Protection of Thyroid Cancer Cells by Complement-Regulatory Factors

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Background. Clinical and experimental studies have suggested that complement activation may play a role in tumor cytotoxicity. Little information is available concerning the presence of complement activation and the localization of complement-regulatory factors in cells or tissues of malignant tumors. The aim of the present study was to examine, using immunohistochemistry and immunoelectron microscopy, whether the complement system is activated in tissues of thyroid carcinoma and whether thyroid carcinoma cells are protected from cell lysis by in situ complement activation.

Methods. Fresh tissues were obtained by thyroidectomy from 15 patients with papillary carcinomas, 7 with follicular carcinomas, and 5 with follicular adenomas. In addition, five specimens of histologically normal thyroid tissue and five specimens of chronically inflamed tissue adjacent to thyroid neoplasms were studied. Immunohistochemical and immunoelectron microscopic localization of complement components, C3d and C5b-9, and the complement-regulatory factors, such as s-protein, decay-accelerating factor (CD55), membrane cofactor protein (CD46), complement receptor types 1 (CD35) and 2 (CD21), and protectin (CD59), were examined in these tissues.

Results. The staining patterns of C3d, C5b-9, and s-protein were positive and homogeneous in the nonneoplastic and most neoplastic thyroid tissues. Immunoelectron microscopy showed these antigens were localized mainly on the subepithelial and vascular basement membranes and attached to the cell surface of thyroid follicular cells. Decay-accelerating factor (CD55) was present homogeneously on the basement membranes, on the basal cell border of the thyroid follicular cells, and often on the luminal surface of carcinoma cells. Both membrane cofactor protein (CD46) and protectin (CD59) were expressed strongly on the cell surface of almost all benign and malignant thyroid follicular cells. Membrane cofactor protein was expressed on both the basal and lateral membrane, showing cell-to-cell interaction, but rarely on the luminal surface, whereas protectin was expressed strongly on the luminal surface and often on the basal cell border but rarely on the lateral membrane. Neither complement receptor type 1 (CD35) nor complement receptor type 2 (CD21) was expressed on any thyroid follicular cells.

Conclusions. The present study confirmed the presence of complement activation with subsequent deposition of C3d and C5b-9 complexes in thyroid carcinomas. It also indicated that thyroid carcinoma cells are protected from cell lysis because of complement activation in multiple phases by complete coverage of the entire cell membrane surface with complement-regulatory factors. These findings were similar to those found in nonneoplastic thyroid follicular cells. Cancer 1994; 73:2808-17.

Key words: thyroid cancer, complement activation, complement-regulatory factors, immunohistochemistry, immunoelectron microscopy.

Clinical and experimental studies have suggested that complement activation may play a role in tumor cytotoxicity. The injection of complement-fixing, tumor-specific mouse monoclonal antibodies produces antitumor effects as well as the localization of both C4 and C3 at tumor sites in patients with neuroblastomas and a variety of other tumor cell types.1-3 Cancer cells, during the processes of invasion and metastasis, also are exposed to complements in blood. If complement-regulatory factors are expressed insufficiently on cancer cells, then the cells can be damaged selectively by complements.

Host cells are exposed continuously to the autologous complement system. Uncontrolled complement activation through C3 can occur by the classic or the alternate pathways, releasing several biologically active factors during the assembly of a common terminal complement complex.
pathway, C5b-9 (the membrane attack complex). Most C3d remains at the site where C3 is activated, but C3c does not. Therefore, detection of C3d as well as C5b-9 indicates in situ complement activation. In the fluid phase, host complement-regulatory factors include C1-esterase inhibitor, C4b-binding protein, factor I, factor H, properdin, and s-protein; in the membrane phase, they include complement receptor types 1 (CR1) (CD35) and 2 (CR2) (CD21), decay-accelerating factor (DAF) (CD55), membrane cofactor protein (MCP) (CD46), and protectin (CD59). DAF is an important attenuator of complement activation, because it blocks the formation of C3/C5 convertases of the alternative and classic pathways, and also hastens the decay of C3 convertase. DAF acts only within the surface of the same cells, that is, it is strictly an intrinsic membrane inhibitor. MCP is a cofactor for factor-I-mediated cleavage of C4b/C3b, but unlike DAF, it does not accelerate the decay of the C3/C5 convertases. CR1 and CR2 protect the cells on which they are expressed as well as the neighboring cells. S-protein (vitronectin) combines with the fluid-phase terminal complement complex at the stage of C5b-7 assembly and inactivates the SC5b-9 complex with regard to membrane insertion and tissue destruction. S-protein also exerts a lesser effect by inhibiting C9 lytic pore formation. Protectin acts at the final stages of membrane attack complex formation, preventing C5b-8-mediated insertion and C9 polymerization in cell membranes.

