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REGULATION OF CD95 (APO-1/ FAS) LIGAND AND RECEPTOR EXPRESSION IN SQUAMOUS-CELL CARCINOMA BY INTERFERON-γ AND CISPLATIN

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CD95 (Apo-1/Fas) ligand (CD95L) expression has been observed in various malignancies. In human primary cell lines from a squamous cell carcinoma (SCC) of the vulva, the effect of cisplatin (CDDP) and IFNγ on the expression of CD95L and its 2 receptor isoforms, CD95 transmembrane (CD95tm) and CD95 soluble receptor, was studied at the mRNA and protein levels. Addition of CDDP and IFNγ increased CD95L mRNA levels in the primary cell line 6-fold and 1.7-fold, respectively. In comparison, CD95tm mRNA levels were diminished by CDDP but increased 8-fold upon IFNγ challenge. CD95L expressed by SCC cells was functionally relevant since these cells were able to induce CD95-specific apoptosis in autologous lymphocytes from the SCC-bearing patient. Thus, CD95L expression in SCC may contribute to tumor-associated immunosuppression, which may be modulated by CDDP and IFNγ. In tumor samples of the primary SCC, CD95L expression was enhanced in the area of the border between invasive tumor tissue and surrounding stroma cells. The locally restricted over-expression of CD95L was congruent with the arrangement of apoptotic stroma cells in the direct vicinity of invading tumor tongues, suggesting a role as invasion factor for CD95L. *Int. J. Cancer* 80:564–572, 1999.
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The CD95 system, consisting of a membrane-bound (CD95tm) isoform, a soluble (CD95sol) receptor isoform generated by alternative splicing and their natural ligand (CD95L), holds a key position in the regulation of the immune response. CD95tm transduces the apoptotic signal after CD95L binding (Trauth *et al.,* 1989). In contrast, CD95sol prevents target cells from undergoing apoptosis by neutralizing CD95L (Hughes and Crispe, 1995). CD95L is expressed on the surface of effector cells but may be cleaved off by specific matrix metalloproteinases. When processed into a soluble form (CD95Lsol), it may cause systemic tissue damage (for review: Hug, 1997). Derangement of this system has been implicated in autoimmune disease (Nakajima *et al.,* 1997; Müschen *et al.,* 1998b) and neoplastic development (Friesen *et al.,* 1996; Hahne *et al.,* 1996). In some malignancies, CD95L contributes to tumor-associated immune privilege, thus protecting tumor cells against attack from tumor-infiltrating lymphocytes (TILs) (for review: Hug, 1997).

Keratinocytes express CD95 constitutively (Wrone-Smith *et al.,* 1995) but not CD95L (Gutierrez-Steil *et al.,* 1998). CD95L expression is restricted to histiocytes in the epidermis (Nakajima *et al.,* 1997), thus contributing to several inflammatory diseases of the skin. Derangement of the CD95 system is critical for tissue damage of UV-radiated skin and for dermic injury originating from cutaneous lupus erythematodes (Nakajima *et al.,* 1997). In contrast to malignant melanoma and basal cell epithelioma, CD95 receptor expression was present in squamous cell carcinoma (SCC) as well as in normal skin (Muraki *et al.,* 1997). High levels of CD95L expression were typically observed during tumor progression and immune evasion of malignant melanoma (Hahne *et al.,* 1996). However, little is known about the role of CD95 receptor and its ligand in SCC.

The findings that chemotherapeutic agents up-regulated CD95L expression in malignant cells and that TILs were killed by CD95L-mediated apoptosis (for review: Hug, 1997) raised the hypothesis that chemotherapy might be a double-edged sword.

Cisplatin (CDDP) is widely used in the treatment of SCC (Welters *et al.,* 1997). However, some malignant cells acquire secondary resistance toward CD95-mediated apoptosis after treatment with cytostatic drugs (Friesen *et al.,* 1996).

In contrast, IFNγ has been shown to sensitize CD95 receptor-bearing cells toward CD95L-mediated apoptosis. Moreover, CD95 receptor-lacking tumor cells lost their resistance toward CD95-mediated apoptosis after treatment with IFNγ (Leithäuser *et al.,* 1993) In basal cell carcinoma, intralesional therapy with IFNα led to tumor regression via CD95-mediated apoptosis (Buechner *et al.,* 1997).

The present study addresses the role of the CD95 system in SCC of the vulva. Vulvar carcinoma is the 4th most common malignancy of the female genital tract and accounts for approximately 4% of all gynecological malignancies. Occurrence of SCC has been associated with granulomatous vulvar diseases, chronic inflammatory disorders of the vulva and different human papillomavirus (HPV) types that have been ascribed an etiological role in squamous cell carcinogenesis (Crum *et al.,* 1997).

MATERIAL AND METHODS

Reagents and antibodies

Oligonucleotides were synthesized by Birsner and Grob (Freiburg, Germany). Anti-vimentin antibody was purchased from Dako (Hamburg, Germany). Rabbit polyclonal antibodies raised against CD3, CD95L and receptor of human origin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytokeratin 7 and anti-cytokeratin 18 antibody, anti-rabbit peroxidase IgG and all other chemicals were from Sigma (Deisenhofen, Germany). Human recombinant CD95L protein was purchased from Alexis (San Diego, USA) and inhibitor of Interleukin-1β converting enzyme (ICE) Ac-YVAD-cmk from Bachem (Basel, Switzerland). BB-3103, an inhibitor of a variety of matrix metalloproteinases, was a kind gift from British Biotech (Oxford, UK).

Cell culture procedures

SCC cell lines were derived from operative tissue of a 73-year-old woman who had a third recurrence of a moderately differentiated invasive SCC of the vulva. At the age of 56 years, the primary treatment for the pT_1,N_0,M_0-staged SCC was surgery. The first recurrence was resected 16 years thereafter, followed by percutane-

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ous radiation of 55 Gy. One year later, a second recurrence arose in the form of a wide, peri-anal ulceration and was operated by resection in sano and creation of a sigma anus-praeter. After 4 months, a third recurrence appeared, presenting in part ulcerated tumors at the transition of the vulva to the vagina. Pre-operative staging did not show any distant metastasis, and a complete radical resection was performed. Tumor samples from this recurrence were used for mRNA isolation, establishment of the SCC cell lines and immuno-histochemistry. Within the next 4 months, a 4th recurrence with pulmonary and cutaneous metastases occurred. The patient is now in a palliative situation.

SCC cells were isolated from tissue fragments as described in Tobias *et al*. (1995). The medium was composed of RPMI 1640 (Seromed, Berlin, Germany), 10% basal medium supplement (Biochrom, Berlin, Germany), non-essential amino acids of MEM (Boehringer-Mannheim, Mannheim, Germany), 10 mmol/l L-glutamine, 5 mmol/l sodium pyruvate, 50 IU/ml penicillin, 50 µg/ml streptomycin, 10 nmol/l estradiol (all from ICN, Eschwege, Germany), 1.8 µg/ml hydrocortisone (Merck, Darmstadt, Germany), 25 µg/ml apo-transferrin (Sigma) and 0.8 IU/ml insulin (Hoechst, Frankfurt, Germany). SCC cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and split for the first time after 28 days.

