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CD95 LIGAND EXPRESSION IN DEDIFFERENTIATED BREAST CANCER

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SUMMARY

CD95 ligand expression has been observed in various malignancies. Studying the CD95 ligand (CD95L) and receptor (CD95) system in eight non-malignant mammary tissues and 40 breast cancer tissues, mRNA and protein expression was determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immunofluorescence. mRNA levels of CD95L correlated positively ($r=0{\cdot}90$; $p<0{\cdot}01$) and transmembrane CD95 inversely ($r=-0{\cdot}88$; $p<0{\cdot}01$) with histopathological grading of the breast tumours: CD95L mRNA levels were low in adenomas, but increased by 20-fold in grade I, 120-fold in grade II, and 310-fold in grade III breast cancer. In contrast, CD95 mRNA levels were low in high-grade carcinomas, but high in benign mammary tissues. Since CD95L acts as an efficient inducer of apoptosis in CD95$^+$ cells, apoptotic cells were identified on the tissue sections. Tumour-infiltrating lymphocytes and stromal cells in close proximity to CD95L-expressing breast cancer underwent apoptosis. As a functional test, CD95$^+$ target cells were cultured on breast cancer tissue sections. The target cells underwent apoptosis when cultured on breast cancer sections, but could be rescued when CD95L was specifically blocked by a CD95–Fc fusion molecule. The data suggest an inverse regulation of CD95 ligand and receptor expression during dedifferentiation of breast cancer. Killing of bystander cells by the CD95L-expressing breast tumour could be involved in tissue invasion. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—**apoptosis; tumour-infiltrating lymphocytes; antitumour immunity; differentiation; breast; carcinoma**

INTRODUCTION

The CD95 (Apo-1/Fas) system consists of a membrane-bound (CD95tm)[1,2] and a soluble (CD95sol) isoform of the CD95 receptor, generated by alternative splicing.[3] Their natural ligand (CD95L) has been shown to hold a key position in the regulation of the immune response.[4] CD95tm transduces the apoptotic signal after CD95L binding.[1,2] On the other hand, CD95sol prevents target cells from undergoing apoptosis by neutralizing CD95L without transduction of the death signal.[3] CD95L is expressed on the surface of effector cells, but may be cleaved off by specific matrix metalloproteinases.[5–8] CD95L processed into a soluble form may cause systemic tissue damage,[5–7] although it is a less potent inducer of apoptosis than its membrane-bound counterpart.[8] Disturbance of this system and imbalance of its constituents have been implicated in autoimmune disease[9] and neoplastic development.[4,10–18]

The expression of the CD95 receptor has been studied in breast cancer cell lines.[10,11] Breast cancer cells are less susceptible to CD95-mediated apoptosis than non-malignant mammary epithelia, either because they lack expression of functional CD95 receptor on the cell surface or because of impairment of the intracellular transduction of the apoptotic signal.[10] On the other hand, soluble CD95 receptor may protect tumour cells from CD95-mediated apoptosis.[12]

The putative role of CD95L in breast carcinoma has not yet been studied. In other malignancies, CD95L has been implicated in the creation of a tumour-associated immune privilege, thus protecting tumour cells against tumour-infiltrating lymphocytes (TILs).[4,14–16] Moreover, CD95L induces localized immune suppression by abrogation of alloantibody production against allografted colon carcinoma cells.[17] In contrast, locally produced CD95L in carcinomas was recently reported to support tumour rejection by the immune system *in vivo*, thus attributing a pro-inflammatory function to CD95L.[18]

The present study shows that CD95L expression correlates with histopathological grading of the tumours and provides breast cancer cells with a highly efficient means of counteracting the host's immune response, by induction of apoptosis in CD95$^+$ lymphocytes.

MATERIALS AND METHODS

Tissue samples

The present study focused on 40 cases of breast cancer and eight cases of benign breast tissue. Among the benign cases, there were one normal mammary tissue sample, two mastopathy III samples, and five fibroadenomas. The breast cancer samples included seven mammary carcinomas of low grade (G1), 19 carcinomas

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Table I—Characteristics of the breast tumour patients involved in the present study*

Grading	*n*	Diagnosis	Age	Staging T	Staging N	Staging M	ER	PgR	CD3δ mRNA
Benign	8	1 normal mammary tissue	59	1 T_0	1 N_0	1 M_0	n.d.	n.d.	5·21
		5 fibroadenomas	37 ± 8	5 T_0	5 N_0	5 M_0	n.d.	n.d.	3·44 ± 1·2
		2 mastopathies III	43 and 62	2 T_0	2 N_0	2 M_0	n.d.	n.d.	0·0 and 1·8
G1	7	2 tubular carcinomas	50 and 83	6 T_{1c}	6 N_0	7 M_0	19 and 23	19 and 0	0·4 and 1·5
		5 IDCs	66 ± 7	1 T_2	1 N_{1b}		241 ± 110	47 ± 25	1·9 ± 0·3
G2	19	3 ILCs	56 ± 18	1 T_{1c} 2 T_2	1 N_0 1 N_1 1 N_2	2 M_0 1 M_1	44 ± 24	23 ± 19	1·5 ± 0·7
		16 IDCs	59 ± 22	2 T_1 1 T_{1b} 2 T_{1c} 8 T_2 1 T_3 2 T_4	11 N_0 3 N_1 2 N_2	14 M_0 2 M_1	30 ± 21	9 ± 11	3·9 ± 1·1
G3	14	1 medullary carcinoma	64	1 T_3	1 N_0	1 M_0	1	3	0·1
		2 ILCs	37 and 83	1 T_{1a} 1 T_3	2 N_1	2 M_0	57 and 45	40 and 51	0·2 and 0·3
		11 IDCs	44 ± 12	1 T_{1c} 8 T_2 2 T_4	3 N_0 5 N_1 3 N_2	7 M_0 2 M_X 2 M_1	13 ± 27	17 ± 29	1·4 ± 0·8

