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Primary gastric apoptosis-rich T-cell lymphoma co-expressing CD4, CD8, and cytotoxic molecules

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Abstract In contrast to primary gastric lymphomas of B-cell type, little is known about primary gastric T-cell lymphomas. We describe three cases with remarkably similar features: diffuse growth, epitheliotropism, medium too large cell size, high apoptotic rates, and a CD3+, CD4+, CD8+, CD45RO+ immunophenotype. Clonal TCRγ gene rearrangement was shown in two cases. Epstein-Barr virus infection was excluded in two cases. Taking advantage of fresh-frozen material, we analyzed two cases further, revealing CD5–, CD16+, CD56–, CD57–, CD25+, CD30+, CD103 (αEβ7)+, bcl-2 protein+, CD95+, CD95 ligand(L)–. CD95L, however, was detected in histiocytic and fibroblastoid by stander cells. The lymphomas expressed granzyme B, perforin, and the TIA-1 antigen in various combinations. All three cases had a very unfavorable clinical course characterized by local recurrence and/or dissemination to other epithelial sites, leading to death within 6–12 months after the initial diagnosis despite surgery and aggressive antineoplastic treatment. These data suggest a novel variant of peripheral T-cell lymphoma operationally characterized as primary gastric, apoptosis-rich, CD103+, EBV-, T-cell lymphoma co-expressing CD4, CD8, CD16 and cytotoxic molecules.

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Introduction

Primary gastric T-cell lymphomas are far more rare than primary gastric lymphomas of B-cell type. Since the first description by Weis et al. in 1986 [40] fewer than 40 cases have been reported [3, 12, 14, 17, 18, 23, 24, 26, 28, 30, 31, 38, 41]. Most of the cases described had a CD4+ helper/inducer phenotype [17, 26, 38]. Only 3 cases in the literature revealed a CD8+ suppressor/cytotoxic phenotype [3, 14, 40]. In this paper we present the clinical features, the course, and the pathology of three primary gastric T-cell lymphomas which co-expressed CD4 and CD8. The tumor cells were medium to large in size, had a clear cytoplasm, and showed a high proliferation rate. The apoptotic index was high, as revealed by in situ nick end labeling (TUNEL). Postoperative survival was poor (6–12 months).

Materials and methods

Tumor specimens

Tumor samples of patients 1 and 2 were drawn from the files of freshly frozen lymphoma tissue collected at our institute over a period of 15 years, which include more than 50 specimens of primary gastric lymphomas of B-cell type. Case 3 was provided by PD Dr. H.P. Spichtin, Basel, Switzerland as a formalin-fixed and paraffin-embedded sample.

Immunohistochemistry

The following monoclonal primary antibodies (mAbs) were used: CD95 (anti-Apo-1) was kindly provided by P.H. Krammer (Heidelberg, Germany). Anti-HLA-ABC (W6/32) and anti-HLA-DR (ISCR3) were obtained from G. Moldenhauer (Heidelberg); CD27 (clone CLB-27) was kindly supplied by R.A.W. Van Lier (Amsterdam, The Netherlands) and CD40 (clone G28–5), by J.A. Ledbetter (Seattle, Wash.); CD1c (clone L161), CD56 (clone T199) and CD103 (HML-1) were obtained from Dianova (Hamburg, Germa-