The VC_1 clone was obained by limiting dilution of VC_0 cells into a 96-well dish. Cells were seeded into the 96-well plate and successively diluted up to a statistical value of 0.0625 cells/100 µl in each well. For single cells, cell culture was continued until a confluent cell layer had been formed; 5,000 cells were seeded into a 96-well dish in 200 µl of medium. Half of the medium was changed daily. During the first 2 weeks, cells were counted by a fluorescence-activated cell sorter (FACS) from Ortho (Neckargemünd, Germany) 7 times. Based on these values, doubling time was determined.

Both cell lines, VC_0 and VC_1, were screened for HPV in a luminometer (Murex, Burgwedel, Germany) using a hybrid capture test kit from Murex. The different HPV DNA species were detected using specific RNA probes. The test discriminates between ''high risk'', ''low risk'' and absence of HPV infection.

Quantitative competitive RT-PCR

Total RNA from cultured tumor cells and the primary tumor was isolated and reverse-transcribed using a total RNA extraction kit (Qiagen, Hilden, Germany) and a first-strand cDNA synthesis kit (Boehringer-Mannheim), respectively. The mRNA levels of CD95L, CD95 isoforms, CD3δ chain and hypoxanthine guanine phosphoribosyltransferase (HPRT), which was used for standardization, were determined by quantitative competitive RT-PCR (Pannetier *et al.*, 1993). As shown in Figure 1, the cDNA to be assayed was co-amplified with known amounts of an internal DNA standard (Δ4), which was apart from a deletion of 4 nucleotides identical to the corresponding fragment of the assayed cDNA. For construction of CD95L, CD95tm, CD95sol, CD3δ chain and HPRT DNA standards, the respective 5′ and 3′ PCR primers (see below) were used to amplify a specific fragment in a human peripheral blood lymphocyte (PBL)–derived cDNA. A 1,000-fold dilution of this product was re-amplified using the respective 3′ PCR primer (see below) and an additional construct primer containing a 4-nucleotide deletion compared with the wild-type (WT) sequence. For quantification of transcripts for CD95L, CD95tm, CD95sol, CD3δ chain and HPRT, respectively, a constant amount of cDNA, corresponding to 50 ng reverse-transcribed total RNA, was mixed with 10^8, 10^7, ...10^3 or 0 copies of the respective standard (Δ4) and then amplified to saturation (40 cycles at 94°C for 20 sec, 58°C for 45 sec and 72°C for 45 sec). The primers used for PCR were 5′-GGCCACCCCAGTCCACC and 5′-CCGAAAAACGTCTGAGATTCC for CD95L, 5′-GGACATGGCTTAGAAGTGG and 5′-GGTTGGAGATTCATGAGAACC for both CD95 receptor isoforms, 5′-CCAGGCTGATAGTTCGGTGACC and 5′-TGTCTGAGAGCAGTGTTCCCAC for CD3δ chain and 5′-CCTGCTGGATTACATCAAAGCACTG and 5′-ACCAGCAAGCTTGCGACC for HPRT.

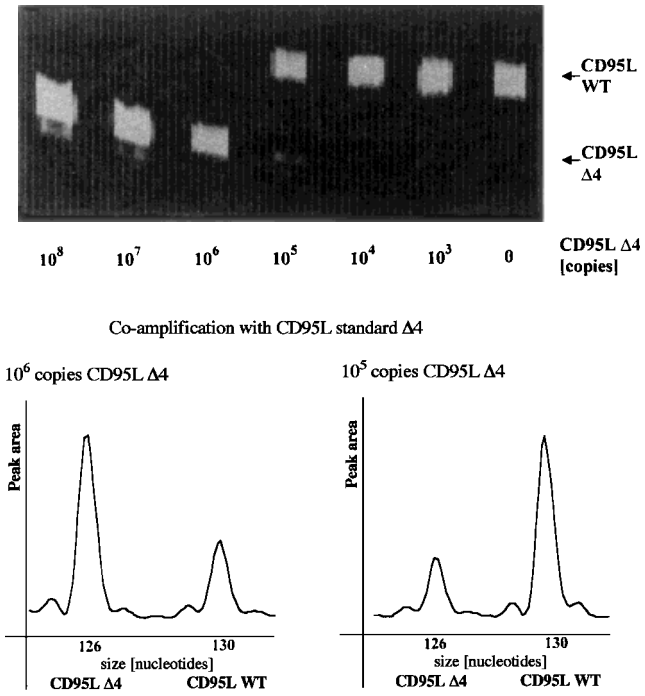

FIGURE 1 – Quantification of mRNA levels by quantitative competitive RT-PCR. The cDNA to be assayed (WT) was co-amplified with known amounts of an internal DNA standard (Δ4), which was apart from a deletion of 4 nucleotides identical to the assayed cDNA. For quantification of CD95L transcripts, a constant amount of cDNA, corresponding to 50 ng reverse-transcribed total RNA, was mixed with 10^8, 10^7...10^3 or 0 copies of the CD95L standard (CD95L Δ4) and then amplified to saturation. The read-out of the amplification involved one additional fluorescent dye-labeled oligonucleotide, which allows discrimination between CD95L wild-type (CD95L WT) and standard (CD95L Δ4) DNA species. PCR amplification products were specifically labeled in run-off reactions. Run-off reactions were loaded on an acrylamide gel and analyzed by an automated sequencer. The fluorescent profiles (see top) were recorded and the profile areas (bottom) analyzed. For co-amplifications with 10^6 and 10^5 copies of the CD95L standard, respectively, the peak area ratios for CD95L WT and CD95L Δ4 were calculated. The number of CD95L WT copies in the cDNA sample was calulated as the mean of WT/Δ4 peak area ratios at 2 standard dilutions [*e.g.*, for the sample shown here: $(0.343 \times 10^6 + 3.461 \times 10^5)/2$, *i.e.*, 344,550 copies].

The read-out of the amplification involved one additional fluorescent dye-labeled oligonucleotide, which allows discrimination between WT and standard (Δ4) DNA species. PCR-amplification products were specifically labeled in run-off reactions, loaded on an acrylamide gel and analyzed by an automated sequencer (ABI 373A; Applied Biosystems, Foster City, CA). The fluorescent dye-labeled (FAM) oligonucleotides used in run-off reactions were 5′-CATTGATCACAAGGCCACCC for CD95L, 5′-TCACCAGCAACACCAAGTGCAA for both CD95 isoforms, 5′-TCTATAGGTATCTTGAAGGGGCTC for CD3δ chain and 5′-CCCCTGTTGACTGGTCATTACAATAG for HPRT. Both CD95 splice variants were simultaneously detected. In comparison with CD95tm, the mRNA encoding for the soluble CD95 isoform (CD95sol) lacking the transmembrane domain was shorter by 62 bases. The fluorescent profiles (Fig. 1, top) were recorded and the profile areas (Fig. 1, bottom) analyzed using the software Immunoscope (Pannetier *et al.*, 1993).

