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ORIGINAL ARTICLE

Serum Containing Inhibitory Factors Which Inhibit Natural Cytotoxicity in Patients With Differentiated Thyroid Cancer

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Differentiated thyroid cancer (DTC), in which histologic examination often reveals evidence of lymphocytic infiltration at the edges of the tumour, is a good model for the interaction between human cancer and the immunocompetent host immune system.

Materials and Methods: We tested sera from patients with DTC for cytotoxicity against cultured human thyroid, eye muscle and K562 cells in antibody-dependent cell mediated cytotoxicity (ADCC) and natural killer (NK) cell cytotoxicity assays.

Results: When cultured thyroid cells were used as targets in ADCC assay, specific cytotoxicity was increased (% specific lysis > mean + 2SD for normals) in only 2 out of 30 patients with DTC but decreased (% specific lysis < mean + 2SD for normals) in 19 patients and mean for the group was significantly less than that for the normals. The same effect was observed using human eye muscle cells as targets in an ADCC assay. Serum from patients with DTC, but not those from normal subjects, inhibited the ADCC activity against normal thyroid cells in serum from patients with Graves' disease, in a dose dependent manner. Serum from patients with thyroid cancer inhibited natural cytotoxicity against human K562 cells while normal serum did not. Pretreatment of normal PBMC with pooled serum from patients with DTC abolished natural cytotoxicity against K562 cells whereas pooled normal serum had no significant effect.

Conclusion: This phenomenon reflected inhibition of the natural cytotoxicity mediated by (NK) cells in the mixed peripheral blood mononuclear cells (PBMC) population by an unknown serum factor or factors. We confirmed this by performing experiments using sera from patients with DTC, human K562 cells as targets in NK cell assay and PBMC from normal subjects as source of NK cells. The inhibitory factor, which probably works at the level of the NK cells and is called natural cytotoxicity blocking factor (NKBF), may be a marker for invasive thyroid cancer, although this needs to be addressed in further studies.

Key Words: Differentiated thyroid cancer, NK cell cytotoxicity, Antibody-dependent cell mediated cytotoxicity, Host immune response, NK cell blocking factor(s)

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Introduction

Differentiated thyroid cancer (DTC) affects both sexes and all ages but is most frequent in middle-aged women. Although relatively uncommon (approximately 30 new cases of thyroid cancer are diagnosed per million people per year), because the life expectancy is usually excellent, it is estimated that as many as 1-2 per thousand people have, or have had, DTC.[1] In addition, microscopic thyroid cancer can be identified in up to 10% of apparently normal thyroid glands at thyroidectomy or autopsy.[1,2] Because single thyroid nodules, of which 5-10% are malignant,[2,3] may be found in life or as an incidental finding at autopsy in as many as 50% of the adult population,[4] the diagnosis and management of thyroid cancer is a major health issue with a huge economic impact. Because the life expectancy is usually excellent, it is estimated that, overall, as many as 2-3 per thousand people have, or have had, DTC.[5]

Host immune response to human cancer is well recognized and likely to be important in determining whether or not the tumour remains localized or spreads to the draining lymph nodes or blood stream.[6] DTC, in which histologic examination often reveals evidence of lymphocytic infiltration at the edges of the tumour,[7] is a good model for the interaction between human cancer and the immunocompetent host immune system. Matsubayashi et al[8] have shown that lymphocytic infiltration around the edge of the papillary thyroid cancer, or inside the tumour, in association with serum antibodies against thyroglobulin and thyroid peroxidase, is associated with a lower probability of local or distant spread. On the other hand, absence of mononuclear cell infiltration in the thyroid is likely to reflect tumour escape from immune regulation and the tendency to extend beyond the thyroid capsule. While the immune mechanisms involved in this process are not well defined, they are expected to include destruction of neoplastic cells by specifically primed cytotoxic T cells and natural killer (NK) cells.[9,10] A simple blood test, which could identify those patients in whom thyroid cancer has extended into, or beyond the capsule of the gland, would be very useful in the management of this disorder.