Very few studies have demonstrated the presence of complement-regulatory factors in malignant tumors. To our knowledge, no reports have described complement activation in tissues or complement-regulatory factors on cells of thyroid cancers, nor has electron microscopy been used to localize the complement-regulatory factors on malignant tumor cells.

Our aim was to examine, by immunohistochemistry and immunoelectron microscopy, whether the complement system is activated in tissues of thyroid cancers and whether thyroid cancer cells are protected from cell lysis by in situ complement activation.

Materials and Methods

Thyroid tissues were obtained from 27 patients after thyroidectomy: 15 patients with papillary carcinomas, 7 with follicular carcinomas, and 5 with follicular adenomas. In addition, five specimens of histologically normal thyroid tissue and five specimens showing chronic inflammation were obtained from areas adjacent to carcinomas and adenomas in these patients. The tissue samples were sliced into sections approximately 5 mm thick, which were fixed in buffered 10% formalin, embedded in paraffin, and cut into 4 μm-thick sections. The thin sections were stained with hematoxylin and eosin, and a histologic diagnosis was made according to the World Health Organization histologic classification.

A portion of each specimen was embedded immediately, frozen in Tissue-Tek optional cutting temperature compound (Miles Inc., Elkhart, IN), and stored at −80°C until they were to be sectioned cytostatically for immunohistochemical study. In addition, some of the trimmed tissues were immersed for 6 hours in a periodate–lysine–paraformaldehyde fixative, rinsed in graded sucrose–0.01 M phosphate-buffered saline (pH 7.4), embedded in the optimal cutting temperature compound, and stored at −80°C until they were to be sectioned by a cryostat. The antibodies used for immunohistochemistry are listed in Table 1. Representative cryostat sections of fresh-frozen material were stained using the avidin–biotin–peroxidase complex technique. The slides were counterstained with methyl green. Negative control sections (phosphate-buffered saline in lieu of antibody) were run concomitantly. Tonsillar lymphoid follicles were used as positive controls for each antibody tested.

After immunostaining, periodate–lysine–paraformaldehyde–fixed thyroid sections were used for immunoelectron microscopy.

The staining intensity was estimated on a five-point scale (0, ±, 1+, 2+, and 3+), and a score was obtained according to that intensity, as described previously. Where a homogeneous pattern was obtained, a visual estimate was made of both the predominant intensity and the minority value.

Statistical analysis was done with Statview II software (Abacus Concepts, Inc., Berkeley, CA). Means were compared by the Kruskal–Wallis test and Bonferroni's methods.