Immuno-cytochemical procedures

VC_0 and VC_1 were seeded on a multitest slide containing 10 wells (Dunn, Asbach, Germany) at 1,000 cells per well. They were incubated without or with IFNγ (100 U/ml) or with CDDP (0.2

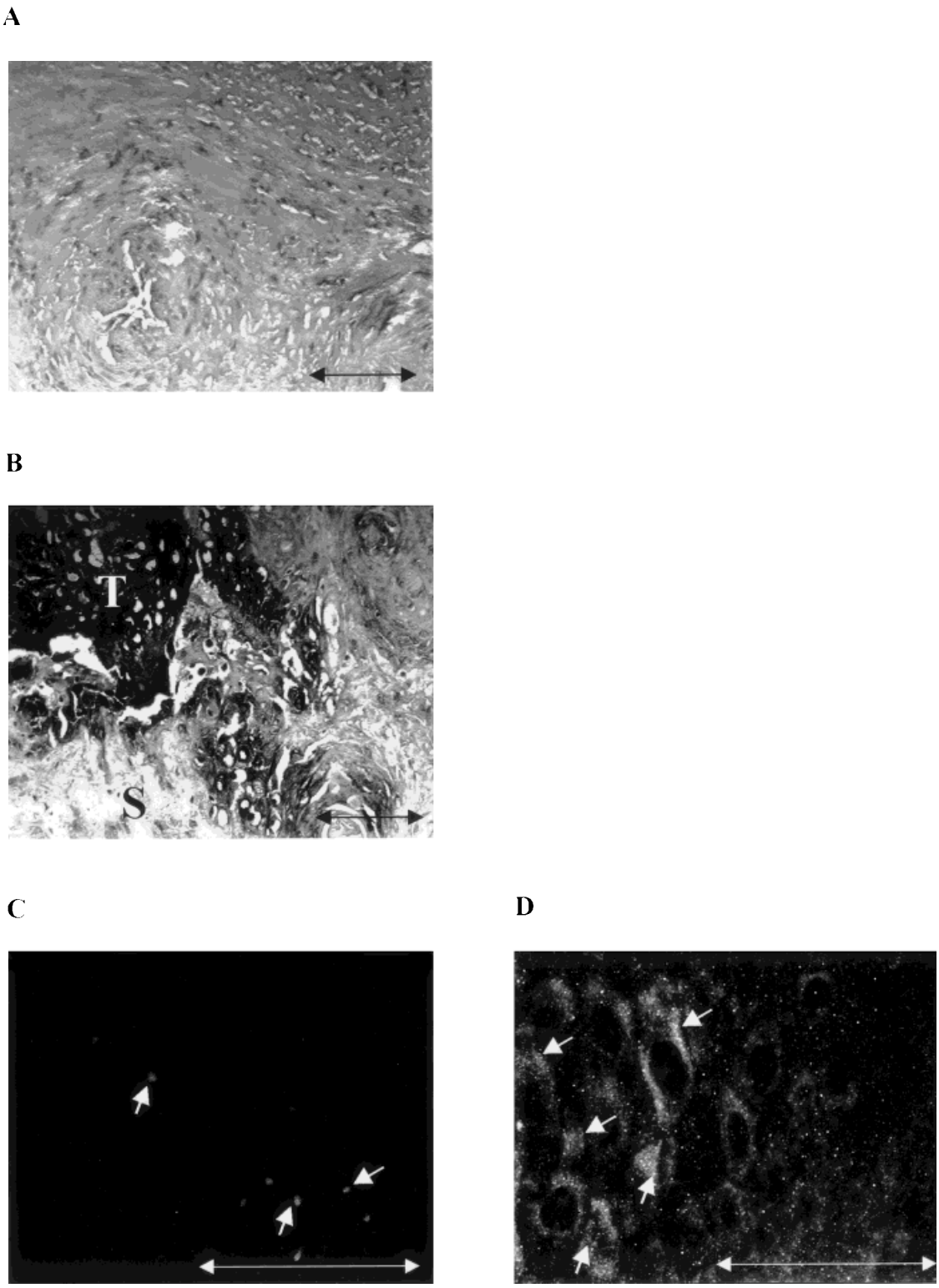

FIGURE 2 – Immuno-histochemical analysis of CD95L and CD3 expression in SCC tissue. Immuno-histochemical procedures were carried out as described in ''Material and Methods''. Cells staining positive for CD95L (dark-colored cells in *a* and *b*, fluorescence in *d*) were detected by light and fluorescence microscopy, respectively. Cells were counterstained by eosin. In healthy vulvar tissue *(a)*, no obvious staining for CD95L was detectable. *(b)* Staining for CD95L expression in vulvar tissue infiltrated by SCC. In *(c)* and *(d)*, double fluorescence staining for CD3 and CD95L was performed on the same SCC tissue section. In *(c)*, some FITC-labeled $CD3^+$ TILs are visible (see arrows); in *(d)*, CD95L-expressing cells are CY3-labeled (see arrows). Scale bar: 100 μm.