*The age of the patients in each group is given in years, and oestrogen receptor (ER) and progesterone receptor (PgR) levels in fmol/mg protein. Quantitatively determinated CD3δ mRNA levels, reflecting the degree of tissue infiltration by T lymphocytes, are given in copies per 1000 copies of HPRT mRNA. Data are expressed as means ± SEM. *n* denotes the number of patients studied for each group. IDC = invasive ductal carcinoma, ILC = invasive lobular carcinoma; n.d. = not determined. The staging gives details about local tumour extension (T), dissemination into lymph nodes (N), and the frequency of metastasis (M).

of intermediate grade (G2), and 14 high-grade carcinomas (G3) (Table I). With the exception of the determination of apoptosis in three breast tumours, none of the tissue samples studied had lymphocytic infiltration or obvious necrosis as assessed by light microscopy.

Reagents and antibodies

Polyclonal rabbit anti-human CD95L IgG1, monoclonal mouse anti-human CD3 IgG1 and monoclonal mouse anti-human mucin-1 (MUC-1) IgG1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Human recombinant CD95 ligand protein corresponding to soluble CD95 ligand (amino acids 103–261) and a CD95:Fc-Ig fusion molecule were from Alexis (San Diego, CA, U.S.A.). RPMI 1640 medium and fetal bovine serum were purchased from Biochrom (Berlin, Germany); phytohaemagglutinin (PHA) from Seromed (Berlin, Germany); and all other chemicals from Sigma (Deisenhofen, Germany). Oligonucleotides were synthesized by Birsner and Grob (Freiburg, Germany).

Cell culture conditions

Jurkat T lymphocytes were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, U.S.A.), cultured in RPMI medium (10 per cent fetal bovine serum), and stimulated with 2·4 μg PHA/ml.

Quantitative competitive RT-PCR

Total RNA from snap-frozen tissue sections was isolated using a total RNA extraction kit (Qiagen, Hilden, Germany) and reverse-transcribed usinga first strand cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). The mRNA levels of CD95 ligand (CD95L), CD95 isoforms, CD3δ chain, and hypoxanthine guanine phosphoribosyltransferase (HPRT), which was used for standardization, were determined using quantitative competitive RT-PCR.[19,20] As shown in Fig. 1, the cDNA to be assayed was co-amplified with known amounts of an internal DNA standard (Δ4), which was identical to the corresponding fragment of the assayed cDNA, apart from a deletion of four nucleotides. DNA standards were constructed essentially as described in Pannetier *et al.*[19] 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-derived cDNA. A 1000-fold dilution of this product was reamplified using the respective 3′ PCR primer (see below) and an additional construct primer containing a four-nucleotide deletion compared with the wild-type sequence. For quantification of the transcripts for CD95L, CD95tm, CD95sol, CD3δ chain, and HPRT, respectively, a constant amount of cDNA, corresponding to 50 ng of 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 of 94°C for 20 s; 58°C for 45 s; 72°C for 45 s with 10 min extention time at 72°C on cycle 40). The primers used for PCR were 5′-GGCCACCCCAGTCCACC and 5′-CC GAAAAACGTCTGAGATTCC for CD95L, 5′-GGAC ATGGCTTAGAAGTGG and 5′-GGTTGGAGATTC