Results

Staining patterns of complement components C3d and C5b-9 and the regulatory factor, s-protein (vitronectin), were homogeneous in the nonneoplastic thyroid tissues (i.e., histologically normal and chronically inflamed tissues) and the majority of neoplastic thyroid tissues (i.e., follicular adenomas and follicular and papillary carcinomas tissues), but were heterogeneous in a minority of neoplastic thyroid tissues. These three antigens sometimes had similar distributions and were present as dense granular deposits along the thin stroma situated between two sheets of both nonneoplastic and neoplastic thyroid follicular cells, being especially marked on vascular walls (Figs. 1 and 2). Often, thyroid follicular
Table 1. Antibodies Used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Immunized animal</th>
<th>Source</th>
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<tbody>
<tr>
<td>C3d</td>
<td>—</td>
<td>Rabbit</td>
<td>Dakopatts, Glostrup, Denmark</td>
</tr>
<tr>
<td>C5b-9 (membrane attack complex)</td>
<td>aEl1</td>
<td>Mouse, IgG2a</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>S-protein (Vitronectin)</td>
<td>8E6 (L8)</td>
<td>Mouse, IgG1</td>
<td>Boehringer Mannheim GmbH, Mannheim, Germany</td>
</tr>
<tr>
<td>Decay-accelerating factor (CD55)</td>
<td>1C6</td>
<td>Mouse, IgG1</td>
<td>Wako, Tokyo, Japan</td>
</tr>
<tr>
<td>Membrane cofactor protein (CD46)</td>
<td>J4-48</td>
<td>Mouse, IgG1</td>
<td>Immunotech S.A., Marseille, Cedex, France</td>
</tr>
<tr>
<td>Complement receptor 1 (CD35)</td>
<td>To5</td>
<td>Mouse, IgG1</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>Complement receptor 2 (CD21)</td>
<td>HB-5</td>
<td>Mouse, IgG2a</td>
<td>Becton Dickinson, Oxford, CA</td>
</tr>
<tr>
<td>Protectin (CD59)</td>
<td>MEM-43</td>
<td>Mouse, IgG1</td>
<td>Serva, Heidelberg, Germany, Funakoshi, Tokyo, Japan</td>
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<tr>
<th>Vectastain Elite ABC Kit</th>
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<tr>
<td>Biotinylated affinity purified goat anti-rabbit IgG</td>
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<tr>
<td>Biotinylated affinity purified horse anti-mouse IgG</td>
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<td>Avidin DH-biotinylated peroxidase H</td>
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Cells were stained weakly on their cell bodies in chronically inflamed tissues and follicular and papillary carcinomas. This positive reaction was prominent in areas densely infiltrated by cells, including lymphocytes. In general, however, it was quite difficult to evaluate by light microscopy whether the basal cell membrane of thyroid follicular cells truly was labeled or merely bore attached reaction products. In a minority of histologically normal thyroid tissues adjacent to thyroid neoplasms, C3d, C5b-9, and s-protein were negative, but, interestingly, in the majority of cases, these antigens clearly were localized along the thin stroma situated between two layers of thyroid follicular cells (Fig. 2a). The staining intensity of C3d was highest in chronically inflamed tissues and lowest in papillary carcinomas, revealing a significant difference between the two ($P < 0.05$) (Table 2). The staining intensity of C5b-9 in chronically inflamed tissues was higher than that in the others, revealing significant differences between chronically inflamed tissue and follicular adenoma ($P < 0.01$), and between chronic inflammatory tissue and papillary carcinoma ($P < 0.05$). The staining intensity of s-protein in papillary carcinoma was significantly lower than that in chronically inflamed tissue ($P < 0.05$) and follicular carcinoma ($P < 0.01$).

The staining pattern of DAF was homogeneous in all thyroid tissues. Like C3d, C5b-9, and s-protein, DAF was observed apparently in the thin stroma, including the vascular walls situated between two layers of thyroid follicular cells. In addition, it was easy to detect the presence of DAF even by light microscopy on the basal cell membrane of thyroid follicular cells, as well as endothelial cells, in all tissues, including the normal ones (Fig. 2). No expression was observed on the lateral surface of the neoplastic thyroid follicular cells. (Left, original magnification ×72; right, original magnification ×180.)
membrane of thyroid follicular cells in any tissues, but weak-to-strong staining was present heterogeneously on the luminal surface of neoplastic thyroid follicular cells, being especially marked in papillary carcinomas (Fig. 3). There was no significant difference in the staining intensity of DAF among the five different histologic groups (Table 2).