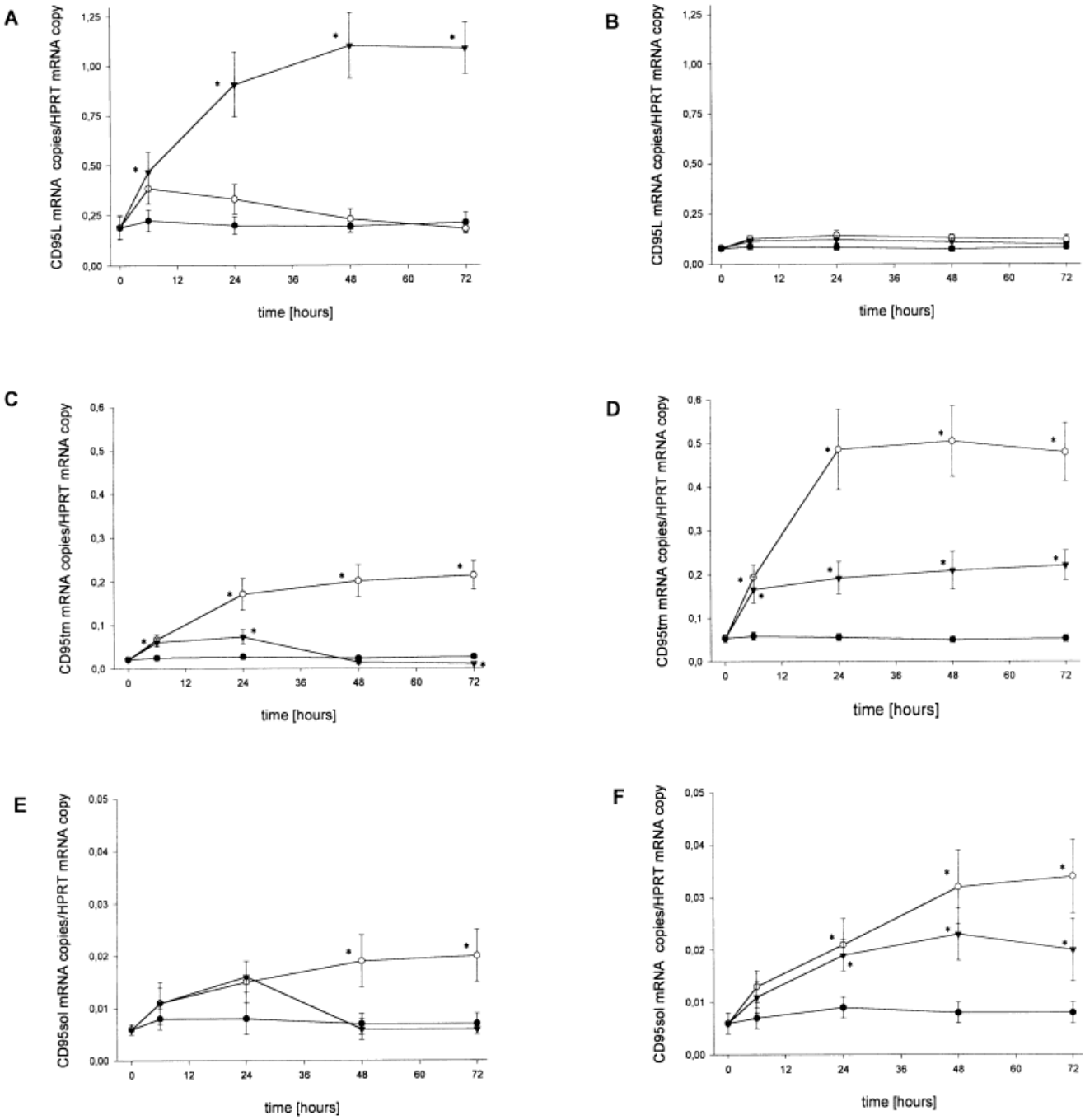

FIGURE 3 – Regulation of CD95L and receptor isoform mRNA expression in SCC cell lines by IFNγ and CDDP. Total RNA was extracted, reverse-transcribed and quantified by competitive quantitative PCR as described in ''Material and Methods''. CD95L, CD95tm and CD95sol mRNA levels are given as mRNA copies/HPRT mRNA copy. The time course of CD95L mRNA expression in response to 100 U/ml IFNγ (open circles) or 0.2 mmol/l CDDP (triangles) and under control conditions (filled circles) in the polyclonal cell line VC_0 *(a)* and its monoclonal derivative VC_1 *(b)* is shown. CD95tm mRNA levels are shown *(c)* for VC_0 and *(d)* for VC_1, CD95sol mRNA levels *(e)* for VC_0 and *(f)* for VC_1. Data are given as means ± SD from 3 independent experiments. *Significantly different from control ($p < 0.05$).

mmol/l) for 48 hr. Tissues from the primary tumor were fixed in 4% *para*-formaldehyde and embedded in paraffin. Sections were cut and deparaffinized by xylene and alcohols, then washed in PBS. All slides were subjected to immunochemical analysis as previously described (Müschen *et al.*, 1998*a*). Experimental handling applies to the ABC immunostain systems (Santa Cruz Biotechnology) following the manufacturer's protocol. For immunofluorescence, tissue sections were double-stained with rabbit anti-human CD95L and mouse anti-human CD3 antibodies. Rabbit and mouse primary antibodies were dye-labeled using CY3- and FITC-conjugated secondary antibodies.

Detection of CD95-specific apoptosis in autologous PBLs induced by IFNγ and CDDP-treated SCC cells

Autologous PBLs were isolated from the patient's venous blood using a Ficoll gradient. Cells of VC_0 and VC_1 were incubated with

IFNγ at 100 U/ml or with CDDP at 0.2 mmol/l or under control conditions for 48 hr. Thereafter, cells were incubated without or with 2 μmol/l of the CD95L-shedding inhibitor BB-3103 for 12 hr. In parallel, autologous PBLs were stimulated with 2 μg/ml phytohemagglutinin (PHA) and half of them were pre-incubated with 500 μmol/l Ac-YVAD-cmk for 12 hr, which blocks the CD95-dependent apoptotic signaling by inhibition of ICE (Tomita *et al.*, 1996). To assess whether shedding of CD95L from cell membranes occured in these cell lines, autologous PBLs were incubated with supernatants derived from SCC cultures or co-cultured with SCC cells at an effector:target cell (E:T) ratio of 10:1. After 24 hr, apoptosis in the PBLs was determined by the TdT-mediated fluorescein-dUTP nick end-labeling (TUNEL) method using an *in situ* detection kit (Boehringer-Mannheim). The percentage of nuclei staining positive for TUNEL was calculated. Specific killing was calculated as the fraction of apoptosis in autologous lymphocytes that was sensitive to Ac-YVAD-cmk.

Statistics

Data are expressed as means ± SD (n = number of independent experiments). Statistical analysis was performed using Student's *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

Growth and cytochemical characterization of the cell lines

The population doubling time was 63 ± 26 hr for VC_0 and 43 ± 5 hr for VC_1 ($p = 0.25$, n = 3). Immuno-cytochemical analysis of the 2 cell lines showed absence of any vimentin expression, which is typically observed in fibroblasts and sarcomatous tissue (Iwaya *et al.*, 1997), indicating that both cell lines were not of mesenchymal origin and that contamination by fibroblasts was negligible. Epithelial markers such as epidermal growth factor receptor (EGFR) were highly expressed. Both cell lines also displayed high expression levels for cytokeratin 7 and cytokeratin 18, which are indicative for SCC (Welters *et al.*, 1997). As several HPV types are known for their etiological role in anogenital SCC (Crum *et al.*, 1997), HPV screening was carried out and showed that both cell lines were infected by HPV types of high risk.

CD95L expression in the primary tumor

Because CD95L expression was previously mainly attributed to T lymphocytes (for review: Hug, 1997), the presence of TILs in the primary tumor was estimated by quantification of CD3δ chain transcripts in the tissue samples. Whereas the number of CD3δ mRNA copies/HPRT mRNA copy was 75.06 ± 9.58 (n = 3) in PBLs (about 70% $CD3^+$ cells), this ratio was 0.02 ± 0.01 in 3 different samples originating from the primary SCC, suggesting that only few TILs were present in SCC tissue. Immunohistochemical analysis of tumor samples showed strong staining for CD95L in carcinoma cells without staining in adjacent stroma cells (Fig. 2*a*,*b*). As shown in Figure 2*c*, there were only few $CD3^+$ T cells present in the area of the tumor spread, further supporting CD3δ quantification by competitive RT-PCR. In addition, CD95L expression in the SCC (Fig. 2*d*) was not co-localized to CD3-expressing TILs, indicating that CD95L expression by TILs in SCC tissue was negligible.

