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Recruitment of T lymphocytes and induction of tumor necrosis factor in thyroid cancer by a local immunotherapy

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Summary. To elucidate the mechanism of action for intratumoral injection of immunopotentiators, infiltrating mononuclear cells and tumor necrosis factor (TNF) were assayed by immunostaining tissue samples of differentiated thyroid cancer resected with or without presurgical local application of OK-432, a streptococcal preparation. Frozen sections of resected specimens were stained with monoclonal antibodies using either a conventional or a modified immunoperoxidase method. The tumors injected with OK-432 showed increased T lymphocyte infiltration and HLA-DR expression on cancer cells as compared to the non-injected controls. Among these T cells, the CD4+ subset was more numerous than the CD8+ population. In four out of the seven cases constituting the injected group, numerous TNF-positive cells were seen in clusters or lines as well as scattered, while none of the seven cases in the control group was associated with a considerable amount of these cells. In their morphology and distribution pattern, these TNF-positive cells appeared to be of macrophage lineage. Thus local injection of OK-432 in thyroid cancer was shown to recruit T lymphocytes of predominantly the CD4+ subset and to induce in situ production of TNF, a known potent tumoricidal cytokine. The present data warrant further studies in this direction besides wider clinical intratumoral application of the reagent.

Key words: T lymphocyte – Tumor necrosis factor – Thyroid cancer

Introduction

Differentiated thyroid cancer is generally regarded as slow-growing and not immediately life-threathening. However, the very fact of having a low proliferation rate makes the cancer resistent to antineoplastic agents and irradiation. Besides surgical removal of the main tumor, high-dose radioiodine is an effective, and sometimes curative therapy for small metastatic foci that can take up iodine, but is not necessarily effective for bulky masses. Hence, unresectable, locally advanced or recurrent tumors are usually difficult to control and often fatal. Recently, intratumoral application of OK-432, a streptococcal preparation, was found to be a promising means of mass control for cancers of the thyroid and other organs [2, 18, 19]. This bacterial reagent has already been widely used as a systemic immunotherapy agent for human cancers [15, 22] as well as in animal models [4, 14]. When locally injected, it attracts numerous mononuclear cells, presumally monocytes/macrophages and lymphocytes [2, 11]. What is the most predominant subtype among these recruited cells? To elucidate the mechanisms of the local effects, immunophenotyping of the infiltrating cells was carried out in thyroid cancer specimens surgically removed after local injection of OK-432 or without pretreatment.

OK-432 has also been known to induce a multitude of cytokines including tumor necrosis factor (TNF), interleukins-1 and -2 and interferon γ [5, 13, 24], and of these, TNF has recently attracted much attention because of its potent, direct tumoricidal action [12, 20]. Is this monokine responsible for the tumor regression seen after topical applications of OK-432? To address this question, TNF-bearing cells were immunostained as well.

Materials and methods

Patients. Fourteen patients with newly diagnosed differentiated thyroid cancer were randomly assigned to either the injected or the control group. Their age, sex, and histological diagnoses are summarized in Table 1. All were euthyroid both physically and biochemically at the initial work-up. To rule out subclinical chronic thyroiditis superimposing on the neoplasmic lesions, patients were excluded from the study when they were positive for thyroid autoantibodies (antithyroglobulin or antimicrosome, as detected in passive hemagglutination assays).

In the injected group, after informed consent had been received, each patient was primed with serially increasing small doses (0.02, 0.05, and 0.1 mg) of OK-432 subcutaneously over 3 weeks. These injections also

