Localization of blood coagulation factors in the germinal centers of human Peyer’s patches

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Summary. The immunohistochemical distribution of 15 blood coagulation factors in the germinal centers (GCs) of human Peyer’s patches (PPs) was studied. Although factor VIII, active alpha-thrombin, and fibrinogen were hardly evident in the GCs, the majority of coagulation factors, such as kallikrein, high-molecular-weight kininogen, factos XII, X, IX, VII, V, XIIIa and XIIIb, prothrombin, anti-thrombin III and inactive alpha-thrombin were found, showing a lace-like staining pattern similar to that obtained with a monoclonal antibody, R4/23, specific for follicular dendritic cells (FDCs) in the GCs. By immunoelectron microscopy, positive reactions for factor X and XIIIa were found on the surfaces of FDCs, GC cells, and/or in the intercellular spaces of GCs, being especially marked on the surface of the labyrinth-like structure of FDCs. It is concluded that a majority of coagulation factors are localized in the GCs of human PPs. Furthermore, it is suggested that some of these coagulation factors have a close topographical relationship with FDCs.

Key words: Follicular dendritic cells, Coagulation factors, Peyer’s patches, Germina1 centers

Introduction

Well-developed Peyer’s patches (PPs) as well as other lymphoid tissues possess germinal centers (GCs) with an accompanying reticular meshwork of follicular dendritic cells (FDCs) (Chen et al., 1978; Smintia et al., 1982). FDCs are believed to play important roles in the regulation and generation of B memory cells, by trapping and retaining immune complexes on the surface of their cytoplasmic processes (Klaus and Humphrey, 1977; Tew and Mandel, 1978; Grey and Skarvall, 1988). Complement components C3b and C3d fragments and their receptors, CR1 and CR2, are indispensable for the trapping and retaining of immune complexes (Reynes et al., 1985; Imai et al., 1986; Kasajima et al., 1987).

Recently, it was demonstrated that some coagulation factors, including factor XIIIa, could be specific markers for the FDCs in paraformaldehyde-fixed freshly-frozen sections of human reactive lymph nodes and hyperplastic tonsils (Nemes et al., 1987; Yamakawa et al., 1991). In hemostasis, factor XIIIa, an active form of factor XIII, is essential for production of cross-linking fibrin in the final step of blood coagulation (Muszbek et al., 1985; Bain, 1986). However, it is not yet clearly understood why coagulation factors are localized on FDCs. Furthermore, it is unknown whether they are localized in the GCs of human PPs.

It is widely reported that coagulation factors are indispensable for the complement-activation cascade (Ghebrehiwet et al., 1981; Mollness and Lachemann, 1988). Alpha-thrombin converts fibrinogen to fibrin, factor XIII to factor XIIa and factor XIIb, and complement component C3 to a hemolytically inactive C3b fragment (Hugli, 1977). It is evident that the factor XIIIb gene is a member of the regulatory complement of activation (RCA) gene family, along with the genes for CR1, CR2, decay-accelerating factor, C4 binding protein and factor H (Rodríguez de Córdoba et al., 1988).

In the present study, the localization of 15 blood coagulation factors in the GCs of human PPs, was investigated immunohistochemically, and its significance was considered.

Materials and methods

Tissue specimens

Tissue samples from the terminal ileum including PPs were obtained at the time of right hemicolecotomy
Coagulation factors in GCs of human PPs

from 10 patients with right colon cancer. None of the patients had any distinct clinical abnormality of complement activation or of the blood coagulation system. Tissue samples for light microscopy were cut into slices approximately 5 mm thick, fixed in B5, embedded in paraffin and cut into 4 μm thick sections. The sections were then stained with hematoxylin-eosin. A portion of each tissue specimen was immersed in periodate-lysine-2% paraformaldehyde (PLP) fixative for 6 h at 4°C, rinsed with graded sucrose in 0.01M phosphate-buffered saline (PBS), pH 7.4, embedded in OCT compound (Miles Elkhart, IN, USA), and stored at -80°C until cryostat sectioning for immunoelectron microscopy (Mclean and Nakane, 1974). The remaining fresh tissues for light microscopic immunohistochemistry were trimmed and immediately frozen in OCT compound.