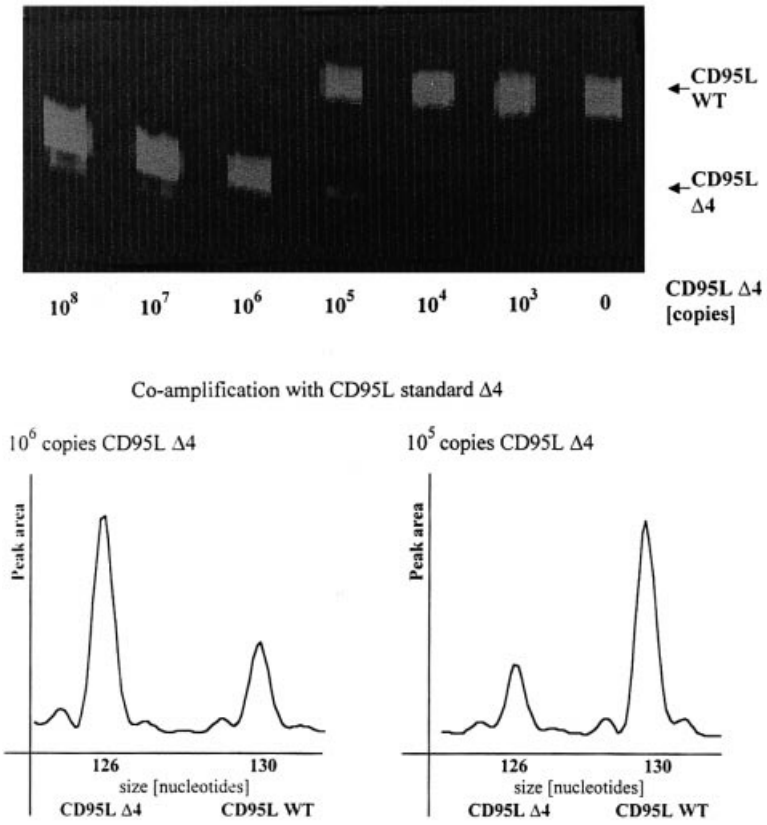

Fig. 1—Quantification of CD95 ligand mRNA levels by quantitative competitive RT-PCR. The cDNA to be assayed (WT) was co-amplified with known amounts of an internal DNA standard (Δ4), which was identical to the corresponding fragment of the assayed cDNA, apart from a deletion of four nucleotides. For quantification of transcripts for CD95L, a constant amount of cDNA, corresponding to 50 ng of 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-labelled oligonucleotide, which allows discrimination between CD95L wild-type (CD95L WT) and standard (CD95L Δ4) DNA species. PCR amplification products were specifically labelled in run-off reactions, loaded on an acrylamide gel, and analysed by an automated sequencer. The fluorescent profiles (see top) were recorded and the profile areas (bottom) were analysed. For co-amplifications with 10^6 and 10^5 copies of the CD95L standard, respectively, the peak area ratios for CD95L wild-type (CD95L WT) and standard (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 two standard dilutions [e.g. for the sample shown here: $(0{\cdot}343 \times 10^6 + 3{\cdot}461 \times 10^5)/2$, i.e. 344 550 copies]

ATGAGAACC for both CD95 receptor isoforms, 5′-CCAGGCTGATAGTTCGGTGACC and 5′-TGTC TGAGAGCAGTGTTCCCAC for CD3δ chain, and 5′-CCTGCTGGATTACATCAAAGCACTG and 5′-CACCAGCAAGCTTGCGACC for HPRT.

The read-out of the amplification involved one additional fluorescent dye-labelled oligonucleotide, which allows discrimination between wild-type (WT) and standard (Δ4) DNA species (Fig. 1). PCR amplification products were specifically labelled in run-off reactions, loaded on an acrylamide gel, and analysed by an automated sequencer (ABI 373, Applied Biosystems, Foster City, CA, U.S.A.). The fluorescent dye-labelled (FAM) oligonucleotides used in run-off reactions were 5′-CATTGATCACAAGGCCACCC for CD95L, 5′-TC ACCAGCAACACCAAGTGCAA for both CD95 isoforms, 5′-TCTATAGGTATCTTGAAGGGGCTC for CD3δ chain, and 5′-CCCCTGTTGACTGGTCATTA CAATAG for HPRT. In comparison to 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) were analysed using the software 'Immunoscope',[19] which was kindly provided by Dr C. Pannetier (Unité de Biologie Moléculaire de Gène, INSERM U277, Institut Pasteur, Paris, France).

Immunohistochemical procedures

For immunofluorescent tissue staining, cryosections (5 μm) were stained with mouse anti-human CD3 IgG1, mouse anti-human MUC-1 IgG1, and rabbit anti-human CD95L IgG1 antibodies as previously

described.[21] Goat anti-mouse IgG1 [fluorescein isothiocyanate (FITC)-labelled] and goat anti-rabbit IgG1 (CY3-labelled) were used as secondary antibodies. Matching mouse anti-human primary antibodies with goat anti-rabbit secondary antibody and rabbit anti-human primary antibody with goat anti-mouse secondary antibody, no cross-reactivity was found. Non-specific binding of primary antibodies was excluded by parallel staining with normal mouse IgG1 and normal rabbit IgG1 instead of the primary antibodies.

Cryosections were fixed with methanol (− 20°C) for 10 min. After three washes with phosphate-buffered saline (PBS), the cryosections were treated with blocking serum for 30 min and washed three times with PBS thereafter. The sections were then incubated for 1 h with primary antibodies at a concentration of 0·5 μg/ml (1:400). After 1 h of repeated washing steps, the sections were incubated with the secondary antibodies (FITC-labelled 1:100; CY3 labelled 1:500) for 1 h. After removal of the secondary antibodies, the sections were washed for 1 h with PBS, air-dried, and covered with mounting medium. Staining was visualized by means of fluorescence microscopy.

Detection of CD95-specific apoptosis in lymphocytes incubated on breast cancer tissues

CD95$^+$ Jurkat T lymphocytes were incubated as described in Strand *et al.*[4] on 5 μm cryosections from mammary tissue. Briefly, cryosections were prepared from four benign mammary tissues and four high-grade carcinomas (G3). For each tissue, two directly adjacent sections were prepared and transferred onto silanized glass slides. Jurkat T lymphocytes were incubated with 2·4 μg/ml PHA for 24 h and 10^6 stimulated Jurkat cells were seeded on each cryosection in 100 μl of RPMI medium. For each mammary tissue, one Jurkat cell culture was supplemented with the CD95:Fc-Ig fusion molecule (100 μg/ml). The ability of CD95:Fc-Ig to prevent CD95 ligand (CD95L)-mediated apoptosis was verified in preliminary experiments using human recombinant soluble CD95L as an inducer of apoptosis in PHA-stimulated Jurkat T lymphocytes. After 24 h, apoptosis in Jurkat T lymphocytes was determined by the TdT-mediated fluorescein-dUTP nick end labelling (TUNEL) method as described in Gavrieli *et al.*[22] Jurkat T lymphocytes were fixed in methanol for 10 min, permeabilized using 0·1 per cent Triton-X solution, repeatedly washed with PBS, and incubated in the presence of the TUNEL reagent (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C in a humidified chamber. After further washing steps, FITC-labelled DNA double strand-breaks were visualized by fluorescence microscopy and the percentages of nuclei staining positive for TUNEL were counted.