ny); CD3 (clone SK7), CD4 (clone SK39), CD8 (clone SK11), CD16 (clone GO22), CD57 (clone HNK-1) were purchased from Becton Dickinson (Mountain View, Calif.); for CD3, CD4, and CD8 staining on paraffin sections a polyclonal CD3 antiserum from Camon (Wiesbaden, Germany), a CD4 mAb (clone 1F6) from Novocastra (Newcastle-upon-Tyne, UK), and a CD8 mAb (clone C8/144b) from Dakopatts (Copenhagen, Denmark) were used. MAbs against CD5 (clone DK23), CD25 (clone ACT-1), CD30 (clone Ber-H2), and bcl-2 (clone 124) were supplied by Dakopatts; anti-perforin (clone 2G9) was obtained from Diagnostic Products Corporation (Los Angeles, Calif.); TIA-1 antibody was purchased from Coulter (Hialeah, Fla.); anti-granzyme B (clone MAB3070) was obtained from Chemicon (Temecula, Calif.); antibody to CD95 ligand (CD95L) (clone G247-4) was obtained from Pharmingen (San Diego, Calif.). A polyclonal biotinylated sheep antibody to mouse Ig (reactive with all mouse isotypes) and a streptavidin-biotinylated peroxidase complex were provided by Amersham (High Wycombe, Bucks., UK) and Dako, respectively; 3-amino-9-ethylcarbazole and *N'N*-dimethylformamide were obtained from Sigma (St. Louis, Mo.). Immunostaining was performed using an indirect streptavidin/biotin-peroxidase method (for details see [22]). The secondary antibody contained 5% pooled human immunoglobulin (Ig)G to inhibit cross-reactions with human surface Ig. Negative controls were performed without the primary antibody and by employing several irrelevant mAbs of different mouse Ig isotypes.

Evaluation of immunostaining

The staining of the tumor cells and of the intraepithelial lymphocytes in the tissue sections was scored as follows: +, all cells strongly positive; (+), all cells weakly positive; –, absence of staining for these cells.

TCRγ gene rearrangement

Paraffin-embedded tissue was deparaffinized in xylene and ethanol and digested overnight in TEN (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, 0.1 M NaCl) buffer containing proteinase K. After heat inactivation, 1 µl of the tissue lysate was subjected to polymerase chain reaction (PCR). DNA from peripheral blood lymphocytes obtained from a healthy donor and water served as controls. Amplification of the TCRγ rearrangement was performed in a 50-µl reaction containing 1 pmol/µl of a 5' (TVg, 5' AGGGT TGTGTTGGAATCAGG 3' and a 3' (TJγ, 5'CGTCGACAACAA GTGTGTTTCCAC 3') consensus primer complementary to conserved sequences of the TCRγ gene [4]. Thirty-five cycles were run at an annealing temperature of 58°C. Ten microliters of the PCR product was separated on an 8% nondenaturing polyacrylamide gel. In a second round, 1 µl of the PCR product was submitted to 20 cycles of a semi-nested amplification at an annealing temperature of 58°C, using the same 5' primer and a nested 3' consensus primer (Jγ INT, 5' GGATCCACTGCCAAAGAGTTT CTT 3') located 17 bp in the 5' direction of TJγ [5]. Then 10 µl of the reaction product was size-fractionated on an 8% polyacrylamide gel and compared with the corresponding PCR product from the first amplification step.

In situ detection of apoptosis

In situ TdT-mediated dUTP-biotin nick end labeling (TUNEL) [13] was performed according to a modification described by Surh and Sprent [35]. Briefly, formalin-fixed and paraffin-embedded tissue sections were deparaffinized, digested with proteinase K (20 µg/ml), and incubated with 0.1% H_2O_2. Samples were then treated with TdT (2.5 U/µl, Promega, Madison, Wis.) and digoxigenin-11-dUTP (2 pmol/µl, Boehringer, Mannheim, Germany) diluted in cacodylate buffer, incubated with anti-digoxigenin sheep Fab antibody fragments (5 µg/ml, Boehringer, Mannheim) and subsequently with horseradish peroxidase-conjugated anti-sheep IgG donkey $F(ab')_2$ (5 µg/ml, Jackson Immunoresearch, West Grove, Pa.). Bound peroxidase was detected with 3-amino-9-ethylcarbazole (0.1 mg/ml), followed by counterstaining in Meyer's hematoxylin.

Southern blot hybridization

Ten micrograms of cleaved cellular DNA was separated by agarose gel electrophoresis and transferred onto a nylon filter. Hybridization was performed in 50% formamide, 2×SSC at 42°C using the ^{32}P-labeled 3.07 kb EBV-Bgl II U fragment as probe. This probe detected the internal repeat I sequence in the EBV genome with a sensitivity better than 0.1 EBV DNA copies per cell.