In the course of our studies of the role of autoimmunity against thyroid and eye muscle autoantigens in autoimmune thyroid disease and thyroid-associated ophthalmopathy, we have identified a factor or factors, in the serum of the majority of patients with DTC which blocks natural cytotoxicity against human thyroid cells and human K562 cells. This factor (or factors), which is presently uncharacterised, may be a marker for invasive thyroid cancer.

Materials and Methods

The studies concerned 30 consecutive patients with differentiated thyroid cancer, 8 men and 22 women, aged 21-63 (mean age 40 years), of whom 20 had papillary cancer, 8 follicular cancer and 2 mixed papillary/follicular cancer. The diagnoses were made from the clinical findings of a hard thyroid nodule or mass sometimes associated with cervical lymph node enlargement, aspiration needle biopsy findings of "malignant" or "suspicious" thyroid epithelial cells and a cold nodule on ^{123}I scanning, and confirmed at open thyroid biopsy. Histological diagnosis of papillary, follicular and mixed papillary/follicular thyroid cancer was based on standard criteria. All patients then underwent completion total thyroidectomy and radioiodine ablation of thyroid remnant and were followed up by one of us (JW). At the time of the study all patients were in remission and on thyroxine suppression, but further follow-

up information about local or distant spread and response to treatment, is not available. As controls, we studied 3 men and 6 women aged 25-51 (mean age 37 years) with no personal or family history of thyroid disease, other autoimmune disease or thyroid cancer.

Target cell preparation

Human eye muscle, obtained fresh at surgery, was washed in phosphate-buffered saline (PBS), separated from fat and connective tissue and cut in $1mm^3$ fragments. The eye muscle fragments were transferred to 24 well tissue culture plates (Falcon Primaria) at one fragment per well and cultured in Minimal Essential Medium supplemented with dialyzed 10% foetal bovine serum (FBS) and D-valine, at 37°C in 5% CO_2 and 95% air for 2 weeks. Cell outgrowth was usually evident by 6-7 days and a confluent monolayer by 2-3 weeks. Cells were then removed from the plate by treatment with 0.125% trypsin in PBS and transferred to 7.5 cm Primaria flasks to expand cell populations. This population, termed extra ocular myoblasts, comprises more than 90% myoblasts. Thyroid tissue was obtained at thyroidectomy from blood type O patients with Graves' disease and as normal tissue adjacent to benign follicular adenoma. Thyroid cells were prepared, characterized, and cultured as primary culture, as described previously.[11] The human erythroleukemia cell line K562 cells were used as target in NK assays.

Antibody-dependent cell mediated cytotoxicity assay

ADCC activity against human eye muscle and thyroid cells was determined using a standard ^{51}Cr release assay, as described previously.[11-13] Briefly, target cells, labelled with 3.7 Mbq of $Na^{51}CrO_4$ (Frost, Kirkland Quebec, Canada) were pre-incubated with patient or normal sera, at an initial dilution of 1/25, for 1 hour at 37°C in 96 well V-bottom plates in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% foetal calf serum, 2mM glutamine, penicillin (50 U/mL) and streptomycin (50 µg/mL). Mixed peripheral blood mononuclear cells (PBMC), as source of killer (K) cells, were separated from whole blood on a Ficoll-Hypaque gradient and added at an effector (Killer cell) to target cell ratio of 25:1. The plates were incubated at 37°C in 5% CO_2 and 95% air for 4 hours, centrifuged for 4 minutes at 250×g and 50µL aliquots transferred to a 96 well flat bottom plates. Radioactivity was counted and percentage specific lysis (%SL) calculated as:

$$\frac{CPM_e - CPM_b}{CPM_{tm} - CPM_b} \times 100$$

where CPM is counts per miute, e=experimental, b=background, and tm=target max. The normal range was determined from age and sex matched normal subjects tested concurrently, as mean ±2SD.