Statistics

Data are expressed as means ± SEM (n is the number of independent experiments). Statistical analysis was performed using Student's t-test. $p<0{\cdot}05$ was considered to be statistically significant.

RESULTS

CD95 ligand and receptor expression in mammary tissue of different histopathological grades

In the present study, eight samples of non-malignant mammary tissues and 40 samples from breast cancer tissues including low- (G1, $n=7$), intermediate- (G2, $n=19$), and high-grade breast cancer (G3, $n=14$) were analysed for membrane-bound and soluble CD95 receptor (CD95tm; CD95sol) isoforms, CD95 ligand (CD95L), and HPRT expression at the mRNA level and for CD95L also at the protein level. CD95 and CD95L mRNA were measured by quantitative competitive RT-PCR and expressed as copies per HPRT copy. In one out of 19 samples from G2 breast cancer, however, mRNA levels could not be detected.

Since CD95L expression was previously mainly attributed to T lymphocytes,[2,3] tissue samples lacking lymphocytic infiltration were selected for this study. The absence of relevant amounts of tumour-infiltrating lymphocytes (TILs) in the tissue samples was controlled by quantification of CD3 mRNA levels and by immunohistochemistry. Whereas CD3δ chain mRNA copies per HPRT copy were 75·061 ± 9·583 ($n=3$) in peripheral blood lymphocytes (PBLs, about 70 per cent CD3$^+$ cells), this ratio was 0·002 ± 0·001 ($n=8$) in the benign breast tissues and 0·001 ± 0·001 ($n=20$) in the breast cancer tissues samples, indicating a negligible presence of TILs (see also Table I). As shown in Fig. 2, there were only a few CD3$^+$ cells on the mammary tissue sections. Moreover, CD3$^+$ cells were not co-localized with sites of CD95L expression (Figs 2A and 2B), suggesting that CD3$^+$ TILs are not a relevant source of CD95L expression in the samples selected for this study. Furthermore, double staining of adjacent sections from the same tissue samples was performed using MUC-1 and CD95L specific antibodies. MUC-1 expression is frequently found in breast cancer cells but is absent in TILs.[23] As shown in Figs 2C and 2D, MUC-1-expressing cells were congruent with sites of CD95L expression.

CD95L mRNA levels inversely correlated with the degree of tissue differentiation (Table II and Fig. 3A; $r=-0{\cdot}90$; $p<0{\cdot}01$). Benign breast tissues displayed low CD95L mRNA levels, whereas CD95L mRNA levels were 20-fold higher in G1, 120-fold in G2, and 300-fold in G3 breast cancers, respectively. Interestingly, CD95L mRNA levels were also correlated with other prognostic markers, as summarized in Table III. Besides a strong correlation with the histopathological grade, high CD95L mRNA levels were also associated with a larger tumour mass, a higher frequency of tumour cell dissemination into the lymph nodes and metastatic spread, as well as decreased levels of oestrogen receptor (ER) expression (Table III).

The correlation between CD95L mRNA expression and the histopathological grade was approximately paralleled at the protein level (Fig. 4). Interestingly, the gain of CD95L expression in less-differentiated breast cancer was accompanied by a loss of the luminal polarity of mucin-1 expression, antibodies against which were used to stain the glandular epithelial cells. Since luminal polarity of mucin-1 expression was fully retained in