Results

Patients and clinical course

Some clinical data of the three patients with primary gastric T-cell lymphoma are given in Table 1. All three patients had had initial leading symptoms of gastric pain and nausea leading to gastroscopy and biopsy. At the time of diagnosis no further neoplastic sides were detected by radiology and echography. The peripheral blood count was normal. A bone marrow biopsy was negative for all three patients. No history of celiac disease is known for any of the three patients. For patients 1 and 3, partial gastrectomy was initially characterized as potentially curative. Patient 2 showed a gastro-colic fistula and local bulky disease and the lymphoma was therefore classified as stage IV. Nevertheless, local recurrence was the cause of death in these patients. Furthermore, the lymphoma of patient 1 disseminated to the lungs and the lymphoma of patient 2 spread to the skin, the nose, and the lungs. The time from diagnosis to death from progressive disease ranged from 6 to 12 months despite combined antineoplastic therapy. Thus, each of these tumors proved to be highly aggressive.

Histology including mitotic and apoptotic indices

Histological features common to the three lymphomas were: diffuse growth pattern, no significant matrix induction, lack of angiocentrism, but clear-cut mucosa association with glandular displacement and marked epitheliotropism, which led to glandular destruction and mucosal ulceration (Fig. 1a, b). There were no typical lymphoepithelial lesions. The lymphoma cells had pale cytoplasm, and mitoses were typical throughout. The gastric mucus cells within the lymphoma showed variable degrees of reactive atypia and featured decreased mucus production. As revealed by a silver stain, there was no *Helicobacter pylori* infection.

Case 1 comprised a mixed population of medium-sized and large lymphoma cells with transparent, very pleomorphic nuclei and small eosinophilic nucleoli. Further peculiarities were: moderate eosinophilia; abundant apoptotic bodies throughout the tumor, corresponding to

Table 1 Clinical data of patients 1, 2, and 3 with primary gastric T-cell lymphoma. Staging according to Radaszkiewicz et al. 1992 [29] (*mL* malignant lymphoma, *RX* radiotherapy, *CHOEP* cyclophosphamide, vincristine, doxorubicin, etoposide, prednisolone)

Case no.	Age (years)	Sex	Duration of symptoms prior to admission	Surgery	Diagnosis	Initial stage	Subsequent therapy	Clinical course	Postoperative survival
1	63	F	5 months	Partial gastrectomy	Gastric T-cell mL, mixed, medium and large	E II 1	RX+CHOP	Gastric relapse 5 months after surgery; pulmonary lymphoma involvement; death from extensive disease and cachexia	6 months
2	33	M	5 months	Gastrectomy, splenectomy, partial pancreatectomy, partial colectomy	Gastric T-cell mL, predominantly medium sized	EIV[a]	CHOEP	Cutaneous metastasis; nasal destruction; pleural lymphomatous involvement	11 months
3	62	M	6 months	Partial gastrectomy	Gastric T-cell mL, predominantly medium-sized	EI1	No further therapy	Local bulky recurrence; death from intestinal perforation	12 months

[a] EIV had local bulk with gastro-colic fistula

about 5% of apoptotic events in TUNEL; and irregular, abscess-like necroses filled with neutrophils. The Ki-67 proliferation rate of the neoplastic component was around 20%. Case 2 had a neoplastic component mainly composed of medium-sized lymphoma cells intermingled with a small subset of large blasts. Nuclei were pleomorphic with very irregular outlines and distinct small nucleoli. Further, there was marked eosinophilia. The Ki-67 proliferation rate of the neoplastic component was around 40–60%. The number of apoptotic events was around 5% and consistent throughout the neoplastic component. Case 3 consisted predominantly of medium-sized lymphoma cells with globular to ovoid nuclei and dense chromatin concealing nucleolar structures. In addition, there was a subpopulation of large cells with a broader rim of cytoplasm and slightly larger nuclei. There was no eosinophilia, but mast cells were numerous. The Ki-67 proliferation rate of the neoplastic component was around 80%. In TUNEL staining, apoptoses were about 5% throughout the lymphoma section (Fig. 2a, b). Outside the neoplastic infiltration the mucosa was not normal. Case 1 had a combination of lymphofollicular and lymphocytic gastritis. Case 2 had a mild chronic gastritis with occasional lymph follicles, and case 3 displayed lymphocytic gastritis.