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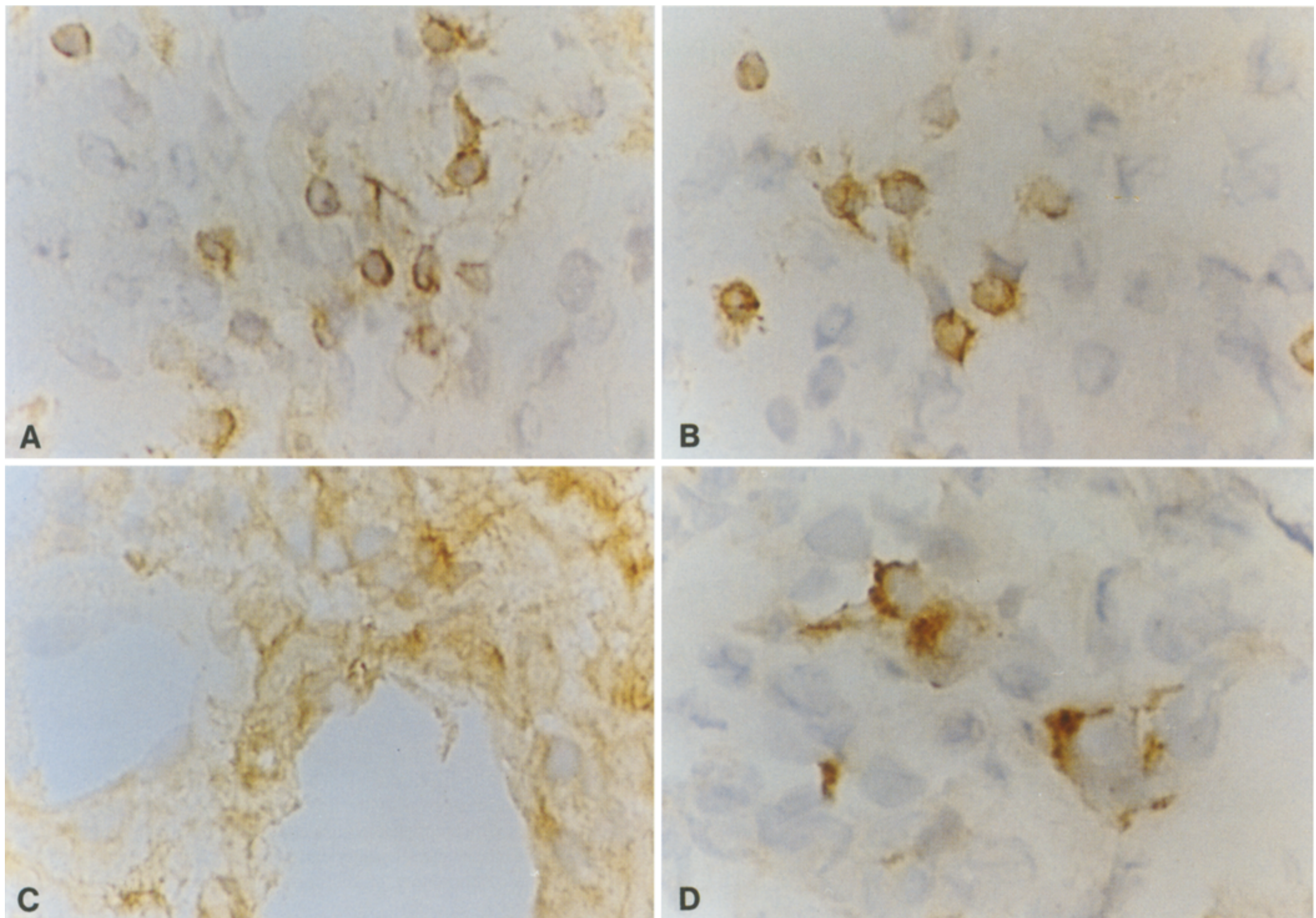

Fig. 1 A–D. Immunoperoxidase staining of thyroid cancer specimens intratumorally injected with OK-432 prior to removal. Magnification ×740. **A** CD4+ T lymphocytes in case 4. **B** CD8+ T cells in case 4. **C** HLA-DR-positive cancer cells in case 4. **D** Scattered macrophages identified with antibody DakoMac in case 2

served to ascertain that none of the subjects was allergic to this bacterial preparation. Seven days after the last subcutaneous dose, 0.5 mg OK-432 was administered directly to the center of the tumor mass, and the cancerous lesion was surgically removed 7 days later. In the control group, the tumors were resected without any immunotherapeutic manipulation, either systemically or locally.