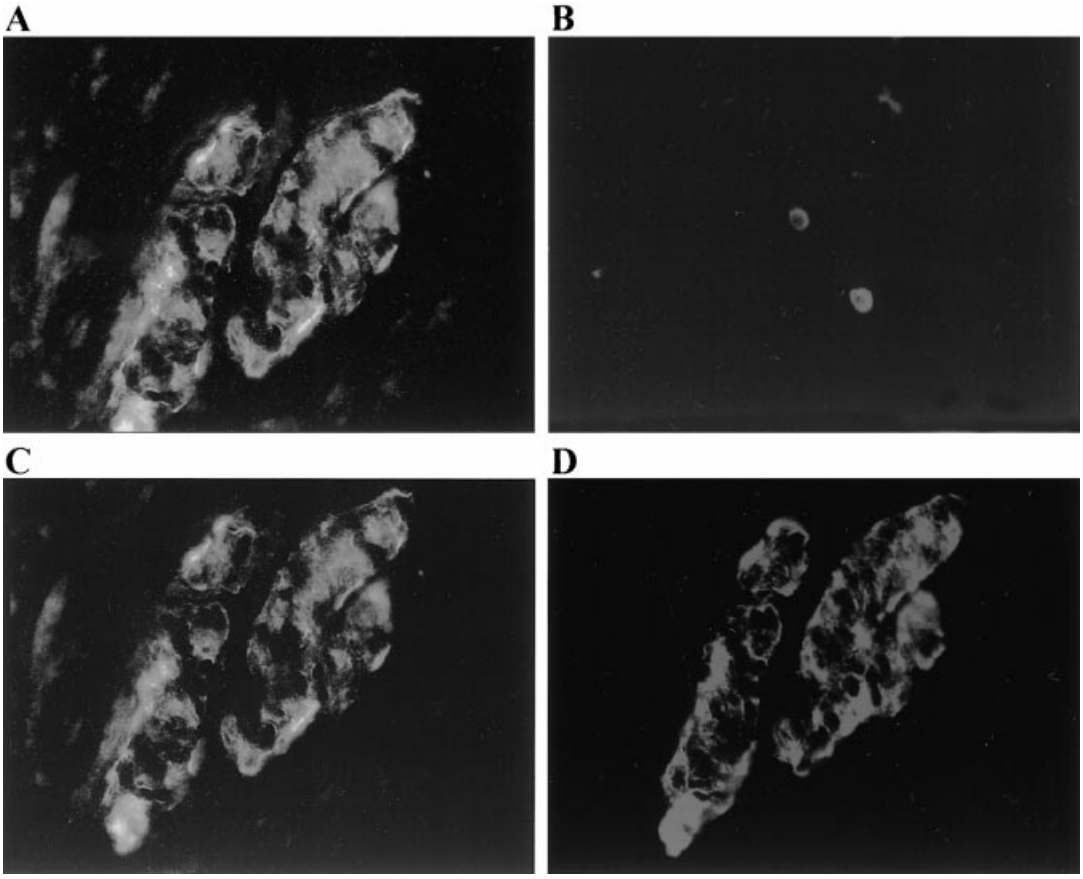

Fig. 2—Identification of CD95 ligand-expressing cells on breast cancer sections. Co-localization of MUC-1 (A; C) expression with CD3- (B) and CD95 ligand-expressing cells (D) was analysed on sections from breast cancer tissues selected for this study. Two directly adjacent 5 mm cryosections were double-stained using FITC-labelled (MUC-1; green fluorescent dye) and CY3-labelled (CD3 and CD95L; red fluorescent dye) secondary antibodies as described in the Materials and Methods section

Table II—CD95 ligand and receptor mRNA levels in mammary tissues of different grades*

	mRNA levels		
	CD95L	CD95tm	CD95sol
	(copies per 10^3 HPRT mRNA copies)		
Benign breast tissues (n=8)	0·4 ± 0·1†	159·3 ± 12·8†	51·6 ± 7·2
G1 carcinomas (n=7)	7·7 ± 1·7†	118·1 ± 10·0†	79·9 ± 8·1†
G2 carcinomas (n=18)	47·2 ± 10·3†	48·8 ± 6·3†	50·2 ± 9·2
G3 carcinomas (n=14)	119·6 ± 19·4†	26·6 ± 5·2†	38·1 ± 10·1

*mRNA levels for CD95 ligand (CD95L) and transmembrane (CD95tm) and soluble (CD95sol) isoforms of CD95 receptor were calculated for benign breast tissues and differently graded breast cancer. Data are given as CD95L, CD95tm or CD95sol mRNA copies per 10^3 HPRT mRNA copies, respectively, and as means ± SEM.
†Significantly different from all other tissues, respectively (p<0·05).

fibroadenomas (Fig. 4A), it was partially lost in low- and intermediate-grade carcinomas (Figs 4C and 4E), and entirely lost in high-grade carcinomas (Fig. 4G).

In contrast to CD95L, CD95 mRNA expression was high in benign breast tissue in favour of the membrane-bound CD95 isoform (CD95tm) (Table II). CD95tm mRNA levels decreased and were inversely correlated with the histopathological grade of the carcinomas (r = − 0·88; p<0·01; Fig. 3B). When compared with benign mammary tissues, in G1 carcinomas CD95tm mRNA levels were diminished by 30 per cent, in G2 carcinomas by 50 per cent, and in G3 carcinomas by 80 per cent (Table II). Consistent with the finding that various malignancies are associated with an elevated expression level of soluble CD95,[12] the contribution of soluble CD95 (CD95sol) to overall CD95 receptor mRNA expression increased in G1, G2, and G3 breast carcinomas, compared with non-malignant tissues