Staining for CD95L among tumor tissue was not homogenously distributed (Fig. 2*b*). Accumulation of CD95L protein was markedly enhanced in invasive tumor spikes, especially in the area of the border between invasive tumor tissue and the surrounding stroma cells (Fig. 2*b*).

CD95L mRNA and protein expression in the cell lines

As assessed by quantitative RT-PCR, the number of CD95L mRNA copies/HPRT mRNA copy in the polyclonal cell line VC_0 (0.19 ± 0.06, n = 3) did not significantly differ from the ratio in 3 different samples of the primary tumor (0.23 ± 0.04, $p = 0.35$). When VC_0 cells were treated with 0.2 mmol/l CDDP, CD95L mRNA levels increased and peaked after 48 hr with a 6-fold augmentation compared to untreated cells. In comparison, IFNγ (100 U/ml)-treated cells showed a slight increase (1.7-fold) of CD95L mRNA expression after 6 hr, which rapidly declined thereafter to control levels (Fig. 3*a*). Immuno-cytochemical analysis confirmed mRNA results (Table I, Fig. 4*a–c*).

TABLE I – IMMUNO-CYTOCHEMICAL ANALYSIS OF CD95L AND RECEPTOR EXPRESSION FOLLOWING TREATMENT WITH IFNγ AND CDDP IN VC_0 AND VC_1 CELLS

Protein	CD95L	CD95
VC_0		
Control	0.3 ± 0.1	0.2 ± 0.1
IFNγ	0.4 ± 0.1	0.8 ± 0.1*
Cisplatin	0.9 ± 0.1*	0.1 ± 0.1
VC_1		
Control	0.3 ± 0.1	0.2 ± 0.1
IFNγ	0.4 ± 0.1	0.7 ± 0.1*
Cisplatin	0.4 ± 0.1	0.3 ± 0.1

SCC cells were incubated for 48 hr under the conditions indicated. Afterward, SCC cells were subjected to immuno-cytochemical analysis for CD95L and receptor (CD95) expression as described in ''Material and Methods''. A score was calculated as the product of the percentage of cells positive for CD95L or CD95 (100% was set as 1) and the intensity of staining, in which 0.25 stands for weak staining, 0.5 for moderate staining, 0.75 for intensive staining and 1.0 for very intensive staining.–*Significantly different from control ($p < 0.05$). Data are given as means ± SD from 3 independent experiments.

In comparison with the parental cell line VC_0, CD95L mRNA levels were less than 50% in the clonal cell line VC_1 (Fig. 3*b*). Furthermore, when VC_1 cells were treated with CDDP or IFNγ, no significant change of CD95L expression was detectable by either quantitative RT-PCR or immuno-cytochemistry (Table I, Fig. 3*b*).

Induction of CD95-specific apoptosis in autologous lymphocytes by SCC cell lines

To assess whether CD95L expression by SCC cell lines was functionally relevant, CD95-specific apoptosis induced in lymphocytes was determined using the TUNEL technique. Polyclonal VC_0 tumor cells pre-treated with IFNγ or CDDP induced significantly more CD95-specific apoptosis in autologous PHA-activated lymphocytes than untreated VC_0 tumor cells (Table II). The extent of CD95-mediated apoptosis induced in lymphocytes by untreated VC_0 cells was related to E:T ratios. At an E:T ratio of 2, about 6% ± 2% (n = 3) of co-cultured PHA-activated lymphocytes underwent CD95-specific apoptosis, whereas 17% ± 4% (n = 3) of the activated lymphocytes were killed at an E:T ratio of 5.

In VC_1 cells, no increase of CD95-specific killing was observed following pre-treatment with IFNγ or CDDP (not shown).

Involvement of CD95L shedding in lymphocyte killing

To estimate whether CD95L shedding from the cell membranes occurred in the SCC cell lines, VC_0 cells were treated with BB-3103, an inhibitor of a variety of matrix metalloproteinases. Exposed to BB-3103-treated SCC cells, the fraction of activated autologous lymphocytes undergoing CD95-specific apoptosis increased 2-fold when compared with control conditions (Table II). Likewise, the effect of IFNγ and CDDP on apoptosis in autologous lymphocytes was about 2-fold enhanced by addition of BB-3103 (Table II). In contrast, the effect of supernatants from pre-treated SCC cells on CD95-specific apoptosis in autologous lymphocytes was completely reversed when SCC cells were supplemented with BB-3103 (Table II).

CD95 receptor expression in tumor cell lines

The mRNA levels for CD95tm as well as for CD95sol were similar in the primary tumor and in the polyclonal cell line VC_0. Expression levels were 0.02 ± 0.01 CD95tm mRNA copies/HPRT mRNA copy and 0.01 ± 0.01 CD95sol mRNA copies/HPRT mRNA copy in the primary tumor as well as in VC_0 cells (n = 3).

The CD95tm mRNA levels in VC_0 increased 8-fold in response to IFNγ and 3-fold in response to CDDP within 24 hr when compared with untreated cells (Fig. 3*c*). However, increased CD95tm mRNA levels following treatment with CDDP declined