NK cell assay

The NK cell assay is also standard and similar to the ADCC assay described above. Briefly, normal PBMC and ^{51}Cr–labelled human K562 cells, as targets, were incubated in complete RPMI medium at an effector to target cell ratio of 25:1 for 4 hours at 37°C in 5% CO_2 and 95% air. Plates were centrifuged for 4 minutes at 250×g and 50 µL aliquots transferred to a 96 well flat bottom plates. Radioactivity was counted and specific lysis calculated as described above.

Other tests

Serum thyroxine, thyroglobulin and Thyrotropin (TSH) levels were measured using standard comnercial radioimmunoassay kits (Travenol Diagnostice Inc., Cambridge, MA, USA) and anti-thyroid peroxidase and anti-

thyroglobulin antibodies were measured using a commenricial hemagglutination kit provided by Fuji Rebio Co. (Tokyo, Japan).

Statistical analysis

Differences between patients with DTC and controls were assessed using Fisher's exact test or paired t tests. In all tests a P value of < 0.05 was taken as significant.

Results

When fresh human thyroid cells were used as targets in ADCC percentasge of specific lysis, %SL was increased (defined as %SL>mean+2 SD for normals), in 2 out of 30 patients with DTC, decreased, (defined as %SL<mean–2SD for normals), in 20 patients, and was normal in 8 patients (Fisher's exact test, $p< 0.001$) (Fig. 1). Mean±SD %SL for patients with DTC (11.2±5%) was significantly less than that for normals (13.9± 1.87%, $p<0.05$). These findings raised the possibility of a serum factor in patients with DTC that inhibited ADCC activity against thyroid cells. As shown in Fig. 2, serum from a patient with DTC, but not that from a normal serum, inhibited the ADCC activity against normal thyroid cell targets by a positive serum from a patient with Graves' disease, in a dose-dependent manner. The same effect was observed using human eye muscle cells (as myoblasts) as targets in an ADCC assay (results not shown). We concluded that this experiment reflected inhibition of the background, or natural, cytotoxicity mediated by NK cells in the mixed PBMC population by serum from patients with DTC. In order to confirm this, other experiments were carried out using human K562 cells –classically used as targets in the NK cell assay– and PBMC from a normal subject as source of NK cells. Results of a representative experiment are shown in Fig. 3. Natural cytotoxicity against human K562 cells was inhibited by serum from 2 patients with thyroid cancer but not

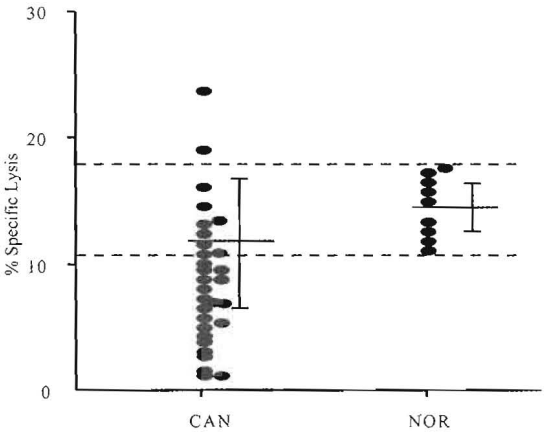

Fig.1. Antibody-dependent cell mediated cytotoxicity against 51Cr-labelled normal human thyroid cells by sera from patients with differentiated thyroid cancer (CAN) and normal subjects (NOR). Serum dilution was 1:25 and effector: target cell ratio 25:1. Results are expressed as % specific lysis and as mean (±2SD) for the groups. The broken horizontal lines, at 17.7% and 10.16%, represent the upper and lower limits of normal (for normal subjects tested concurrently).