Both MCP and protectin always were expressed strongly in a homogeneous pattern in all benign and malignant tissues, and, moreover, were expressed on the surface of almost all cells, including thyroid follicular cells and vascular endothelial cells, but not on the subepithelial and endothelial basement membranes. Light microscopy revealed distinctions in the localizations of MCP and protectin on thyroid follicular cells: MCP was expressed simultaneously on both the basal membrane and the lateral membrane, showing cell-to-cell interaction, but very rarely on the luminal membrane (Fig. 4), whereas protectin also was expressed strongly on the luminal membrane and often weakly on the basal membrane, but very rarely on the lateral membrane (Fig. 5). No significant difference in staining intensity of either MCP or protectin was evident among the five different histologic groups.

CR1 and CR2 were not present on any thyroid follicular cells but were found on the surfaces of cells in the lymphoid germinal centers in chronically inflamed tissues, as they are in cases of Hashimoto's thyroiditis.

The ultrastructural localization of complement components and regulatory factors on thyroid follicular cells also was observed with immunoelectron microscopy. The results are summarized in Table 3.

Distinct localization of C3d, C5b-9, and s-protein was evident on the subepithelial and subendothelial basement membranes (Fig. 6), corresponding to the light microscopic observations showing stromal staining, including that of vascular walls. An often faint positive reaction was observed on the basal cell border of not only nonneoplastic thyroid follicular cells but also neoplastic thyroid follicular cells (Fig. 6a), but immunoelectron microscopy failed to detect a distinct positive reaction on thyroid follicular cells in chronically inflamed tissues and carcinomas.

DAF was present predominantly on the subendothelial and vascular basement membranes (Fig. 7). In addition, this factor frequently was observed on the basal cell border of thyroid follicular cells in all tissues and frequently on the apical cell border of carcinoma cells. It rarely was found, however, on the lateral membrane of thyroid follicular cells in any tissues.

The ultrastructural localizations of MCP and protectin corresponded approximately with the findings of light microscopy; MCP apparently was present simultaneously on the basal cell membrane and the lateral membrane, showing cell-to-cell interaction, but not on the luminal membrane of thyroid follicular cells (Fig. 8), whereas protectin was localized predominantly on the luminal surface and often weakly on the basal membrane, but not on the lateral membrane of thyroid follicular cells (Fig. 9).

Discussion

The present study confirmed the presence of complement activation with subsequent deposition of C3d and C5b-9 complexes in thyroid carcinomas and some histologically normal thyroid tissues, chronically inflamed thyroid tissues, and follicular adenomas both in the stroma and on the tumor cell membrane. The data also indicate that thyroid carcinoma cells are protected from in situ complement-mediated cell lysis by expressing complement-regulatory factors, just as benign thyroid follicular cells do. Furthermore, interesting findings related to the ultrastructural localization of complement-regulatory factors were obtained.

S-protein (vitronectin) consistently was localized in

Table 2. Staining Intensity of Complement Components and Regulatory Factors in Thyroid Tissues

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<tr>
<td>Normal tissue</td>
<td>3.75 ± 0.88</td>
<td>3.16 ± 1.32</td>
<td>5.73 ± 0.48</td>
<td>4.32 ± 1.56</td>
<td>5.70 ± 0.45</td>
<td>0</td>
<td>0</td>
<td>5.80 ± 0.25</td>
</tr>
<tr>
<td>Chronically inflamed</td>
<td>5.18 ± 1.17</td>
<td>4.66 ± 1.73</td>
<td>5.85 ± 0.55</td>
<td>4.78 ± 1.88</td>
<td>5.80 ± 0.56</td>
<td>0</td>
<td>0</td>
<td>6.00 ± 0</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>2.82 ± 0.91</td>
<td>2.88 ± 1.96</td>
<td>4.82 ± 0.92</td>
<td>4.22 ± 1.67</td>
<td>5.60 ± 0.65</td>
<td>0</td>
<td>0</td>
<td>5.90 ± 0.22</td>
</tr>
<tr>
<td>Follicular carcinoma</td>
<td>2.26 ± 1.04</td>
<td>3.24 ± 2.46</td>
<td>5.29 ± 0.76</td>
<td>5.24 ± 1.70</td>
<td>5.79 ± 0.39</td>
<td>0</td>
<td>0</td>
<td>6.00 ± 0</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>1.72 ± 0.82</td>
<td>2.73 ± 2.38</td>
<td>4.61 ± 1.51</td>
<td>5.17 ± 1.96</td>
<td>5.74 ± 0.51</td>
<td>0</td>
<td>0</td>
<td>5.87 ± 0.30</td>
</tr>
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</table>