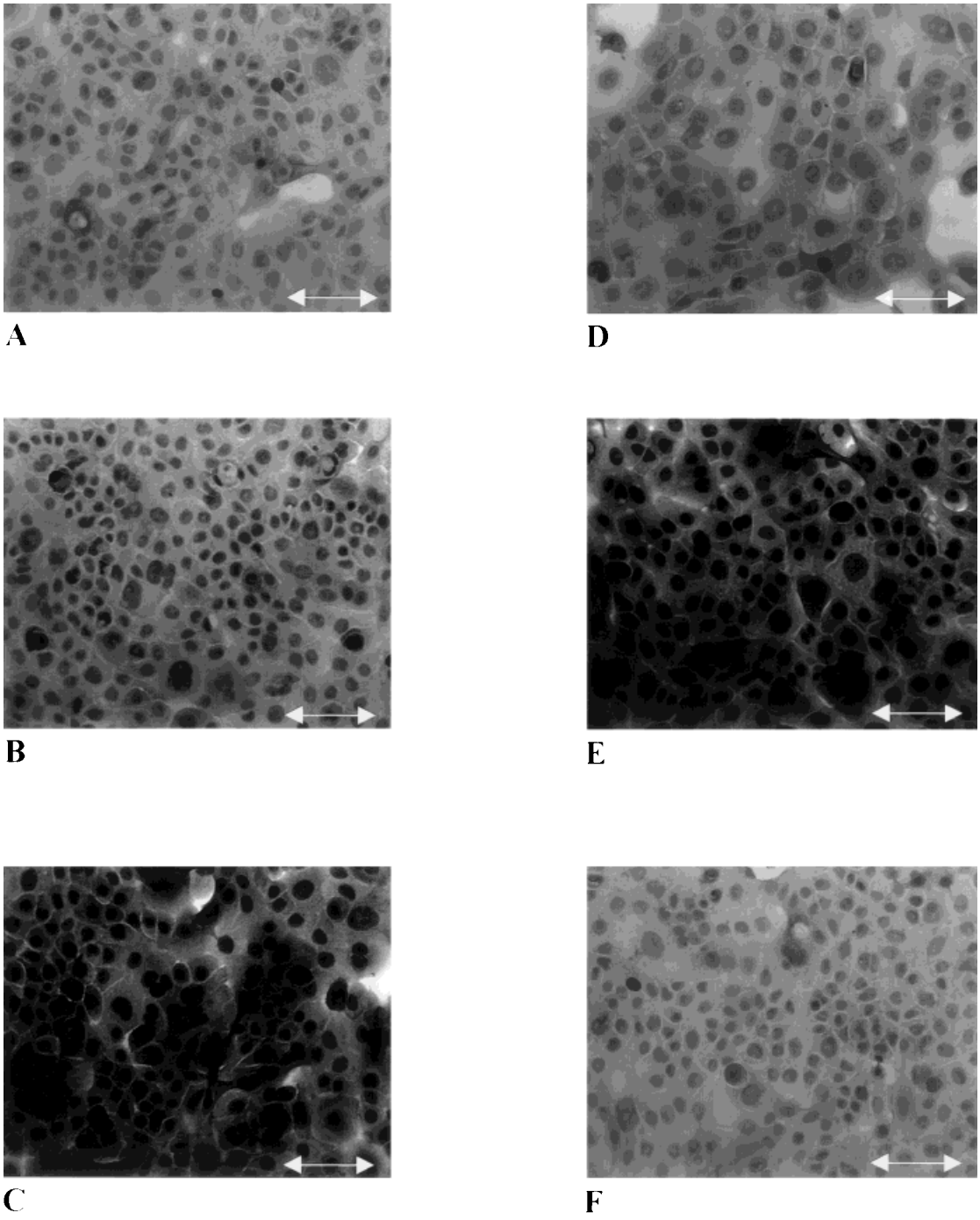

FIGURE 4 – Immuno-cytochemical analysis of CD95L and receptor expression in VC_0 cells. Immuno-cytochemical procedures were carried out as described in ''Material and Methods''. *(a–c)* CD95L expression in VC_0 cells without *(a)* or with 100 U/ml IFNγ *(b)* or with 0.2 mmol/l CDDP treatment *(c)* for 48 hr. *(d–f)* Staining for CD95 receptor: *(d)* control, *(e)* IFNγ *(f)* CDDP-treated cells. Scale bar: 75 µm.

rapidly thereafter and were diminished by 60% below control levels after 72 hr (Figs. 3*c*, 5).

In the clonal cell line VC_1, CD95tm mRNA expression was 2.6-fold higher than in the polyclonal parental cell line VC_0 (Fig. 3*c*,*d*). IFNγ and CDDP treatment increased CD95tm mRNA levels 10-fold and 4-fold in VC_1 cells after 48 hr, respectively (Fig. 3*d*). In contrast to VC_0 cells, high CD95tm mRNA levels were maintained even after 72 hr of CDDP exposure (Fig. 3*d*). CD95tm mRNA levels were 10-fold higher than those of CD95sol (Fig. 3*d*,*f*).

Immuno-cytochemical results, though only reflecting the presence of the membrane-bound and the cytoplasmic fractions of the soluble CD95 receptor isoform, were consistent with mRNA data (Table I, Fig. 4 *d–f*).

CD95-mediated apoptosis in SCC cells and in the primary tumor

Tumor cells were incubated with an agonistic anti-CD95 antibody in several concentrations, and apoptosis was then detected by TUNEL staining. In untreated SCC cells, the extent of apoptosis was correlated with the concentration of the agonistic anti-CD95 antibody (Fig. 6*a*,*b*). At an antibody concentration of 100 ng/ml, the number of apoptotic VC_0 cells was significantly increased when primed with IFNγ or CDDP compared to untreated cells (Fig. 6*a*).

TABLE II – INDUCTION OF CD95-SPECIFIC APOPTOSIS IN AUTOLOGOUS LYMPHOCYTES BY CD95L-EXPRESSING SCC CELLS

Pre-treatment	CD95-specific apoptosis (%)	
	Co-culture	Supernatants
Control	12 ± 2	9 ± 3
IFNγ	22 ± 4*	19 ± 5*
CDDP	28 ± 6*	25 ± 4*
BB	25 ± 5*	3 ± 2*
IFNγ + BB	50 ± 6*	2 ± 1*
CDDP + BB	66 ± 7*	4 ± 2

VC_0 SCC cells were pre-treated without (control) or with 100 U/ml IFNγ (IFNγ) or with 0.2 mmol/l CDDP for 48 hr. Afterward, cells were incubated without or with 2 µmol/l of the CD95L-shedding inhibitor BB-3103 (+BB) for 12 hr. After activation with PHA for 12 hr, autologous peripheral blood lymphocytes were either co-cultured with pre-treated SCC cells or treated with supernatants from SCC cells for 24 hr. Apoptosis in lymphocytes was assessed using the TUNEL technique. CD95-specific apoptosis was calculated as the fraction of apoptotic autologous peripheral blood lymphocytes sensitive to Ac-YVAD-cmk. Data are given as means ± SD from 3 independent experiments.

*Significantly different from control ($p < 0.05$).

