregulate a set of anti-apoptotic genes [Ghosh et al., 1998]. Interestingly, IκBα is also a substrate for FAP-1, which dephosphorylates IκBα at Tyr 42, and renders IκBα more susceptible to subsequent phosphorylation on serines 32 and 36 and its ubiquitination [Nakai et al., 2000], which in turn, allows for NFκB the p65 subunit translocation to the nucleus. Therefore, it appears that FAP-1 overexpression and its anti-apoptotic effects observed in the selected Fas-resistant PCI-15A sublines were related to activation of the NFκB pathway. To confirm this hypothesis, phosphorylation of IκBα and NFκB activity were measured in FAP-1^{+} selected and in FAP-negative parental PCI-15A cells. Indeed, IκBα phosphorylation in the selected PCI-15A cells was accompanied by NFκB activation and higher expression of Bcl-2. Therefore, FAP-1 overexpression and its anti-apoptotic activity are a part of the mechanism involving NFκB activation in SCCHN cells. Ivanov et al. [2005] postulate a casual relationship between high basal NF-κB activity seen in normal fibroblasts and melanoma cell lines and NF-κB-dependent transcriptional regulation of *FAP-1* gene expression. Our evidence suggests that FAP-1 activates NF-κB through the interaction with IκBα. In addition, downregulation of Fas expression on the tumor cell surface that accompanies FAP-1 overexpression may play a protective role. It is possible that more that one molecular mechanism regulates FAP-1-mediated protection from apoptosis. Modeling of FAP-1 related molecular events in SCCHN cell lines has uncovered an evasion mechanism, which may be relevant to escape of these tumors from immune surveillance and their tendency to reoccur.

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