Abstract. Background: Medullary thyroid carcinoma (MTC) is a calcitonin-producing tumor of the parafollicular C-cells, accounting for 5-10% of all thyroid tumors. To date, the only effective treatment is the early and total surgical removal of all neoplastic tissue. As the prognosis of patients with advanced MTC, unresectable or distant metastases is poor, and chemotherapy or irradiation is of no significant value, alternative strategies have been sought. Materials and Methods: A promising treatment approach for human MTC, that has already been introduced at our facility, is based on vaccination with autologous dendritic cells (DCs). Strong evidence that vaccination with autologous tumor lysate-pulsed DCs induces a specific immune response in vivo has been provided. However, the therapeutic success of this approach is sometimes critically limited by the small amount of tumor material available, especially from patients operated at an early tumor stage. Thus, it would be to the best advantage to have sufficient amounts of autologous tumor cells available for DC pulsing. Results: A method to generate viable autologous tumor cell cultures from a variety of MTC tissue samples, even when the sample size is small, has been successfully established. These cell lines maintain their neuroendocrine phenotype. In addition, it can be shown that these cells also display the biological features of neuroendocrine tumor cells at the molecular level. Conclusion: The unlimited availability of these MTC cell lines makes it possible to specify cancerogenesis of MTC. In addition, the availability of sufficient amounts of tumor lysate from these cell lines offers the advantage of prolonged immunotherapy. Finally, these cell lines could be elegantly used as read-out system to monitor the in vivo immune response during immunotherapy with DC cell-based vaccination in patients suffering from MTC.

Human medullary thyroid carcinoma (MTC) is a calcitonin-producing tumor of the parafollicular C-cells accounting for 5-10% of all thyroid tumors. MTC may occur sporadically, in a familiar form without associated endocrinopathies or as part of multiple endocrine neoplasia type 2A or 2B with autosomal dominant inheritance (1, 2). In MEN2A, MTC occurs in association with pheochromocytoma and hyperparathyroidism; in MEN2B, MTC occurs in association with pheochromocytoma and mucosal neuromas. In addition, there is an unusual variant of MEN2A associated with skin lesions called cutaneous lichen amyloidosis (3). The clinical varieties are associated with distinct types of mutation in the RET proto-oncogene in chromosome 10. RET mutations have been found in patients with MEN2 syndromes, the related sporadic tumors MTC and pheochromocytoma, and in familial and sporadic Hirschsprung's disease (4, 5).

The prognosis of patients with advanced MTC is very poor. Surgical treatment offers the only chance of a cure, while chemotherapy or irradiation is of no significant value (6). Intrinsic drug resistance was attributed to an overexpression of the mdrl gene (7). For this reason, alternative strategies have been sought. A promising approach is the activation of the patient's immune system against malignant cells. One of these strategies deals with the stimulation of resting T-cells by autologous dendritic cells (DC) (8, 9).

DCs are the most potent antigen-presenting cells for naïve T-cell activation and play an important role in the induction of primary and secondary immune responses. DCs are generated in vitro from peripheral blood mononuclear cells,
using granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) (8). These immature DCs can be further induced to mature by inflammatory stimuli, such as tumor necrosis factor alpha (TNF-α), IL-1β or CD40-ligand (10). Pulsing these DCs with tumor lysates enhances the cytolytic activity of T-cells (11, 12).

There have already been several successful clinical trials using tumor antigen-pulsed DCs for such tumors as melanomas (13), renal cell carcinoma (14), or prostate carcinoma (15). The development of dendritic cell vaccination for endocrine malignancies has been described, among others, in 7 MTC patients (16-22). Treatment with carcinoembryonic antigen and calcitonin-loaded DCs produced immunological and clinical responses.

Our group established new treatment approaches that focus on the ability of tumor cell lysate-pulsed DCs to induce antitumor T-cell responses against autologous tumor cells (23, 24). We developed an experimental tumor model for MTC therapy, using DCs, T-lymphocytes and MTC cell lines in vitro under autologous conditions (25). We demonstrated that mature tumor lysate-pulsed DCs obtained from patients with MTC could prime a HLA-class I-restricted antitumor T-cell response against autologous tumor cells. In a phase II clinical trial, we demonstrated a tumor marker response in 7 out of 10 patients with MTC and we observed an objective radiological decrease of tumor lesions in 5 out of 10 patients (26). However, the therapeutic success in the case of MTC is critically limited by the small size of these tumors, resulting in insufficient tumor lysate for the repeated treatments. An attractive alternative could be the in vitro cultivation and enrichment of autologous tumor cells. However, only a few MTC cultures have been established due to the usually small tissue samples and poor initial proliferation in vitro. The TT cell line was the first human MTC cell line established (27). It originated from a MEN2A case with an exon 11/codon 634 mutation (28). For almost two decades this was the only cell line available.