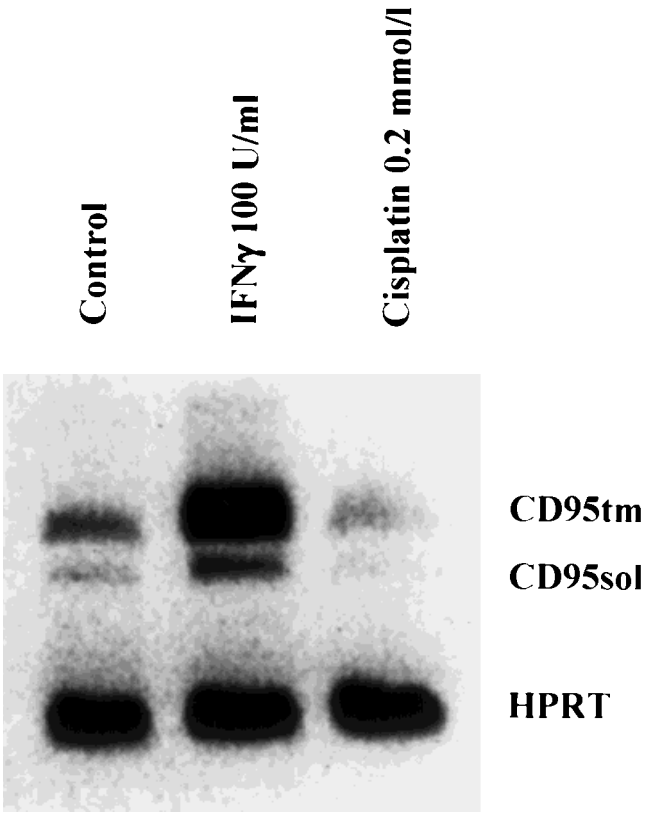

FIGURE 5 – Regulation of CD95 receptor isoform mRNA expression in SCC cells by IFNγ and CDDP. CD95tm and CD95sol receptor splice variants differ by 62 bases, the soluble isoform lacking the transmembrane domain of CD95. RT-PCR products for the 2 CD95 receptor splice variants expressed by VC_0 cells are shown under control conditions and when treated with IFNγ or CDDP for 48 hr. PCR products for CD95tm (392 bp), CD95sol (330 bp) and HPRT (263 bp) were separated on a 2% agarose gel and visualized by ethidium bromide staining. The results are representative of 3 independent cell preparations.

Interestingly, almost 70% of CDDP-treated VC_0 cells became apoptotic even without addition of the anti-CD95 antibody (Fig. 6*a*). Compared to this, in its clonal derivative VC_1, more cells were apoptotic when treated with CDDP (90%, Fig. 6*b*).

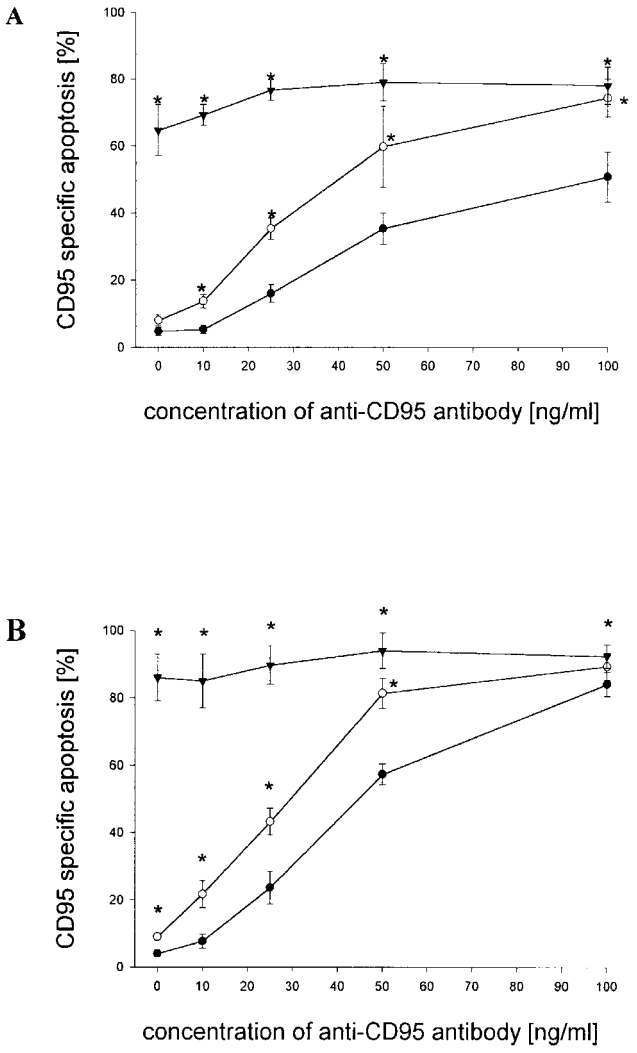

FIGURE 6 – Susceptibility of VC_0 and VC_1 cells to anti-CD95-mediated apoptosis. SCC cells were incubated with an agonistic anti-CD95 antibody (concentrations indicated) for 24 hr. CD95-specific apoptosis *(a)* in the polyclonal cell line VC_0 and *(b)* in the clonal cell line VC_1. SCC cells without treatment are depicted by filled circles, IFNγ (100 U/ml)-treated cells by open circles and CDDP (0.2 mmol/l)-treated cells (pre-incubation for 48 hr) by triangles. Data are given as means ± SD from 3 independent experiments for each set of conditions. *Significantly different from control ($p < 0.05$).

Whereas the clonal cell line VC_1 was entirely killed after treatment with 0.2 mmol/l CDDP for 6 days, a fraction of about 30% of the polyclonal VC_0 cell line survived CDDP treatment. In VC_0 cells surviving after 6 days of CDDP treatment, neither 0.2 mmol/l CDDP (9% ± 4 %, n = 3) nor 100 ng/ml of the agonistic anti-CD95 antibody (12% ± 5 %, n = 3) induced further significant CD95-specific apoptosis, indicating that these cells acquired secondary cross-resistance toward CD95- and CDDP-mediated cytotoxicity.

In the primary SCC, only stroma cells in the direct vicinity of invasive tumor tongues stained positive for apoptosis. The apoptotic nuclei of stroma cells formed a lining which was congruent with the arrangement of the locally restricted over-expression of CD95L protein (Fig. 7).

DISCUSSION

Studying the CD95 system in primary SCC cell lines, CD95 receptor and ligand expression were differentially regulated by