DAF: decay-accelerating factor; MCP: membrane cofactor protein; CR: complement receptor.

* P < 0.05.
† P < 0.01.
Figure 6. Immunoelectron micrograph of C5b-9 staining in chronically inflamed tissue (top) adjacent to papillary carcinoma (bottom). Positive reaction (arrows) is evident predominantly on the basement membranes, partly attached to the basal surface of the thyroid follicular cell in top of figure. (Bar = 1 μm; top, original magnification ×5400; bottom, original magnification ×3600.)

Table 3. Ultrastructural Localization Sites of Complement Components and Regulatory Factors on the Cell Membrane of Thyroid Follicular Cells

<table>
<thead>
<tr>
<th>C5b-9</th>
<th>s-protein</th>
<th>DAF (C3b)</th>
<th>MCP (C4b)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>A B C D E</td>
<td>A B C D E</td>
</tr>
<tr>
<td>Basal membrane</td>
<td>(++)</td>
<td>(+)</td>
<td>(++)</td>
</tr>
<tr>
<td>Lateral membrane</td>
<td>(++)</td>
<td>(+)</td>
<td>(++)</td>
</tr>
<tr>
<td>Luminal membrane</td>
<td>(++)</td>
<td>(+)</td>
<td>(++)</td>
</tr>
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</table>

DAF, decay-accelerating factor; MCP, membrane cofactor protein; ( ) : negative; (+) : weakly positive; (++) : moderately to strongly positive; A, histologically normal thyroid tissue; B, chronically inflamed thyroid tissue; C, follicular adenoma; D, follicular carcinoma; E, papillary carcinoma.

the thin stroma and around carcinoma cells, in a distribution similar to that of C3d and C5b-9. These types of deposits probably represent SC5b-9 (m) complexes that are cytolytically active; however, immunohistochemical analysis has not excluded the presence of cytolytically inactive SC5b-9 complexes. Interestingly, these three antigens were observed even in the majority of histologically normal thyroid tissues. Generally, normal thyroid and muscle tissue are negative for C5b-9, but even histologically normal muscle fibers in autoimmune myositis are sometimes positive for C5b-9. Moreover, C3d and s-protein normally are present in glomeruli. The present study demonstrated that even
though thyroid tissues adjacent to thyroid neoplasms seemed to be histologically normal, stimulation due to the presence of thyroid neoplasms may evoke in situ complement activation.

DAF was particularly abundant on vascular walls.
proteins but also on the epithelial surface of the gastrointestinal mucosa, exocrine glands, and others. The present immunoelectron microscopic observations demonstrate that DAF was present consistently on the subepithelial and vascular basement membranes in all tissues and on the basal cell border of all thyroid follicular cells, and almost constantly on the luminal surface of thyroid follicular cells in chronically inflamed tissues and neoplastic tissues. The presence of DAF was especially marked in cases of carcinoma and showed attenuated expression on the luminal surface of carcinoma cells. DAF was absent, however, on the lateral membrane of all thyroid follicular cells.