Immunohistology

The immunohistochemical data of antigen expression of all three cases are given in Table 2. For case 3 only paraffin-embedded tumor tissue was available, showing positive staining for CD3, CD4, CD8 and CD45R0. On comparison of the immunoprofile of cases 1 and 2 a very similar expression pattern of the studied antigens emerged: both cases were HLA-ABC positive but lacked HLA-DR. T-cell differentiation antigens CD3, CD4 and CD8 were expressed in both cases (Fig. 1c, d). CD45RA was negative while CD45RO was positive in both cases. The natural killer (NK) differentiation antigen CD16 was expressed in both cases, whereas CD56 and CD57 were both absent. Regarding activation markers, CD25 was positive in cases 1 and 2 and CD30 was detected in all three cases. Expression of CD40 was limited to case 1. The mucosa adhesion molecule CD103 (which is the integrin receptor $\alpha E\beta 7$) was positive in cases 1 and 2. Molecules involved in cell survival and death were expressed as follows: the bcl-2 protein was positive in all three cases. Both CD95 and a 15-kDa protein associated with cytolytic granula (TIA-1) were expressed in cases 1 and 2 (Fig. 2e, f). Perforin was positive in case 1 (Fig. 2c). Granzyme B was positive in cases 1 and 3 (Fig. 2d). The CD95L was negative in all three cases. Intermingled macrophages and fibroblastoid cells in the lamina propria, however, were positive for CD95L (Fig. 2g). Cases 1 and 3 showed an accompanying lymphocytic gastritis. Paradigmatically, for case 1 the immunophenotype of the intraepithelial lymphocytes was investigated. The intraepithelial lymphocytes re-