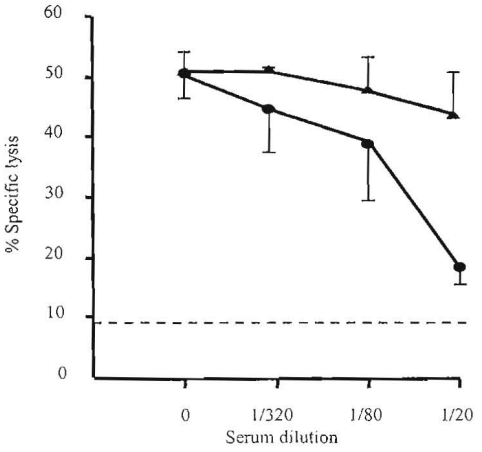

Fig. 2. Effect of serum from a patient with differentiated thyroid cancer (▲) and a normal subject (●) on antibody-dependent cell mediated cytotoxicity against ^{51}Cr-labelled normal human thyroid cells mediated by serum from a patient with Graves' disease. Effector: target cell ratio was 25:1. Results are expressed as % specific lysis (±SD) of triplicate samples at different serum dilutions. The broken line, at 9.3% specific lysis represents background lysis with lymphocytes only (natural killer cell cytotoxicity).

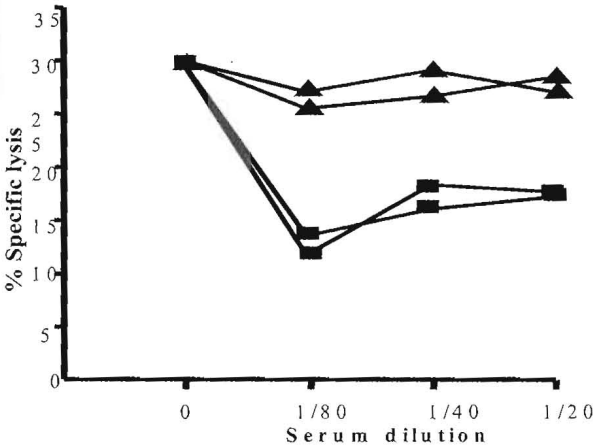

Fig. 3. Effect of serum from two patients with differentiated thyroid cancer (▲) and two normal subjects (■) on NK cell cytotoxicity against ^{51}Cr-labelled human K562 cells. PBMC from a normal subject were used as source of normal NK cells. Effector: target cell ratio was 25:1. Results are expressed as mean % specific lysis of triplicate samples at different serum dilutions.

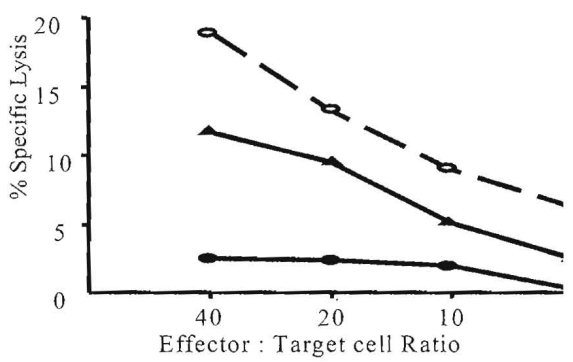

Fig. 4. Effect of pre-treatment of NK cells with pooled serum from patients with DTC (●), normal subjects (○) and medium (▲) on natural cytotoxicity against 51Cr-labelled human K562 cells. PBMC from a normal subject were used as source of normal NK cells Effector: target cell ratio was 25:1. Results are expressed as mean % specific lysis of triplicate samples at different effector: target cell ratios.

by serum from 2 normal subjects. Pretreatment of normal PBMC with pooled serum from patients with DTC containing the putative inhibitory factor abolished natural cytotoxicity against K562 cells, whereas pooled normal serum or culture medium had no effect (Fig. 4), suggesting that the effect was at the level of the NK cells rather than the target.

Discussion

To summarise the main findings, we have identified a serum factor (or factors) in the majority of patients with DTC which inhibits killer/NK cell cytotoxicity against thyroid, eye muscle and K562 target cells, and may be a marker for tumor escape from host immune recognition, although this needs to be confirmed in further studies. This factor (or factors), which we call "natural cytotoxicity-blocking factor (NKBF)", appears to be reactive with the NK cell rather than the target cell, and is inhibitory to natural cytotoxicity against several targets. Interestingly, this effect was also observed with sera from 43% of patients with subacute thyroiditis (Hiromatsu et al unpublished observation) suggesting that host response to thyroid cells which have been transformed by a viral infection may also be compromised.