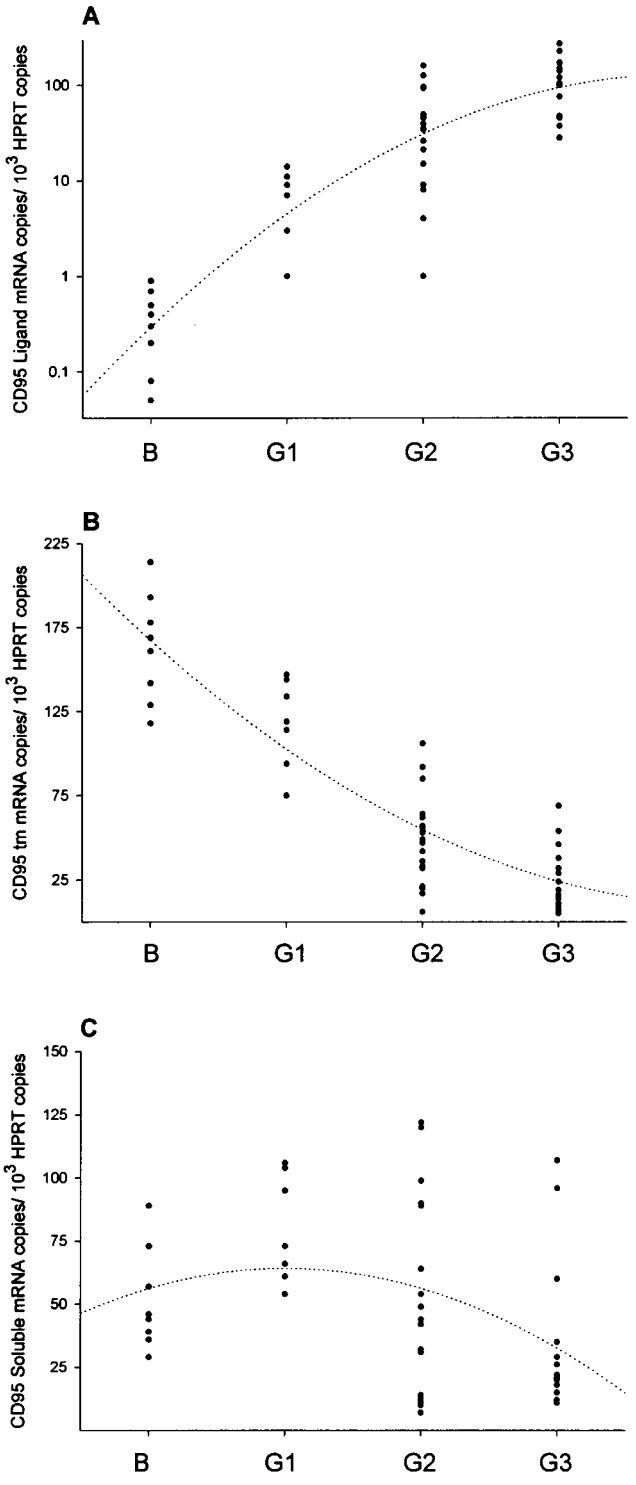

Fig. 3—Correlation of CD95 ligand and receptor mRNA expression with the histopathological grade of mammary tissue. The *x*-axis shows benign mammary tissue (B) and differently graded breast cancer (G1–G3). Filled circles reflect CD95 ligand (A; CD95L), membrane bound CD95 (B; CD95tm), and soluble CD95 (C; CD95sol) mRNA levels as determined by quantitative competitive RT-PCR in mammary tissue samples from 47 patients studied

(Table II). However, there was no obvious correlation between CD95sol mRNA levels and histopathological grade ($r=0{\cdot}35$, $p>0{\cdot}05$; Fig. 3C).

Table III—Correlation of CD95 ligand mRNA levels with prognostic markers of breast tumours*

Correlation between CD95L mRNA expression and	*n*	*r*	*p*
Grade (benign, G1–G3)	47	0·90	<0·01
T (T_0–T_4)	47	0·69	<0·05
N (N_0–N_3)	47	0·72	<0·05
M (M_0/M_1)	47	0·74	<0·05
ER expression	39	0·65	<0·05
PgR expression	39	0·38	n.s.

*CD95 ligand (CD95L) mRNA levels were studied in 47 breast tumours and correlated with histopathological grade, staging parameters (T, N, and M), and receptor expression for oestrogen (ER) and progesterone (PgR). *n*=number of patients; *r*=correlation coefficient; *p*=level of statistical significance; n.s.=not statistically significant.

Detection of apoptosis in mammary tissue of different histopathological grades in situ

The target cells of the CD95L action *in vivo* were detected by TUNEL analysis of benign and grade III breast cancer tissues. In benign breast tissues ($n=3$), apoptotic cells were hardly detectable. In contrast, induction of apoptosis was shown in bystander cells in proximity to CD95 ligand-expressing breast cancer. In infiltrated (Figs 5A–5D) and non-infiltrated breast cancer tissue (Figs 5E–5G), bystander cells underwent apoptosis. As shown by double staining using anti-CD3 antibodies, the apoptotic bystander cells on infiltrated breast cancer sections were mainly tumour-infiltrating T cells (TILs; Figs 5A and 5B), whereas apoptotic cells at the edge of invading tumour tongues on non-infiltrated sections were stromal cells or sporadic lymphocytes lacking both CD3 and CD95L expression (e.g. B cells). The absence of $CD3^+$ TILs was verified by RT-PCR and immunofluorescence (not shown).

Induction of CD95 specific apoptosis in Jurkat T lymphocytes cultured on mammary tissue sections of high grade

In order to assess whether CD95L expression in breast cancer was functionally relevant and bystander cell apoptosis observed on the sections could be induced by CD95L expression in breast cancer, $CD95^+$ target cells (Jurkat T cells) were incubated on cryosections from mammary tissue. Only a few Jurkat T lymphocytes (5 ± 2 per cent, $n=4$) underwent apoptosis when cultured on non-malignant tissue, which was not significantly different from the rate of apoptosis when CD95L-mediated apoptosis was blocked by CD95:Fc-Ig (2 ± 1 per cent, $n=4$) or when Jurkat T lymphocytes were cultured alone (3 ± 1 per cent, $n=3$). In comparison, the extent of apoptosis was increased 10-fold (58 ± 5 per cent, $n=4$) when Jurkat T lymphocytes were cultured on sections from G3 tumours. This was largely inhibited by CD95:Fc-Ig (23 ± 5 per cent, $n=4$), indicating