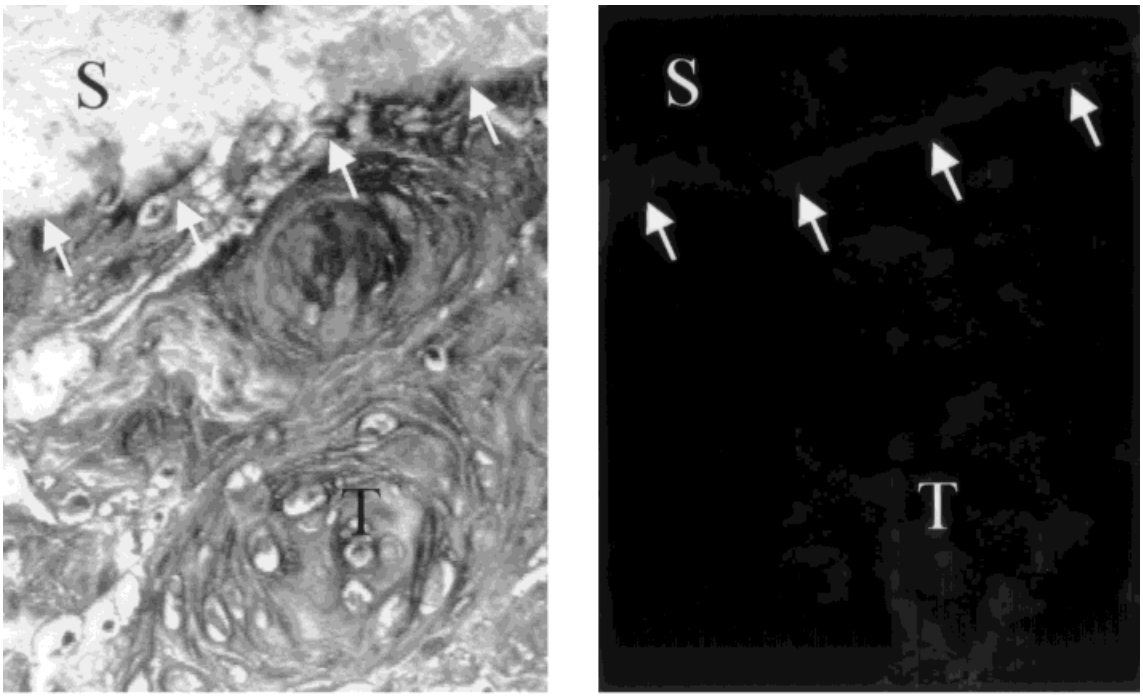

FIGURE 7 – Immuno-histochemical analysis of CD95L expression and TUNEL staining for apoptotic nuclei in primary SCC. Immuno-histochemical procedures and TUNEL staining were performed successively for the same tissue section as described in ''Material and Methods''. Cells staining positive for CD95L (dark-colored cells) or for apoptosis were detected by light or fluorescence microscopy, respectively. Cells were counterstained by eosin staining. *(a)* Distribution of CD95L expression and enhanced CD95L expression along the invasion front of the SCC (see dark-colored cells). *(b)* Arrangement of apoptotic stroma cells (S) in the vicinity of the SCC (T) (fluorescence signals, see arrows).

IFNγ and CDDP. While CD95L mRNA and protein levels were strongly increased by CDDP treatment in the parental cell line VC_0, there was only little augmentation upon IFNγ challenge. The finding of over-expression of CD95L mRNA levels after CDDP exposure was consistent with observations in other malignancies, in which CD95L levels increased after cytostatic drug treatment, such as doxorubicin and bleomycin (for review: Hug, 1997).

CD95tm and CD95sol mRNA levels were increased by IFNγ in both SCC cell lines but diminished below control levels by CDDP in polyclonal VC_0 cells after 48 hr of incubation.

In the present study, CD95L expression was functionally relevant since CD95L-expressing SCC cells were able to induce apoptosis in autologous lymphocytes, thus indicating potential consequences for anti-tumor immunity. Functional significance of CD95 receptor expression was demonstrated using an agonistic anti-CD95 antibody that induced apoptosis in SCC cells. Sensitivity of untreated polyclonal VC_0 and clonal VC_1 cells to CD95-mediated apoptosis was correlated with CD95tm mRNA levels in the cell lines.

For several malignancies, up-regulation of CD95L mRNA expression by chemotherapeutic drugs has been described (Friesen *et al.*, 1996; Hug, 1997). However, Landowski *et al.* (1997) report on CD95 receptor down-regulation in response to cytostatic drug treatment as a mechanism of selection for drug resistance. One might speculate that the increase in CD95L mRNA levels observed here in CDDP-treated VC_0 cells was also due to selection of drug-resistant and, moreover, CD95L-over-expressing cells by cytotoxic drug treatment. Polyclonality was a prerequisite of selection for drug resistance (Friesen *et al.*, 1996; Landowski *et al.*, 1997). In the present study, CDDP led to an augmentation of CD95L mRNA quantities only in the polyclonal parental SCC cell line VC_0. Likewise, CD95L up-regulation in human leukemia cells by doxorubicin was limited to a polyclonal parental cell line, whereas CD95L expression in its monoclonal derivative was not changed (Friesen *et al.*, 1996).

CDDP treatment increased CD95tm mRNA levels in the VC_1 clone, whereas CD95tm mRNA levels were significantly diminished in VC_0 cells after 48 hr (Fig. 5). CDDP treatment led to killing of the entire VC_1 clone within 48 hr, whereas the parental cell line VC_0 was not affected by CDDP-mediated cytotoxicity. In line with the findings of Landowski *et al.* (1997), CDDP treatment increased CD95tm mRNA levels in the CD95-sensitive VC_1 clone as a whole with subsequent elimination of CD95-sensitive cells, *i.e.*, extinction of the entire VC_1 clone. However, about 30% of VC_0 cells were not killed by CDDP treatment within 48 hr and were not susceptible to CD95-mediated apoptosis. After treatment with CDDP for 6 days, about 90% of the remaining VC_0 cells were affected by neither CDDP- nor anti-CD95-mediated apoptosis. Thus, we speculate that survival of CDDP-treated SCC cells was coupled with resistance toward CD95-mediated cytotoxicity. Accordingly, Friesen *et al.* (1996) as well as Landowski *et al.* (1997) observed a close relationship between doxorubicin resistance and CD95 resistance in leukemia cells. The cross-resistance toward both chemotherapeutic drug treatment and CD95L-mediated cytotoxicity in SCC cells may be of clinical relevance. Esaki *et al.* (1996) reported that SCC cells acquired secondary resistance to CDDP, thus limiting the efficiency of chemotherapeutic drug treatment.

In tissue sections from the primary SCC, apoptotic nuclei of stroma cells formed a lining at the border to the SCC, which was congruent with the arrangement of the locally restricted over-expression of CD95L protein. From these observations one might hypothesize that, in the zone of tumor invasion, adjacent stroma cells were targeted by tumor cells bearing CD95L on the cell membrane. CD95L-expressing tumor cells may up-regulate CD95L mRNA levels by direct contact with CD95-bearing cells from the

antigenic host organism, thus taking advantage of CD95L expression as a factor of tissue invasion.

Mononuclear $CD3^+$ cells and CD3δ mRNA were hardly detectable in the SCC samples by immunofluorescence or by quantitative RT-PCR, indicating that the presence of TILs was negligible. This might be explained by the disappearance of nearly all $CD3^+$ mononuclear cells by tumor-associated cytotoxicity. However, one might hypothesize that the tumor tissue was *a priori* hardly infiltrated by TILs and, thus, not the main site of lymphocyte killing. However, autologous lymphocytes strongly underwent CD95-specific killing after incubation on tumor cell lines.

Our results show an inverse effect of CDDP and IFNγ on the CD95 system in cell culture with a likely impact on anti-tumor immunity. CDDP was highly efficient at killing tumor cell lines in the present experiments but enhanced lymphocyte killing by SCC cells. This might have caused selection of cells that were resistant to both CDDP and CD95-mediated cytotoxicity. However, IFNγ had little effect on lymphocyte depletion by tumor cells and sensitized SCC cell lines to CD95-mediated apoptosis. Further studies will be required to establish the potential clinical relevance of these observations.

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