When serum from patients with Hashimoto's thyroiditis or Graves' hyperthyroidism is incubated with PBMC from normal subjects and ^{51}Cr-labelled normal human thyroid target cells in an ADCC assay, lysis of the thyroid cells typically occurs due to the presence of cytotoxic antibodies reactive with a cell surface antigen, probably thyroid peroxidase. In one representative study[11] sera from 65% of patients with autoimmune thyroid disease lysed human thyroid cell targets in ADCC. The finding of suppressed ADCC activity against thyroid, eye muscle cell targets with sera from patients with DTC that are thyroid antibody negative in the present study, was therefore unexpected.

NK cell cytotoxicity is a well-recognised host defense mechanism against a wide range of viral infections and transformed and virally infected target cells without prior exposure to antigen and without restriction by major histocompatibility complex antigens. Indeed, it has been shown that early tumor-infiltrating NK cells are critical for the generation of tumor specific cytotoxic T lymphocytes.[14] Decreased NK/killer (K) cell activities have been demonstrated in patients with thyroid cancer,[15,16] other cancers,[17] systemic lupus erythematosus,[18] Graves' disease[19] and in pregnant women,[20] possibly caused by serum inhibitory factors. Lee et al[21] showed that thyroid-derived lymphocytes displayed "intense cytotoxic activity against NK-sensitive cells including K562 cells: while Boros et al[22] found that the NK activity of PBMC from thyroid cancer patients was elevated in tumour free patients and in cases of highly differentiated histologic type compared to patients with metastasis or anaplastic tumour. In these patients NK activity was significantly less in the progressive phase of the disease compared to the healthy controls but there was no relationship between the natural killer activity and the time elapsed since the initial surgery. Our study provides further evidence for such a factor in DTC.

There is good evidence that specific antibodies against cell surface antigens, particularly the CD16 protein,[23] may influence NK activity against tumor cells and aggregated IgG can suppress the activity of the related K cells.[24] Endo et al[25] reported that the observed decrease in peripheral K cell numbers in patients with autoimmune thyroid diseases may be due to saturation of Fc receptors on K cells by binding with immune complexes. Immune complexes are known to influence immune function[26] and host response to infection and cancer (reviewed in Kniker[27]). Fukazawa et al[28] reported a significant correlation between elevated levels of the monokine immunosuppressive acid protein (IAP) and decreased number of K cells, measured in a plaque assay, in subacute thyroiditis. We have recently shown that decreased cytotoxicity in SAT may also be due to a serum factor (Hiromatsu et al unpublished observation). IAP suppresses K cell function as well as the mixed lymphocyte reaction and phytohemagglutinin (PHA)-induced lymphocyte blast formation[29] and serum levels correlate with spread of cancer.[30] While some cytokines (IL-1α, IL-2) enhance NK activity in infection, autoimmunity and cancer,[31-33] others (IL-4, IL-12, IFN-γ, TGF-β) depress this form of cytotoxicity.[6,24,34] Another good candidate is the sialosyl-Tn antigen (STnAg), a mucin-associated carbohydrate antigen expressed by a variety of carcinomas, notably colon, which is markedly inhibitory to NK cell cytotoxicity against K562 cells in the presence of ammonium ions.[32] In a recent study, Chen et al[35] found that 14-deoxy-delta 12,14 prostaglandin J2 induced apoptosis of a thyroid papillary cell line through increased intracellular iron and oxidative stress. All of these agents are candidates for the NKBF activity which we have observed in this study. Further characterisation of the factor and elucidation of its clinical significance in patients with thyroid cancer are the subjects of our ongoing studies. In conclusion, we have identified a factor in the serum from most patients with DTC that inhibits Killer/NK cell mediated cytotoxicity against a variety of human cell targets. While others have shown abnormalities of NK cell numbers or function. Additional studies are needed to determine its significance, including its relationship to disease stage, response to treatment and long term prognosis

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