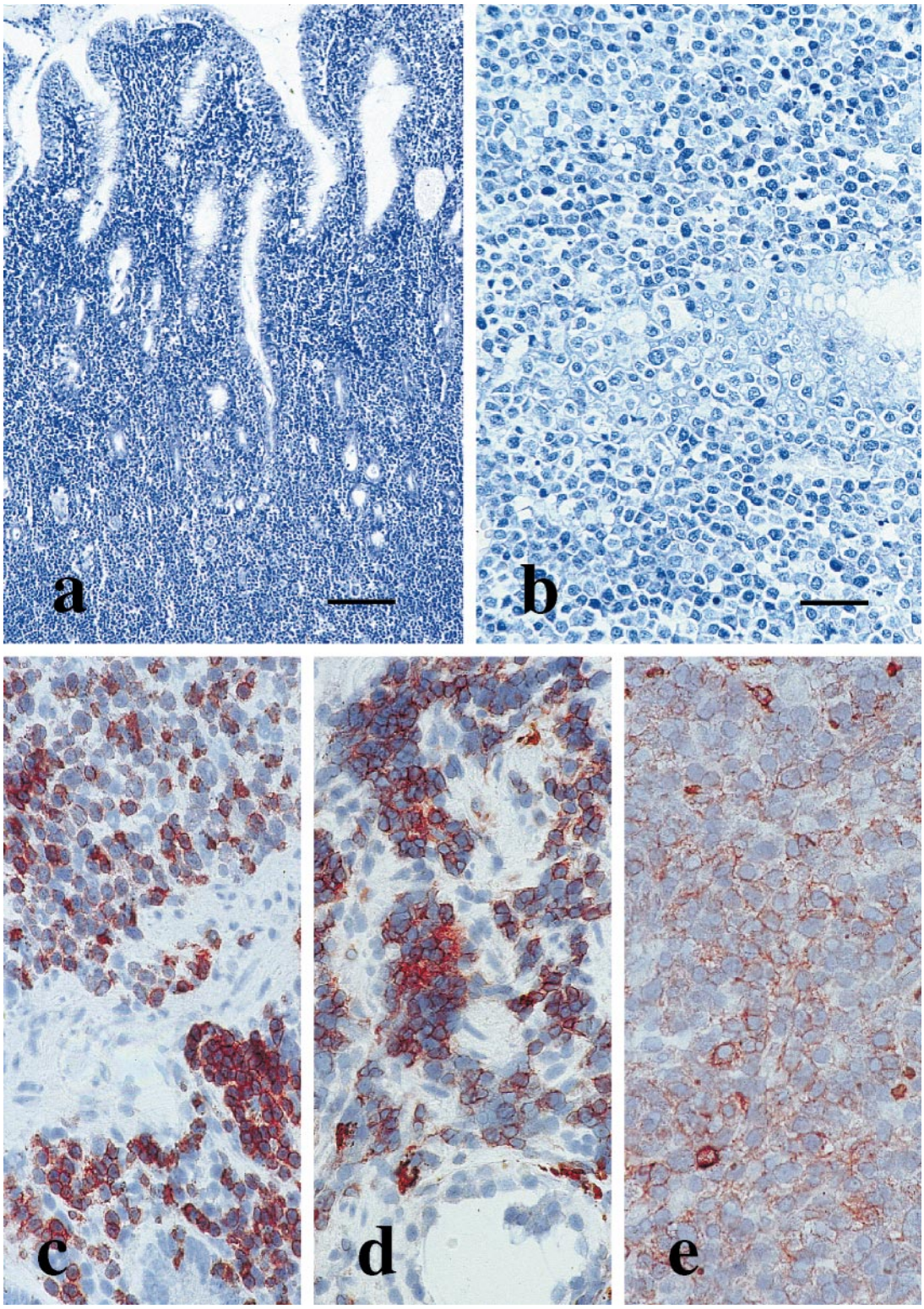

Fig. 1a, b Histomorphology of case 3. **a** The growth pattern is diffuse, with infiltration and destruction of the gastric mucosa. Giemsa, ×78; *scale bar* 100 µm **b** The lymphoma cell population is made up of mainly medium-sized cells with a small subset of blasts. A marked epitheliotropism of lymphoma cells is present. Giemsa, ×314; *scale bar* 25 µm **c**–**e** Immunohistology, case 2. **c** The lymphoma cells are weakly CD3 positive. Strongly staining cells are intermingled reactive T-cells. **d** CD4 is strongly expressed by the lymphoma cells. **e** CD8 is weakly but consistently expressed by the lymphoma cells. **c**–**e** ×314