Here, we describe a method of generating viable autologous tumor cell cultures from a variety of MTC tissue samples, even small ones. We demonstrated that these cell lines maintain their neuroendocrine phenotype. In addition, we were able to show that these cells also display the biological features of neuroendocrine tumor cells at the molecular level. The availability of sufficient amount of cells could serve as the basis for further studies, as well as for clinical trials such as DC-based immunotherapy of patients with MTC.

Materials and Methods

Tumor sampling. Tumor samples obtained during primary surgery and/or re-operation of patients with MTC were transported within 24 h in Ham’s F12 nutrient mixture (Biochrom AG-Seromed, Berlin, Germany) without serum, containing 100 U penicillin/ml and 100 μg streptomycin/ml.

Primary cell culture. After removal of necrotic, fatty and fibrous tissue, the tumors were roughly cut and transferred to an antibiotic mix (PBSA, containing 1000 U penicillin/ml and 1000 μg streptomycin/ml) for 20 min. When excess tumor tissue was available, tissue fragments were cryopreserved. Biopsies with distinct amounts of erythrocytes were incubated in erythrocyte lysis buffer for 15 min. The tissue was transferred to Ham’s F12 medium supplemented with 20% fetal bovine serum (FBS) (PAA Laboratories, Exton, PA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Biochrom AG). Further disaggregation was done mechanically by chopping the tissue finely, as described earlier (29). Enzymatic disaggregation was never done, to avoid potential alterations of the tumor antigens. Cells were incubated at 37°C in a 5% CO2 and 95% humidity incubator, using Cell+ culture flasks (Sarstedt, Nümbrecht, Germany).

Cryopreservation. Tumor tissue: Each biopsy was chopped into 3- to 4-mm pieces, suspended in medium with 10% dimethylsulfoxide (DMSO, SIGMA-Aldrich, Vienna, Austria), frozen and stored in liquid nitrogen. This valuable material provided the opportunity to repeat the initiation of cultures with unsatisfactory growth.

Tumor cells: Whenever excess cultured cells became available, they were frozen in batches of 1x10⁷ cells/ml.

Establishment of MTC cell lines. The originally heterogeneous cultures were exposed to collagenase (0.05% in complete medium; SIGMA-Aldrich) in order to separate tumor cells from fibroblasts (30). Antibiotics were omitted after a few passages.

Passages: Since the cell lines grew in suspension, trypsinization was not required. The appropriate volume of the cell suspension was diluted in fresh medium and seeded into new flasks at a final density of 2-5x10⁶ cells/ml medium.

Cell proliferation was controlled by a Casy-1® Cell Counter and Analyzer, Model TTC (Scharfe System, Reutlingen, Germany).

Adaptation to serum-free conditions. Primary MTC cultures were grown in Ham's F12 medium (Biochrom AG) with L-glutamine, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) (all by PAA Laboratories, Pasching, Austria). After the first passages, one portion was continued in Ham’s F12 + FBS; the other portion was adapted stepwise to serum-free conditions in QUANTUM 263 Medium with L-glutamine (PAA Laboratories).

Mycoplasma testing. The cultured MTC cells were tested for Mycoplasma contamination using Hoechst 33258 (SIGMA-Aldrich).

Immunocytochemical characterization of MTC cell lines. The cultured MTC cells were seeded into Flexiperm® chambers (Kendro, Vienna, Austria), which had been mounted on Super-Frost® Plus slides (Menzel, Braunschweig, Germany), centrifuged and fixed in formalin (10% v/v, in 0.01M PBSA) for 30 min. Normal human skin fibroblasts served as controls. Thereafter, the slides were washed twice in PBSA and subjected to indirect ICC. Primary antisera were applied overnight at 4°C at a dilution of 1:500 in 0.1M PBSA containing 0.3% Triton X-100 and 1% normal goat serum, followed by two washes with PBSA containing 0.2% Triton X-100 for 10 min each. FITC- or TRITC-conjugated secondary antisera from goat and directed against IgG of different species according to the primary antisera were diluted 1:400 and applied at room temperature for 45 min, followed by two washes with PBSA containing 0.2% Triton X-100. After a
final rinse with plain PBSA, the slides were mounted with glycerol containing paraphenylenediamine and examined for fluorescence with an Olympus Provis AX70 fluorescence microscope equipped with a Kodak DCS 40 digital camera.

The following antisera were used: Rabbit anti-calcitonin (Calbiochem, Vienna, Austria), rabbit anti-dopamine β-hydroxylase (Oncogene, Vienna, Austria), sheep anti-tyrosine hydroxylase (Calbiochem), vasopressin (Bachem, Bubendorf, Switzerland) guinea pig anti-vimentin (Oncogene), dilution 1:500. All secondary antisera were raised in goat against rabbit, sheep and guinea pig IgG (H+L chain specific), and conjugated either with FITC or TRITC (Calbiochem).