Immunostaining. Thyroid cancer specimens obtained at surgery were embedded in OCT compound (Miles Lab., Elkhart, Indiana) and snap-frozen in liquid nitrogen. Surface markers of lymphocyte subsets and macrophage were detected with an indirect immunoperoxidase method as described for autoimmune thyroid tissues [9]. The monoclonal antibodies used were DakoMac (Dako Reagents, Santa Barbara, Calif.) for macrophage, and the Leu series (Beckton-Dickinson FACS Systems, Oxnard, Calif.) for lymphocytes (Leu4 for CD3+ whole mature T cells, Leu3 for CD4+ helper/inducer T cells, Leu2 for CD2+ suppressor/cytotoxic T cells, and Leu12 for CD19+ mature B cells). In addition, tumor cells with aberrant HLA-DR expression were identified with a Beckton-Dickinson monoclonal antibody (clone L243). For TNF, the initial trials with conventional methods using labeled second and/or third antibodies were all unsatisfactory even in lymphoid tissues. Hence, to increase the sensitivity of staining, we adopted a modified immunoperoxidase technique, the labeled streptavidin/biotin method. In separate experiments with other antigens, we found this method 4–16 times more sensitive than the usual two-step indirect immunoperoxidase system. For the staining, air-dried, 6-μm-thick cryostat sections were fixed in cold acetone for 10 min, rinsed in 0.05 M TRIS-buffered saline (TBS, pH 7.6), and preincubated with 10% normal goat serum for 20 min at room temperature. After tapping-off excess serum, a murine monoclonal antibody to human TNFα (MTN-1, Hayashibara Biochemical Laboratories, Okayama, Japan), diluted 1 : 100, was applied overnight at 4° C, and then for 1 h at room temperature. Slides for the negative control were incubated with the dilution buffer (1% bovine serum albumin in TBS) or with a control monoclonal antibody, anti-(human chorionic gonadotropin), instead of with the specific antibody. After washing in TBS, optimal dilutions of biotinylated goat anti-(mouse immunoglobulins) and peroxidase-labeled streptavidin (both from Dako) were applied serially – 20 min for each reagent – at room temperature. To minimize cross-reactivity of the biotinylated second antibody to human immunoglobulins in the tissue, normal human serum was added to the antibody solution to a final concentration of 5%. The sections were developed in diaminobenzidine solution, counterstainted with hematoxylin and mounted as reported [9]. Since there is only negligible endogenous peroxidase activity in the thyroid tissues examined, no specific inhibitory treatment was performed.

As a positive control, sections of tonsils, obtained at tonsillectomy for reactive hyperplasia, were run parallel to the thyroid slices in each experiment.

To evaluate the density of immunostained cells, at least ten representative high-power fields (hpf) for each specimen were examined in a Nikon light microscope. The mean number of positive cells per field was calculated, and classified into the following scores: –, if no more than 1/hpf; +, more than 1 but no more than 5; ++, more than 5 but no more than 10; +++, more than 10. The counting was done by a single observer (T. M.) in a blind fashion without knowing the patient's identity or the kind of antibody applied for each slide.