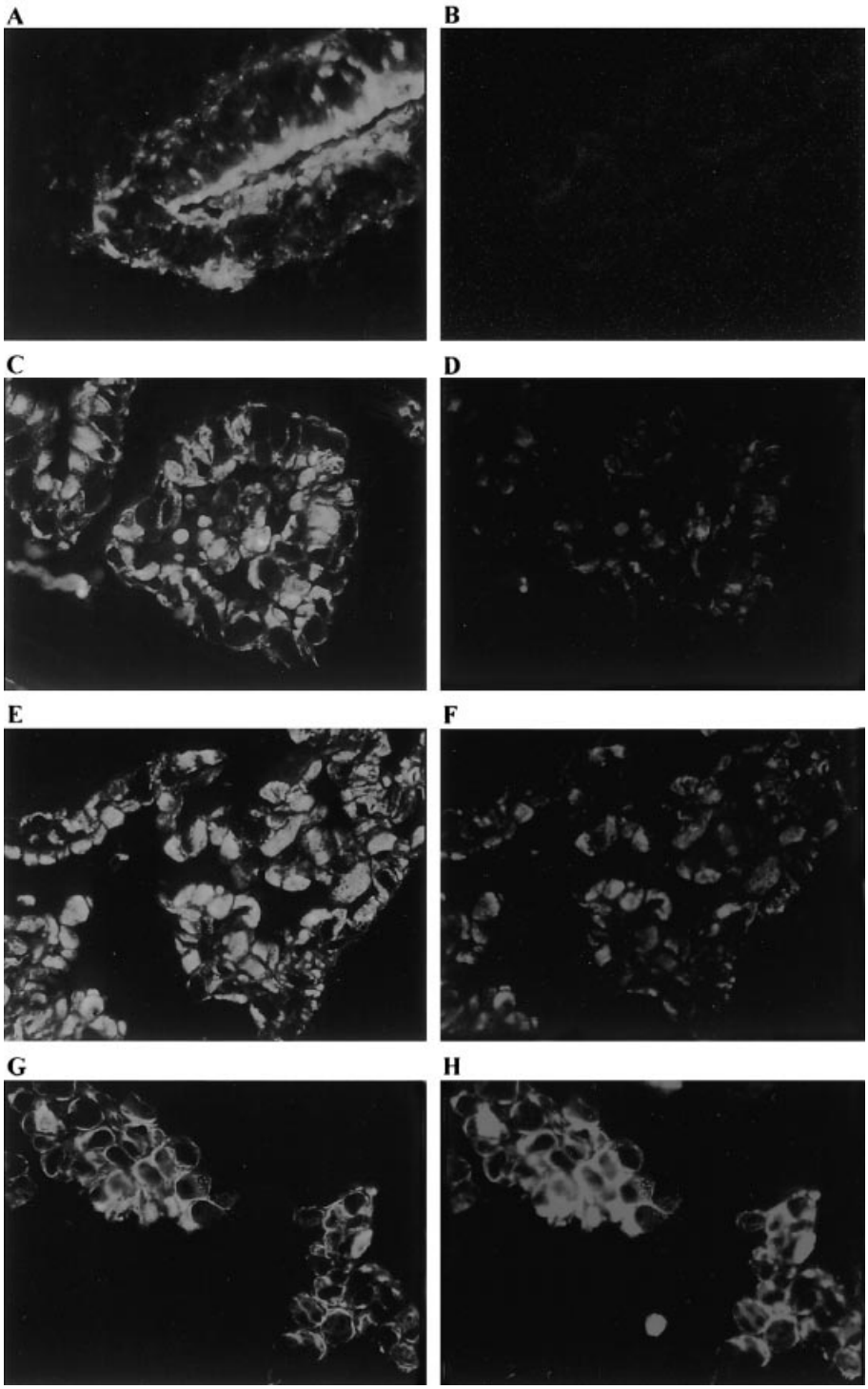

Fig. 4—Immunohistochemical analysis of CD95 ligand expression in breast tissues. Mammary tissues were double-stained by immunofluorescence, as described in the Materials and Methods section, with antibodies raised against mucin-1 (green fluorescent stain; see A, C, E, G) and CD95 ligand (red fluorescent stain; see B, D, F, H). In benign breast tissue (A, B: fibroadenoma), CD95 ligand expression is virtually absent. C and D show a grade I, E and F a grade II, and G and H a grade III breast cancer section. Whereas fibroadenomas retained luminal polarity of mucin-1 expression (A), this was partially lost in low- (G1; C) and intermediate-grade (G2; E) carcinomas and entirely lost in high-grade (G3; G) carcinomas

that Jurkat T-cell apoptosis induced by breast cancer was mediated to a large part by a CD95-dependent pathway.

DISCUSSION

Studying the CD95 system in breast cancer, we found that CD95 ligand mRNA expression was