MCP primarily protects host cells from alternate pathway-mediated C3 targeting. The presence of antibodies directed against the surface antigens of tumor cells would activate the complement system through the classic pathway. It is still unknown whether complement activation through the alternate pathway occurs in thyroid carcinoma tissues or whether MCP protects thyroid carcinoma cells from such activation. It has been suggested that in pathologic states such as autoimmune disorders, the binding of an autoantibody to a target may overcome this protective effect of MCP via the classic pathway. As shown in this study, the tissues surrounding thyroid neoplasms sometimes contain a chronic inflammatory reaction similar to that evident in Hashimoto’s thyroiditis, and furthermore, that some papillary carcinomas were infiltrated by cells composed mainly of lymphocytes. It therefore is easy to suggest the occurrence of a pathologic state such as an autoimmune thyroid disease in these tissues.

Chronically inflamed tissues contained more intense deposits of C3d, C5b-9, and s-protein than did papillary carcinomas. The significance of this finding is not clear, but it is likely that complement activation occurs frequently and is widely distributed in chronically inflamed tissues like those in seen in cases of Hashimoto’s thyroiditis. MCP exists ubiquitously on most human nucleated cells exposed to blood, including the epithelial cells of the normal thyroid gland. It has been reported that the intensity of staining of exocrine gland epithelial cells is strongest on the luminal surfaces of the cells and may be due to cell differentiation and/or maturation or to polarization of membrane proteins to the exterior cell surface. Alternatively, it may reflect the particular need for regulators of complement activation on cells exposed to extracellular fluids other than serum or indicate that MCP has another, as yet unidentified, physiologic role. Furthermore, similarities in the tissue distribution of MCP, especially on the luminal surfaces of exocrine glandular cells, to that of DAF are suggested. To our knowledge, no data concerning the localization of MCP or DAF in thyroid carcinomas, however, have been reported. In the present ultrastructural study, MCP was present consistently on both the basal cell border and lateral membrane, showing cell-to-cell interaction, but not on the luminal surface of thyroid follicular cells.

CR1 is expressed on most peripheral blood cells, follicular dendritic cells, and other cell types. CR2 is expressed by mature B-lymphocytes, follicular dendritic cells, and pharyngeal epithelial cells. In the present study, thyroid follicular cells were found to express neither CR1 nor CR2.

Protectin is distributed widely and normally on the membranes of human blood cells, vascular endothelial cells, thyroid follicular cells, and other cell types. Tandon et al. studied two membrane attack complex-inhibiting proteins, protectin and membrane attack complex-inhibiting protein/homologous restriction factor, in various benign thyroids and found that protectin is more important in mediating resistance to complement attack. They found that protectin was localized mostly on the luminal surface of thyroid follicular cells in normal thyroids and in those obtained from patients with Hashimoto’s thyroiditis, Graves’ disease, and benign thyroid nodules. Their study did not include malignant tissues. Our present ultrastructural study also demonstrates that, unlike MCP, protectin is present consistently on the luminal surface and less frequently on the basal cell border, but is absent on the lateral membrane.

Our ultrastructural observations demonstrate an interesting distinction in the distribution of complement-regulatory factors on thyroid carcinoma cells; that is, s-protein, DAF, MCP, and, frequently, protectin are all present on the basal membrane, and that MCP and, frequently, s-protein are present on the lateral membrane. Furthermore, protectin and, frequently, s-protein and DAF are present on the luminal surface, revealing that the entire surface membrane of each thyroid follicular cells is surrounded with complement-regulatory factors. It is unclear what these phenomena imply, but it is suggested that complement-regulatory factors protect thyroid carcinoma cells against cell lysis by in situ complement activation.

There were some differences in the staining intensity of C3d, C5b-9, s-protein, and DAF among the five different histologic tissue types. As in the cases of follicular and papillary carcinoma, specimens of chronically inflamed tissue often showed intense complement activation, indicated by the presence of heavy deposits of complement components and regulatory factors, suggesting more frequent and intense stimulation of these
tissues than of histologically normal tissues and follicular adenomas.

Clinical administration of murine monoclonal antibodies against various tumor antigens, so-called missile therapy, can produce antitumor effects leading to tumor regression. Our data may be of use in subsequent studies of missile therapy for certain thyroid malignancies.

References


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