Abstract Molecular analysis of T-cell receptor (TCR) chain rearrangement has recently become an attractive tool for demonstrating the clonal origin of cutaneous T-cell lymphoma (CTCL) and for identifying the malignant clone at the molecular level. Over the past decade a number of attempts have been made to culture malignant CTCL cells using standard procedures and these attempts have resulted in several cell lines from the peripheral blood of Sézary syndrome, mycosis fungoides and CD30+ lymphoma patients. However, so far it has not been proven by sequence analysis that the cultured T cells truly represent the malignant cells. Aiming to functionally analyze the malignant T cells at a clonal level, we generated a total of 150 T-cell clones (TCC) from lesional skin and peripheral blood of three patients with mycosis fungoides and one patient with a CD30+ lymphoma. Cells were grown either in the presence of autologous irradiated peripheral blood feeder cells using various conditions for T-cell stimulation by direct outgrowth or from skin specimens with various cytokine combinations. In order to identify the malignant TCC we used N-region-specific PCR and compared TCR γ-chain sequences from clones of lesional skin with in vitro-generated TCC. With the methods employed, none of the 150 established cell lines was found to be identical to the malignant TCC which was readily detected in lesional skin. Our results indicate that standard cell culture methods are not suitable for growing low-grade CTCL cells from the skin but give rise only to benign infiltrating T cells.

Keywords T-cell clones · Skin · T-cell lymphoma · T-cell receptor-γ PCR

Introduction

Cutaneous T-cell lymphomas (CTCL) are a heterogeneous group usually of low-grade lymphomas primarily involving the skin. Among these, mycosis fungoides (MF) is the most frequently occurring type of low-grade CTCL [1]. Despite its rather benign clinical course, MF inevitably progresses to tumor stage. To better understand the biology of this disease and to develop specific therapeutic strategies, a number of attempts involving cell culture, cytogenetic and molecular methods have been made to further characterize the malignant cells themselves [2, 3, 4, 5, 6]. All of these strategies have been hampered by the fact that presently no definitive molecular or phenotypical marker for the identification of malignancy at the single-cell level exists in low-grade CTCL. Although CTCL cells characteristically display a convoluted nuclear morphology, it is often impossible to clearly distinguish them from a large number of accompanying benign infiltrating T cells by routine histopathology or immunohistochemistry [7].

In the absence of a clear concept for the optimal growth requirements of CTCL cells, a variety of cell culture methods have been explored over the past two decades. So far this has resulted in the growth of a small number of documented cell lines only, which were derived from lesional skin or peripheral blood of patients with Sézary syndrome [3, 4, 8], MF [9], CD30+ cutaneous large cell lymphoma [10] or, more recently, pleomorphic T-cell lymphoma [11]. Proving that the cultured lines are identical to the dominant, and by definition malignant, T-cell clone (TCC) in vivo has rarely been attempted at the molecular level, although proof of malignancy appears to be crucial considering the large number of inflammatory T cells in the malignant lesions.

Based on T-cell receptor (TCR) gene rearrangement also in CTCL, PCR analysis of TCR-γ gene segments has become widely used for investigating clonality [12, 13]. It is generally accepted that the existence of a single clone in CTCL lesions provides substantial evidence of malig-
nancy. To date, this important aspect has only rarely been analyzed by TCR-β or TCR-γ rearrangement in T-cell lines established from patients with CTCL.

Abrams et al. succeeded in establishing a cell line from the blood of a patient with Sézary syndrome that showed the same TCR rearrangement as the patient’s native peripheral blood mononuclear cells (PBMC) [4]. In contrast, investigating cultured TCC derived from lesional MF by TCR-γ chain sequencing, Ho et al. were not able to confirm malignancy of clones cultured from the skin of patients with this type of CTCL [9]. More recently, having established two most probably malignant clones from a pleomorphic large T-cell lymphoma, Bagot et al. succeeded in proving cytogenetic alterations identical to those in the original malignant TCC in lesional skin [11]. However, sequence data for the TCR gene were not provided.

The ability to regularly culture malignant lymphocytes from CTCL skin lesions would provide a unique opportunity for studying the biology of CTCL cells in vitro including various aspects of antitumor immunity. Therefore, the aim of the present study was to establish cultures from those T lymphocytes which, based on molecular sequencing, were the dominant and by definition malignant cells in lesional skin. Using IL-4 and IL-7, cytokines that have been reported to promote the growth of CTCL cells, we established a large series of TCC from the skin and blood of four patients with different stages of CTCL. For identification of malignant T cells in culture, the sequenced TCR-γ chain gene of the cultured clones was compared with the sequence of the clone that was found to dominate the original skin lesion of the donating patient.