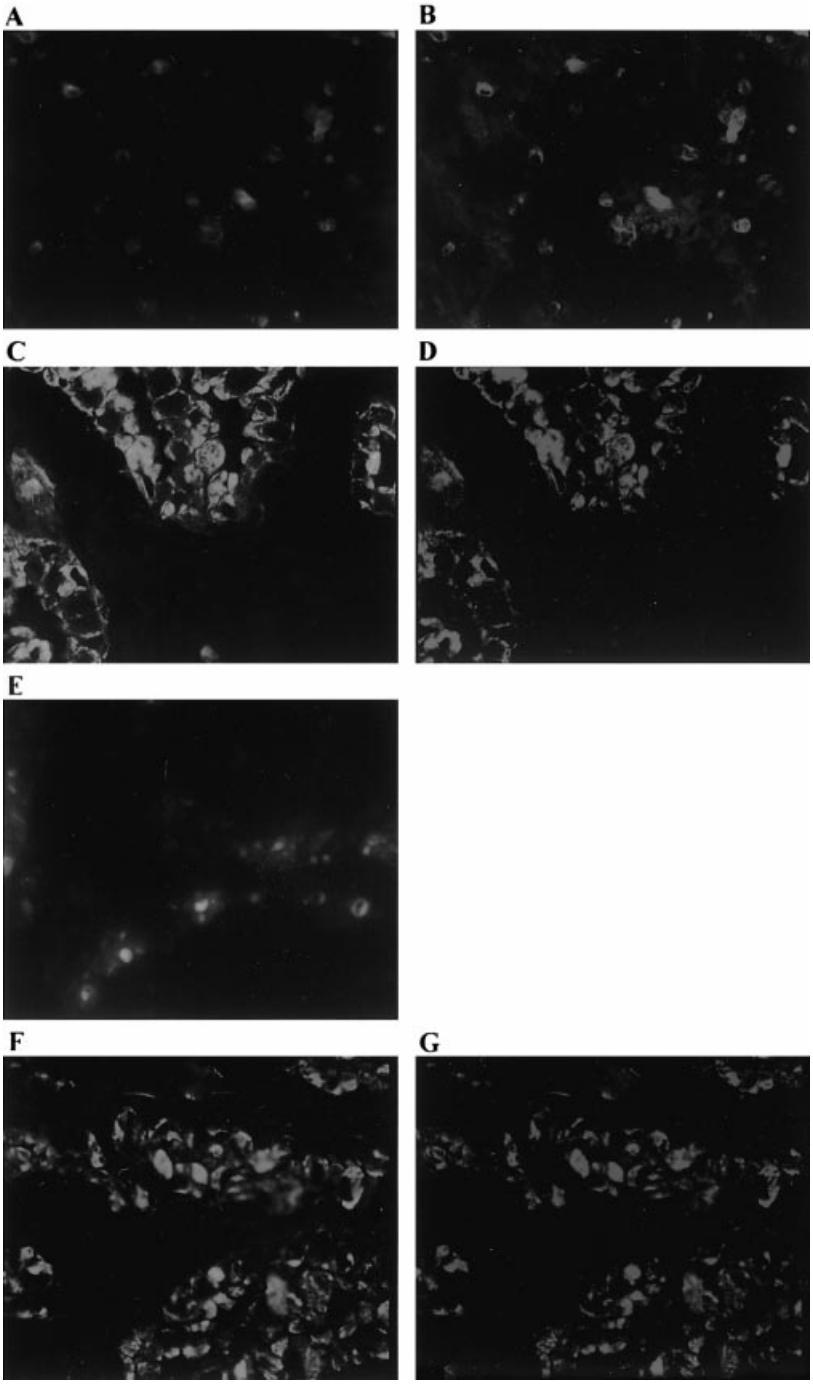

Fig. 5—Induction of apoptosis in bystander cells in the proximity of CD95 ligand-expressing breast cancer. A–D show grade III breast cancer tissue with tumour-infiltrating T cells and E–G a non-infiltrated section. In both cases, two directly adjacent 5 mm cryosections were double-stained for detection of apoptosis using the TUNEL method (A and E, blue fluorescent dye), CD3 (B, red fluorescent dye), Mucin-1 (C and F, green fluorescent dye), and CD95L expression (D and G, also red fluorescent dye), respectively, as described in the Materials and Methods section. For the tissue section shown in E–G, CD3 expression was detectable neither by RT-PCR at the mRNA, nor by immunofluorescence at the protein level (not shown)

inversely ($r = -0{\cdot}90$, $p<0{\cdot}01$) and CD95 receptor mRNA expression positively ($r=0{\cdot}88$, $p<0{\cdot}01$) correlated with the degree of tissue differentiation. Since CD95 receptor expression is correlated with sensitivity towards CD95L-mediated apoptosis in malignant cells,[24,25] it appears conceivable that the loss of CD95 expression during dedifferentiation of breast cancer could result in a loss of sensitivity towards CD95L-mediated apoptosis, e.g. induced by cytotoxic TILs. This is in line with findings in embryonal

carcinoma cells.[24] During retinoic acid-induced differentiation, embryonal carcinoma cells acquired sensitivity towards CD95-mediated apoptosis. In differentiated embryonal carcinoma cells, CD95tm mRNA levels were increased and CD95L mRNA expression was diminished when compared with undifferentiated cells.[24]

High-grade carcinomas expressed high levels of CD95L with potentially detrimental effects on the host organism, since both TILs and stromal cells in the proximity of CD95L-expressing breast cancer underwent apoptosis. Although it cannot be ruled out that these bystander cells were killed by factors other than CD95L, the capacity of frozen breast cancer sections to induce CD95L-specific apoptosis in $CD95^+$ target cells is suggestive of a role for CD95L in tumour-associated bystander cell cytotoxicity. Killing of $CD3^+$ TILs by breast cancer might favour 'immune escape' of the tumour, as described for other malignancies,[4,13–16] whereas elimination of surrounding stromal cells might favour tissue invasion. CD95L originating from advanced breast cancer could either damage the local environment or cause systemic tissue damage when CD95L was cleaved off from the cell membrane by matrix metalloproteinases.[5,6] Benign mammary epithelia, however, also express CD95L mRNA, although at levels hardly above the detection limit and CD95L protein could not be detected on the tissue sections by immunofluorescence (Fig. 4B).

Interestingly, CD95L mRNA expression was also correlated with the local tumour extension (T), dissemination into lymph nodes (N), higher frequency of metastasis (M), and decreased oestrogen receptor expression (Table III). This may possibly indicate that CD95L is more generally implicated in progression of breast cancer. On the other hand, high-grade carcinomas highly expressing CD95L tend to high proliferation with rapid metastatic spread and early loss of oestrogen receptor expression, which by itself is a criterion of dedifferentiation in breast cancer. Given the dependence of growth pattern, metastatic spread and oestrogen receptor expression on tissue differentiation of breast cancer, it remains unclear whether their correlation with CD95L expression is merely a consequence of the relationship between CD95L expression and histopathological grade, or might be considered independently.

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