Immunohistochemistry

To evaluate the immunoreactivities of all the antisera listed in Table 1 in the same GC, 5 μm-thick serial cryostat sections from fresh-frozen tissues were prepared. The sections were treated in order to block endogenous peroxidase activity with 5 mM H104 in PBS for 15 min at 4°C, and then immunostained using the indirect or direct immunoperoxidase method (Farr and Nakane, 1981). To visualize peroxidase activity, 0.02% 3,3'-diaminobenzidine (Dojin Chemicals, Tokyo, Japan) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.01% H2O2 was used. Nuclear staining was performed with methyl green. FDCs were identified by immunostaining with a mouse monoclonal antibody, R4123 (Naiem et al., 1983). Each of the other immunostaining patterns were estimated light microscopically by comparison with that of R4123. At least 20 different GCs were evaluated.

For immunoelectron microscopy, cryostat sections, 8-10 μm thick, were prepared from PLP-fixed tissues and immunostained with anti-human factor X and factor XIIa antibodies, using the indirect immunoperoxidase method. Sections were fixed with 0.1% glutaraldehyde in PBS for 10 min at room temperature, refixed with 1% osmium tetroxide in PBS for 1 h at room temperature, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections were prepared using an LKB Ultratome III and observed using a Zeiss EM 109 electron microscope.

Control study

Control studies for immunohistochemistry were carried out as follows: 1) Instead of the first-step antisera, normal rabbit, goat or mouse immunoglobulin was used; 2) Non-specific staining was checked by omitting the first-step antisera or second-step antisera; 3) Endogenous peroxidase activity was checked using only the diaminobenzidine reaction; 4) Vascular endothelial cells and GCs in human lymphnodes (n = 2) obtained from patients with gastric carcinoma at operation were estimated (Yamakawa et al., 1991) for positive control; 5) Physiological irrelevant tissues for blood coagulation factors, including normal colon (n = 2), uterine leiomyoma (n = 3) and primary follicles without GCs of PP (n = 3) were used for negative control in order to check the specific reactions only in the GC.

Results

Localization of coagulation factors in the GCs of PPs

The results are summarized in Table 2. All GCs were reactive with monoclonal antibody R4/23 specific for FDCs, showing a lace-like staining pattern especially in the light zone and the mantle zone (Fig. 1). As shown in Table 2, the majority of coagulation factors, including factor XII, high-molecular-weight kininogen (HMWK), kalikrein, factors X, IX, VII, V, XIIIa and XIIIb, prothrombin, inactive alpha-thrombin (EST1) and anti-thrombin III (ATIII) were restricted to the GC and showed a lace-like staining

![Fig. 1. Immunostaining of R4/23. Note a lace-like staining pattern in the GC especially in the light zone (LZ) and in the mantle zone (MZ). Fresh-frozen section counterstained with methyl green. × 127](image-url)
Coagulation factors in GCs of human PPs

Figs. 2a - 2e.
Immunostaining in Peyer's patches using fresh-frozen cryostat sections. All slides are counterstained with methyl green.
2a. Immunostaining of factor VII. Note a lace-like staining pattern in a GC and part of a mantle zone. The stronger reaction is marked in a light zone (LZ) × 167.
2b. Immunostaining of the factor XIIIb. The strong, lace-like staining pattern is seen not only in a germinal center (GC) but also in the mantle zone (MZ). The vascular endothelia (arrows) are also positive. × 127.
2c. Immunostaining of prothrombin. Note the stronger positive reaction in a whole germinal center, being marked in the light zone (LZ). × 127.
2d. Immunostaining of factor VIII. No marked positive reaction is found in a GC. Only the vascular endothelia (arrows) reacted. × 127.

pattern. However, there were some differences in the positive reaction patterns. Factor XII, HMWK, factor VII (Fig. 2a), factor XIIIa and factor XIIIb (Fig. 2b) were seen not only in the GC but also in the mantle zone. On the other hand, factors X, IX, V and prothrombin (Fig. 2c) were found throughout the whole GC, but not in the mantle zone. In some positive cases, immunostains to HMWK, kallikrein, factors X, V, XIIIa, XIIIb, and prothrombin were also found in the cytoplasm of small round cells both within and without the GCs. Factor VIII (Fig. 2d) and fibrinogen were hardly evident in the GCs and mantle zone, but both were localized in the vascular walls and/or lumens in the PPs. ATIII and inactive
alpha-thrombin (EST1), the latter recognizing only the thrombin-antithrombin III Complex, were detected in a whole GC, being marked in the light zone (Fig. 3a). The localization of prothrombin was very similar to that of ATIII and EST1. However, active alpha-thrombin (EST4), which recognizes only the active enzyme sites of thrombin, was not observed distinctly (Fig. 3b).