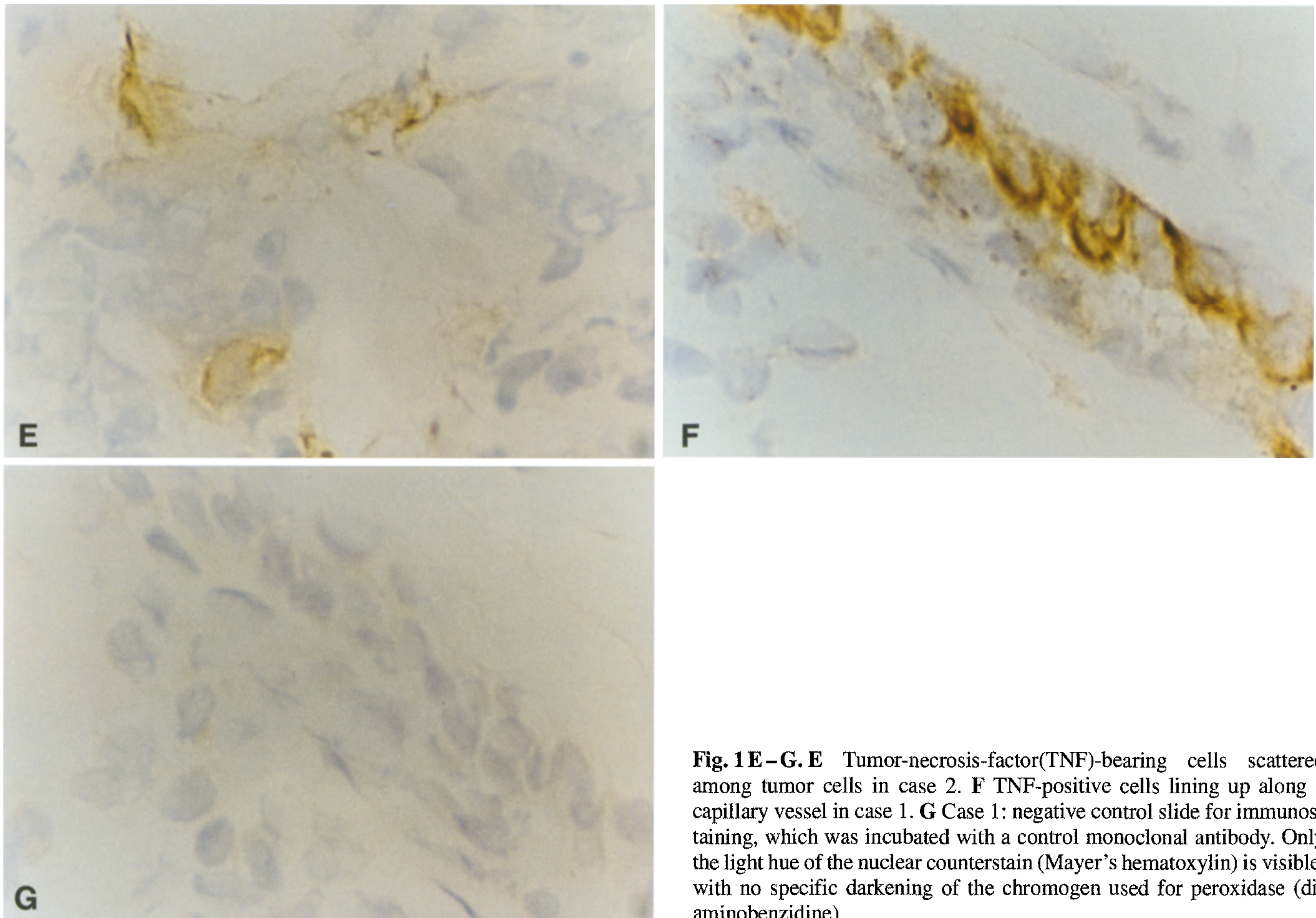

Fig. 1 E–G. E Tumor-necrosis-factor(TNF)-bearing cells scattered among tumor cells in case 2. **F** TNF-positive cells lining up along a capillary vessel in case 1. **G** Case 1: negative control slide for immunostaining, which was incubated with a control monoclonal antibody. Only the light hue of the nuclear counterstain (Mayer's hematoxylin) is visible, with no specific darkening of the chromogen used for peroxidase (diaminobenzidine)

Results

In the routine histopathological specimens stained with hematoxylin/eosin, the thyroid tumors injected with OK-432 showed various degrees of necrosis and infiltration of mononuclear cells, whereas these changes were absent or minimal in the control non-injected cancer specimens.

Lymphocyte subsets, macrophages and HLA-DR-positive tumor cells appeared clearly in the conventional immunostaining (Fig. 1 A–D), and the labeled streptavidin/biotin technique, the first incubation being performed overnight in the cold, reproducibly and distinctively demonstrated TNF in situ without increased background staining. Figure 1 E, F demonstrates two patterns of distribution for TNF-positive cells in OK-432-treated thyroid cancer tissue. When abundant, TNF-bearing cells were scattered (Fig. 1 E) or clustered, or sometimes aligned along and within small vessels (Fig. 1 F). In the control specimens, solitary TNF-positive cells or small clusters of up to several cells were seen only rarely. The specific stain for TNF was seen in the cytoplasm of cells with macrophage morphology when viewed closely in high-power fields. On the other hand, tissue macrophages identified with DakoMac antibody, were found in considerable number over whole tissue slices in every specimen of both OK-432-injected and control cases (Fig. 1 D). No significant background staining was seen in the negative control slides (Fig. 1 G).

Table 1 gives a semiquantitative assessment of the T lymphocyte subsets, DR-positive cancer cells and TNF-bearing cells, and the extent of tumor necrosis in the thyroid specimens studied. The Leu12$^+$ B cell type is not listed, because it was almost absent in the tumors examined, except in rare clusters of lymphocytes. Four out of seven cases in the injected group showed ++ or +++ infiltration of CD3$^+$ T cells, while none of the control cases did. Among the subsets, the CD4$^+$ population was generally more abundant than CD8$^+$ cells. The amount of DR-positive neoplastic cells tended to reflect the density of CD3$^+$ lymphocytes. Collectively, the injected group also showed an increased number of TNF-positive cells compared to the control group. However, when seen individually, the frequency of TNF-bearing cells correlated only roughly with that of T lymphocytes and not with that of DR-expressing cancer cells or the extent of tumor necrosis.

Discussion

In the present study, OK-432-treated thyroid cancer specimens showed increased infiltration of T lymphocytes as well as DR antigen expression on tumor cells and potentiation of TNF production in situ. The near absence of B lymphocytes between cancer cells implies that humoral immunity played only a minimal or negligible role in the effect

Table 1. Clinical background and immunohistochemical profiles of the thyroid specimens[a]