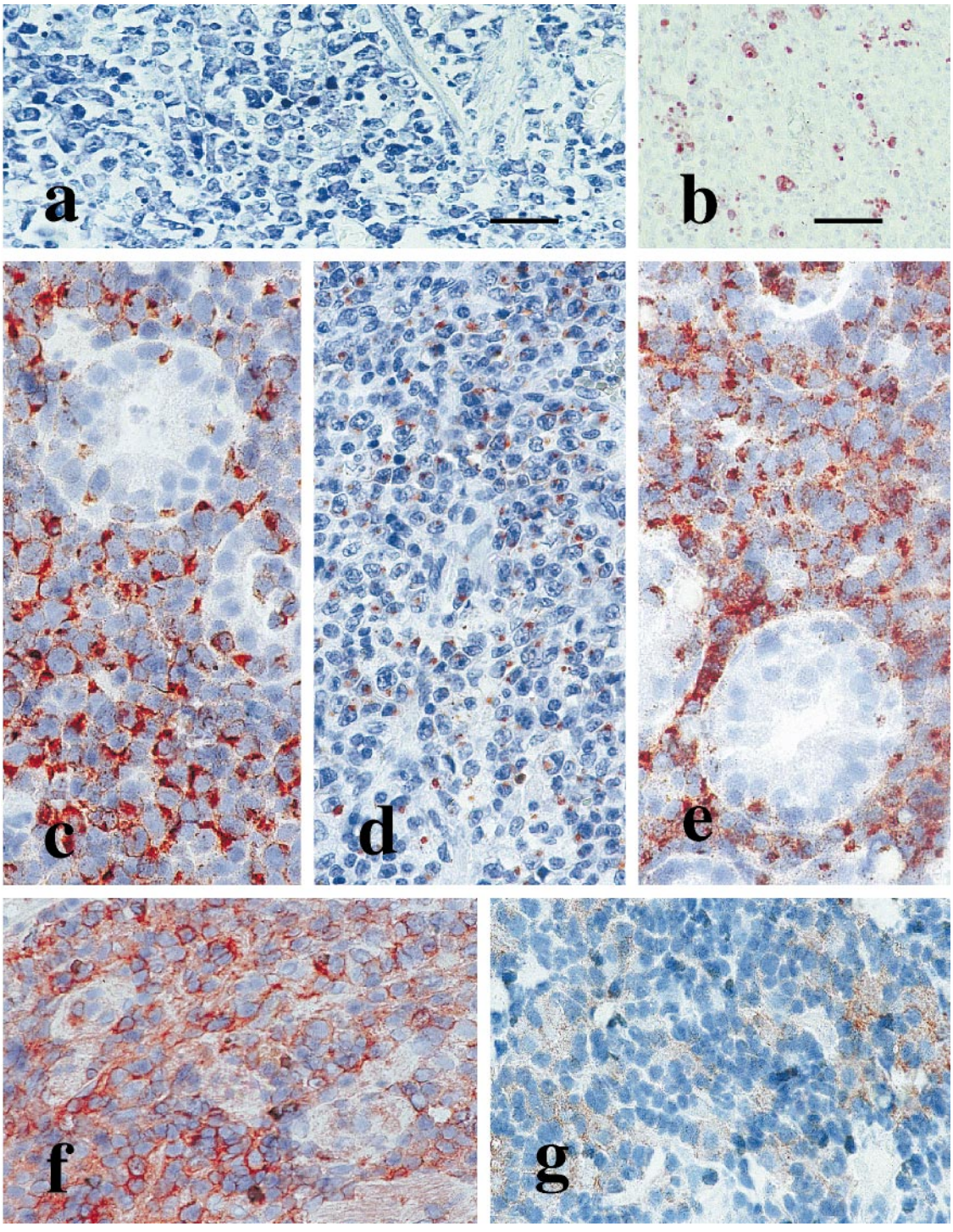

Fig. 2 **a** Case 3. Histology reveals partial tumor breakdown with multiple apoptotic bodies throughout the lymphomatous infiltrate. Giemsa, ×314, *scale bar* 25 µm. **b** In situ nick end labeling (TUNEL) confirms a high apoptotic index within the lymphoma population as shown by stained apoptotic nuclei. ×184, *scale bar* 50 µm **c** Case 1. Lymphoma cells are consistently perforin positive. Epithelial cells of the gastric glands are perforin negative. Intraepithelial lymphoma cells are perforin positive. **d** Case 3. Granzyme B is detected in a dot-like fashion in the lymphoma cells. Staining on paraffin section **e** Case 2. Immunohistology shows a strong dot-like cytoplasmic staining for TIA-1. Epithelial cells of the gastric glands are TIA-1 negative, whereas intraepithelial lymphoma cells are TIA-1 positive. **f** Case 2. The lymphoma cells are heterogeneously positive for CD95. **g** Case 2. Lymphoma cells are negative for CD95L. Intermingled histiocytes and fibroblastoid cells are CD95L positive. **c**–**g** ×314

Table 2 Immunohistochemical profiles of the three primary gastric T-cell lymphomas studied [+ positive, (+) weak expression, – negative, *n.a.* not analyzed]