Tumorigenicity testing. Tumor cells from each MTC cell line were suspended in PBSA and injected subcutaneously into the flanks of 10 nude mice (nu/nu-BALB/c), age 4 weeks, at a cell number of 3x10^7 cells per animal. The arising tumor nodules were harvested, partly transplanted into other nude mice and partly returned to culture conditions.

Results

Establishment of autologous MTC cell lines. In primary cell cultures, outgrowths of cells were evident 1-2 weeks after set up. Mixed monolayers developed, comprising tumor cells and stromal fibroblasts. The tumor cells were not fully adherent, and were regularly separated from fibroblasts until the fibroblasts became reduced and senescent.

Both adherent and non-adherent tumor cells were seen. Additionally, the monolayers showed multicellular areas that piled up and detached from the growing surface. Finally, each cell line grew as a suspension with a population doubling times of 2-3 days.

A stepwise transition to serum-free conditions was tolerated well by each cell line. However, the requirements varied from tumor to tumor: while several cell lines showed optimal growth results in Ham’s F12 ± 10% FBS, for other cell lines the growth promoting activity was higher in serum-free Quantum 263 (see examples in Figure 1).

Mycoplasma testing. All cultures were Mycoplasma-negative.

Immunocytochemical characterization. The MTC cultures showed inhomogeneous reactions to antibodies. While some cells revealed positive immunoreactivity with antibodies to calcitonin (Figure 2A), dopamine β-hydroxylase (Figure 2B), tyrosine hydroxylase (Figure 2C) and vasopressin (Figure 2D), other cells were negative or showed vimentin-positive reactivity (Figure 2E). Control cultures of normal skin fibroblasts stained prominently for vimentin (Figure 2F).
Tumorigenicity testing. Five out of 8 cell lines were tumorigenous. The cells formed slow-growing tumors which were subsequently transplanted to mice. No relationship between in vitro proliferation and the stages of tumors or to the origin from primary tumors or metastases was detectable. Subsequent mouse passages improved the growth rates and the grade of differentiation, such as the amounts of neuroendocrine granules.

Discussion

For many years, the main hope in MTC therapy has been early diagnosis and the development of effective chemotherapeutic agents. Only recently was it found that DC-based vaccination holds promise in endocrine malignancies. The ability of in vitro expanded DCs to serve as vaccine in endocrine malignancies was first demonstrated in one patient.
with a parathyroid hormone-secreting carcinoma, and another patient with a metastasized neuroendocrine pancreas carcinoma that was strongly positive for chromogranin A (17, 19). Furthermore, it was demonstrated that MTC patients treated with carcinoembryonic antigen and calcitonin-loaded DCs showed immunological and clinical responses (18). We initiated a study of 10 patients with advanced MTC, in whom autologous DCs were pulsed with autologous tumor lysate. As the vaccinations were well tolerated in all patients, and a positive immunological response was induced, we concluded that dendritic cell-based vaccination is particularly suited to the treatment of advanced MTC (23, 25, 26). However, the therapeutic success of DC-based immunotherapy is sometimes critically limited by the small size of available tumors resulting in insufficient lysate for repeated treatments. The induction of antitumor response by DC-based immunotherapy must be prolonged. With an optimized protocol for culturing MTC cells, it should be possible to establish long-term cultures from each tumor sample.

For many years, the in vitro growth of malignant cells from solid tumors has been a problem. Proliferation can be limited by variation both among and within samples of tumor tissue, even from the same tumor type (31). Stromal cell overgrowth (32) and senescence of tumor cells in culture (33) seriously limit or prevent the growth of tumor cells. The cultivation procedure will end in a selection of certain cell populations, while other populations will die out. Which tumor cell population survives depends on the technique of cultivation and on the choice of an optimal nutrient mixture. Despite these problems, we were able to establish a method to generate viable autologous tumor cell cultures from a variety of MTC tissue samples, even when the sample sizes were very small (29, 34-36).

In this study – in contrast to all earlier projects – we aimed at the extension of the lifespans of the autologous tumor cells by optimizing culture conditions. We found that the lysates gained from cultured tumor cells were as effective as the lysates from freshly-frozen, non-cultured tumor tissue. Based on this experience, a clinical trial was initiated using tumor lysate from cultured autologous MTC cells.

Cultivation in the conventional nutrient mixture with fetal bovine serum was optimal for the initiation of MTC cultures. Later, the transition to serum-free conditions was well tolerated. We attempted to standardize the experimental and application protocols. Batch-to-batch variation of serum was excluded, variables were minimized and clearly defined conditions were achieved.

Although cultures of tumors should be useful tools, they have not been used in DC immunotherapy before. Based on our experience in the cultivation of MTC cells, sufficient tumor lysate will be optimal for analyzing the cytotoxic effects in a clinical study, using tumor lysate from cultured autologous MTC cells for each individual patient. We suggest that DC therapy combined with the establishment of autologous MTC cell lines will offer unlimited availability of tumor lysate for prolonged immunotherapy.

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References