Patient	Age (years)/sex	Pathology	CD3+	CD4+	CD8+	DR+	TNF+	Necrosis
Injected group								
1. N. T.	51/F	PAC	+	+	+	++	++	Absent
2. N. S.	39/M	PAC	+++	+++	++	++	++	Minimal
3. H. N.	65/M	PAC	+++	+++	+++	+++	–	Prominent
4. N. T.	73/F	PAC	++	++	+	+++	+	Absent
5. K. M.	45/M	PAC	+	+	+	+	–	Mild
6. K. K.	47/M	PAC	++	+	–	++	+++	Minimal
7. N. M.	73/F	PAC	+++	+++	++	+++	++	Absent
Control group								
8. K. H.	67/M	PAC	–	–	–	–	–	Minimal
9. M. S.	72/F	PAC	+	+	–	–	+	Absent
10. I. T.	75/M	FAC	–	–	–	–	+	Absent
11. N. E.	41/F	PAC	–	–	–	–	–	Absent
12. S. H.	33/M	PAC	+	+	+	–	–	Minimal
13. N. E.	29/F	PAC	–	–	–	+	–	Minimal
14. H. T.	44/F	PAC	+	++	+	–	–	Absent

[a] PAC, papillary adenocarcinoma; FAC, follicular adenocarcinoma; TNF, tumor necrosis factor

of intratumoral injection. Among the intratumoral T cells recruited, the CD8+ subset may be directly cytotoxic to malignant thyrocytes. However, unlike the situation in gastric cancer [2, 11], this was not the major population. Alternatively, these infiltrating T cells could very likely produce lymphokines that could act synergistically with TNF in tumor killing [17]. In this context, although the aberrant DR expression on cancerous thyrocytes strongly implies local interferon γ production [7, 10], our attempt to immunostain this cytokine, using antibodies currently available to us, was unsuccessful, even with the sensitive labeled streptavidin/biotin method described above. Similarly, our attempt to identify NK cells in the thyroid tumors is not included in this report because of technical problems.

TNF is a cytokine mainly produced by cells of macrophage/monocyte lineage, and is probably one of the key mediators of host defense against malignant neoplasm. Its antineoplastic action has been widely documented, as well as its diverse toxic effects as "cachexin" (see [1, 12, 20] for review). Although the possibility of cancer immunotherapy with this cytokine has attracted much attention, the initial clinical trials of its systemic use encountered adverse effects so severe that the doses had to be limited [16]. Apparently the unwanted side of a double-edged sword had the greater impact in that case. Meanwhile, the local injection of TNF itself, or of immunopotentiators able to induce it, seems promising as a way of topical mass control if the target tumor is within reach of the needles. Systemic side-effects should be much less if the cytokine is administered in this way. In clinical practice, for example, we have seen significant mass reduction and symptomatic relief after intratumoral injections of OK-432 in two cases of locally advanced thyroid carcinoma [8]. This streptococcal preparation has already been proven to stimulate release of TNF into serum or pleuroperitoneal effusion [6, 21, 24].

The present investigation disclosed that, if unstimulated, only a rare fraction of cells of macrophage lineage constitutively expresses TNF immunoreactivity, whereas TNF-positive cells are plentiful in some subjects after intratumoral injection of OK-432. This augmentation of local TNF production may have been achieved by stimulating the preexisting tissue macrophages which, with DakoMac antibody, were shown to be abundant in thyroid cancer tissue, or by recruiting activated monocytes from the circulation as suggested by the pericapillary or intracapillary TNF-bearing cells.

There could be at least two explanations of why not all the cases in the injected group showed a significant number of TNF-positive cells in their thyroid tissues. First, genetic variation between subjects in the sensitivity to OK-432 could be an influencing factor [23]. Second, the timing between intratumoral injection and tumor removal might not be ideal for TNF immunostaining in some cases. The 1-week interval employed in this protocol was chosen because this was reported to be the optimal timing to induce maximal immune cell infiltration in gastric cancer tissue endoscopically injected with OK-432 [11]. However, results of induction experiments in vitro suggest that the peak for TNF production may have passed earlier [3]. We might have seen more TNF-bearing cells in the injected group had the interval be shorter, though this is not practically feasible nor ethically permissible from the surgical point of view, because of the excessive local inflammatory reaction associated with the injection. A similar argument for timing could be made to account for the discrepancy between the extent of tumor necrosis and the density of TNF-possessing cells. Alternatively, significant necrosis may require multiple factors besides TNF, such as other cytokines and various killer cells.

Besides the mechanism of immediate mass reduction, it is also important to know of any possible long-term prophylactic effects of the intratumoral therapy. We are thoroughly following-up all the participants to see if the rate of local recurrence and metastasis is different between the injected group and the control.

In conclusion, our data indicate that recruitment of T cells and TNF production "on the spot" are important factors for antitumoral effects of locally injected OK-432, a known multicytokine inducer. The results clearly warrant further investigations with a larger number of subjects, and also provide a rationale for wider clinical applications of this treatment.

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