MHC	Clone	Case 1	Case 2	Case 3
HLA-ABC	W6/32	+	+	n.a.
HLA-DR	ISCR3	–	–	n.a.
T-cell differentiation				
CD1c	L161	–	–	n.a.
CD3	SK7/CD3 antisera	+	(+)	+
CD4	SK3/1F6	(+)	+	(+)
CD5	DK23	–	–	n.a.
CD8	SK11/C8/144β	(+)	(+)	+
CD27	CLB-27	–	–	n.a.
CD45RA	4KB5	–	–	n.a.
CD45R0	UCHL1	+	+	+
NK cell differentiation				
CD16	GO22	(+)	(+)	n.a.
CD56	T199	–	–	n.a.
CD57	HNK-1	–	–	n.a.
Activation markers				
CD25	ACT-1	(+)	(+)	n.a.
CD30	BerH2	(+)	(+)	–
CD40	G28-5	(+)	–	n.a.
Mucosal adhesion molecule				
CD 103 (αEβ7)	(HML-1)	+	+	n.a.
Molecules involved in survival and death				
bcl-2 protein	124	(+)	(+)	+
CD95	anti-Apo-1	(+)	(+)	n.a.
CD95L	G247-4	–	–	n.a.
Perforin	2G9	(+)	–	–
15-kDa protein associated with cytolytic granula	TIA-1	+	+	–
Granzyme B	Mab 3070	+	–	+

vealed the following immunophenotype: HLA-ABC+, HLA-DR+, CD3+, CD4–, CD8+ and CD57–.

TCRγ gene rearrangement

In cases 2 and 3, PCR analysis of the TCRγ rearrangement revealed single bands of about 190 bp each after polyacrylamide gel electrophoresis, indicating clonal origin. Specificity of the signal was confirmed by a slightly shorter PCR product after semi-nested amplification, corresponding to the internal location of TJγ INT in relation to TJγ. A polyclonal pattern was detected in case 1, which yielded a diffuse smear, similar to the PCR product amplified from peripheral blood lymphocytes.

EBV status

The two cases which could be examined to this end (cases 1 and 2) were both negative for EBV sequences by Southern blot analysis.

Discussion

The vast majority of primary gastric lymphomas are of B-cell type [15]. Primary gastric T-cell lymphoma is exceedingly rare. Eight out of 13 cases from the literature with a CD4+ phenotype and a reported follow-up died within 39 months (median 15 months) after diagnosis, while 1 was alive with recurrence after 20 months. Four patients had no residual disease after surgery and chemotherapy [17, 23, 31, 38, 41]. Two out of 3 reported patients with a CD8+ phenotype died within 6 months after diagnosis, while 1 had no residual disease after 6 months of follow-up [3, 14, 40].We present three cases of primary gastric T-cell lymphoma with a CD3+, CD4+, CD8+ phenotype with a very aggressive course and the death of patients 6–12 months after surgery and subsequent chemo/radiotherapy.

Immunohistology of the three cases revealed an almost identical immunophenotype. CD4/CD8 co-expression is a novel finding in primary gastric T-cell lymphoma. Those reported in the literature were either of CD4+ or of CD8+ phenotype. One of the CD8-positive cases was CD4 negative [5]. For the other two cases no CD4 data were available. This is due to the fact that CD4 reagents working on paraffin-embedded tissue were not available at the time of publication. Remarkably, both cases had an unfavorable clinical course [14, 40]. Our cases were CD45R0 positive, underlining the T-cell lineage of these lymphomas. There was no prominent NK phenotype, since of the NK cell differentiation antigens analyzed, only CD16 was expressed. The lymphoma cells further expressed CD25, CD30 and, in one case, CD40 molecules and therefore correspond to activated T-cells. The presence of CD103 in two of the three tumors may explain the epitheliotropism of the lymphoma cells, and it supports the hypothesis that they originate from the intraepithelial T-cell component and/or T-cells from the lamina propria [6]. CD103 expression might also account for the high propensity of all three tumors to recur locally and to disseminate to other epithelial sites, such as nose, lung and skin. The expression of various molecules involved in cell survival and death merits attention. The lymphoma cells expressed the bcl-2 protein on the one hand and, on the other, a variety of pro-apoptotic molecules. The death receptor CD95 was expressed. The lymphoma cells were equipped with cytotoxic molecules such as perforin, TIA-1 and granzyme B. The presence of cytotoxic molecules underlines the high cytolytic potential of these lymphoma cells. These molecules are mediators of the apoptotic cascade in target cells [36, 37] and are largely restricted to cytotoxic T-cells [2, 16, 20]. Furthermore, these molecules have been identified in a variety of peripheral T-cell lymphomas [9, 10]. In our study the apoptotic index revealed by TUNEL was about 5%. This has to be regarded as a high proportion of apoptotic cells compared with the T-cells in the thymus, which show an apoptotic rate below 1% [34], or apoptotic indices in highly malignant B-cell lymphomas with a maximum value of 2.5% [18]. The high apoptotic index