Material and methods

Patients

Four patients were enrolled in this study, one with patch-stage MF (patient RM), two with tumor-stage MF (patients AR and HT) and one with cutaneous large-cell anaplastic CD30+ lymphoma (patient EO). All patients showed a typical clinical picture and the diagnosis was confirmed by histopathology including immunohistochecmistry which disclosed a CD4+ phenotype of atypical cells in all cases. Also, clonal rearrangement studies of the TCR-γ chain gene in skin biopsies supported the diagnosis in each case. Biopsies were taken either before a specific therapy had been started or at least 2 months after cessation of specific therapy.

Preparation of single cells from skin biopsy specimens

A 4-mm punch biopsy was taken from lesional skin for routine histology and PCR, and to establish TCC, another 4-mm punch biopsy was obtained. All patients gave written consent. Epidermis and dermis were separated by overnight incubation (4 °C) with dispase (2.4 U/ml; Boehringer Mannheim, Germany) as described previously [14]. The epidermis was incubated with 0.25% trypsin (Sigma, Deisenhofen, Germany) for 20 min at 37 °C and a single-cell suspension was prepared by gentle pipetting. Cells were washed in modified Hank’s solution with 50% heat-inactivated fetal calf serum (FCS) and 0.1% DNase (Boehringer). The epidermal cell yield varied between 0.2 and 0.7 × 10^6 cells per biopsy. The percentage of T cells was determined by flow cytometry (PasIII; Partec, Münster, Germany) using a monoclonal antibody (mAb) to CD3 (Ancell Corporation, Bayport, Minn.). The percentage of T cells ranged between 0.6% and 17%, correlating with the degree of epidermotropism of T cells found in the individual’s histology. The dermis was incubated for 5 h at 37 °C with an enzymatic cocktail containing collagenase (0.1%), DNase (0.2%), dispase (1.2 U/ml), hyaluronidase (0.1%; Sigma), EDTA (Biochrom, Berlin, Germany) and 10% FCS. The dissociated cells were washed and filtered through a sterile 50-µm nylon gauze. Absolute cell yields obtained from the dermal compartment ranged between 0.6 and 2.5 × 10^6 T cells (mean 18%). The viability of cells was at least 90% as determined by trypan blue exclusion.

Limiting dilution procedure and culture of TCC

Direct cloning of T cells was performed as described previously [15]. Different dilutions of epidermal and dermal cells were cocultured with 2 × 10^6 autologous irradiated (55 Gy) PBMC (Ficoll-Hypaque separation) in limiting-dilution plates (Nunclon, 96-well, round-bottomed plates; Nalge Nunc International, Denmark) in Iscove’s medium with 10% FCS, 1% penicillin/streptomycin (both Biochrom), IL-2 (20 U/ml), IL-4 (5 ng/ml), IL-7 (1 ng/ml) (all cytokines PBH, Hannover, Germany) and phytohemagglutinin (10 µg/ml; PHA; Sigma). TCC from patient HT were grown in the presence of IL-2, IL-4 and IL-7 at the concentrations given above and with additional stimulation by incubation with anti-CD3 (20 µg/ml) and soluble anti-CD28 (1 µg/ml). For stimulating the TCR, plates were coated with OKT3 mAb (Becton Dickinson) overnight at 4 °C prior to the addition of the cells. On day 14 of culture those wells containing more than 50 lymphoblasts were scored positive. Only cultures with a high probability of clonal growth (dilutions with less than 37% of growing cultures) [15] were expanded further.

TCC were expanded in 24-well plates in Iscove’s medium and subcultured every 14 days with 1 × 10^6 allogeneic irradiated PBMC as feeder cells supplemented with PHA, IL-2, IL-4 and IL-7 (patients RM and AR) and anti-CD3 anti-CD28 in combination with cytokines (patient HT), using the concentrations given above. TCC of patient EO were grown as bulk culture from a whole 4-mm punch biopsy in the presence of IL-2, IL-4 and IL-7 with different cytokine combinations at the concentrations given above. In order to sustain proliferation of TCC, allogeneic irradiated feeder cells were added together with PHA after 6 weeks and every 2 weeks from then on. The numbers of TCC generated from each skin biopsy and from peripheral blood are shown in Table 1. Only CD4+ TCC were subjected to TCR analysis.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>TCC (n = 150)</th>
<th>Culture conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM</td>
<td>MF (patch stage)</td>
<td>36 / 7</td>
<td>PHA, IL-2, IL-7, ± IL-4</td>
</tr>
<tr>
<td>AR</td>
<td>MF (tumor stage)</td>
<td>21 / 0</td>
<td>PHA, IL-2, IL-7, ± IL-4</td>
</tr>
<tr>
<td>HT</td>
<td>MF (tumor stage)</td>
<td>11 / 6</td>
<td>IL-2, IL-4, IL-7, OKT3, αCD26</td>
</tr>
<tr>
<td>EO</td>
<td>CD30+ large-cell anaplastic lymphoma</td>
<td>Three bulk cultures / 0</td>
<td>IL-2, IL-4, IL-7</td>
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</tbody>
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