within the three cases of primary gastric T-cell lymphomas presented is remarkable and might suggest some intraneoplastic suicide/fratricide. The frequency of apoptotic events might have been further augmented by CD95 ligand expression in macrophages and fibroblastoid cells within the lymphomatous tissue. Evidence for a CD95-based cell death has been shown for cytotoxic T-cells killing CD95-expressing T-cells and CD95-positive tumor cells from a lymphoma T-cell line [25, 42]. In addition, CD95 ligand can be shed from the cell surface and then activate the CD95 receptor from solution [11]. The expression of bcl-2 protein does not necessarily argue against this view, since it has been shown that a caspase-cleaved fragment of bcl-2 can even trigger programmed cell death [8].

PCR of the TCRγ rearrangement showed clonality only in cases 2 and 3, whereas in case 1 a polyclonal pattern was obtained. However, the absence of a distinct band does not generally exclude T-cell neoplasm. Failure to demonstrate clonality by TCR-PCR has been described in cases that were unambiguously diagnosed as T-cell lymphoma by means of histology and immunohistochemistry [21, 32]. Possible explanations for false-negative results include imperfect rearrangements with incorrect or no juxtaposition of V-J elements [1] and technical reasons, such as poor PCR priming, failure of the amplification reaction, degradation of the underlying DNA and small lymphoma cell population in relation to a polyclonal background [39]. The last, however, could be excluded for case 1, since hematoxylin/eosin staining of an adjacent section of the material investigated showed that the proportion of tumor tissue was well above the detection level of 3% [4].

The immunophenotypic characteristics of our gastric T-cell lymphoma cases are at variance with enteropathy-associated intestinal T-cell lymphoma, which is generally CD4 negative, shows heterogeneous expression of CD8, and is positive for EBV in up to 36% of cases [27, 33]. Our cases also seem to differ from nasal and nasal-type NK/T cell lymphomas in being CD16 positive and CD56 negative. Typically nasal and nasal-type NK/T cell lymphomas have a strong association with EBV and have their TCR genes in germline configuration [7].

In conclusion, we report three cases of primary gastric T-cell lymphomas co-expressing CD3, CD4, CD8, and CD103 with strikingly similar morphologic and immunohistological features, such as high apoptotic indices and “necrotic” lymphoma breakdown associated with the presence of the cytotoxic molecules, e.g., granzyme B, perforin and TIA-1. Furthermore, in the two cases in which fresh-frozen tissue was available, the death receptor CD95 was expressed and the lesions contained CD95L-expressing stroma cells. This setting might also have contributed to the high apoptotic rates. Nevertheless, all three patients presented with a very unfavorable clinical course owing to recurrence and dissemination to other epithelial sites. These commonly found characteristics suggest the existence of a rare mucosa-associated T-cell lymphoma variant differing from enteropathy-associated T-cell lymphoma and nasal and nasal-type NK/T cell lymphomas. This variant can be defined operationally as primary gastric apoptosis-rich, CD103+, EBV–, T-cell lymphoma co-expressing CD4, CD8, CD16 and cytotoxic molecules.

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