**Immunoelectron microscopy**

The localization of factor X (Fig. 4a) and factor
Coagulation factors in GCs of human PPs

Table 1. List of antisera used in this study

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Source</th>
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<tbody>
<tr>
<td>[first-step antisera]</td>
<td></td>
</tr>
<tr>
<td>monoclonal mouse anti-human prothrombin&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>alpha-thrombin EST-1&lt;sup&gt;2&lt;/sup&gt;, EST-4&lt;sup&gt;3&lt;/sup&gt;</td>
<td>BML-JPN</td>
</tr>
<tr>
<td>R4/23</td>
<td>Bioscot-UK</td>
</tr>
<tr>
<td>polyclonal rabbit anti-human factors X, VII, VIII and V, fibrinogen factors IX, Xila and Xlllb, kallikrein, antithrombin III</td>
<td>DAKO-JPN</td>
</tr>
<tr>
<td>polyclonal goat anti-human factor XII, high-molecular-weight kininogen</td>
<td>Hoechst-JPN</td>
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</tbody>
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<sup>1</sup> This antisera has a cross reactivity to human thrombin.

<sup>2</sup> EST1 binds the thrombin-antithrombin III complexes (T-AT), but binds thrombin only minimally. EST1 also recognizes a site on prothrombin although with a low affinity.

<sup>3</sup> EST4 binds thrombin but not the T-AT complex. EST4 recognizes an epitope of the enzyme active site.

HPR = horseradish peroxidase

Table 2. Results of immunohistochemical staining of coagulation factors in germinal centers of human Peyer’s patches

<table>
<thead>
<tr>
<th>factor XII</th>
<th>HMWK</th>
<th>kallikrein</th>
<th>factor X</th>
<th>factor IX</th>
<th>factor VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td></td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>factor VII</td>
<td>+</td>
<td>factor V</td>
<td>factor Xlia</td>
<td>+</td>
<td>fibrinogen</td>
</tr>
<tr>
<td>++</td>
<td>+</td>
<td></td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>prothrombin</td>
<td>inactive thrombin (EST1)</td>
<td>active thrombin (EST4)</td>
<td>ATIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>+ +</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

++: strong positive, +: weak positive, -: negative

HMWK, high-molecular-weight kininogen; ATIII, antithrombin III.

XIIIa (Fig. 4b) was examined by electron microscopy. Both were observed on the surface of GC cells, FDCs, and/or in the intercellular spaces of the GC. They were particularly evident on the surface of the labyrinthine-like cytoplasmic extensions of FDCs (Fig. 4b). No distinct immunostaining, however, was found in perinuclear cisternae or rough endoplasmic reticulum of any lymphocytes or FDCs.

Control study

No immunostaining was found when the first- and/or second-step antisera were omitted, and when normal mouse, goat or rabbit serum was incubated instead of the first-step antisera. All coagulation factors were immunopositively detected in vascular walls and/or GCs of human lymphnodes. No marked lace-like immunostaining of all antisera was found in any tissues of normal colon, uterine leiomyoma, or primary follicles without GCs of PP.

Discussion

Generally, it is evident that blood coagulation factors are activated via the intrinsic or extrinsic pathway. The intrinsic pathway is initiated when factor XII is activated with a cascade of catalytic reactions by exposure to a foreign surface, subsequently followed by cleavages of factor XI, factor IX, factor X and prothrombin. In this cascade, HMWK, factor VIII and factor V are cofactors which are indispensable for activation of factor XII, factor X and prothrombin, respectively (Bain, 1986). On the other hand, the extrinsic pathway is initiated when factor VII is exposed to tissue factor (Nemerson and Gentry, 1986; Fair and MacDonald, 1987). Although factor VIII is essential for activation of factor X in the intrinsic pathway (Hultin, 1982; Mertens et al., 1985), factor VII can satisfy activation factor X without factor VIII in the extrinsic pathway (Bom and Bertina, 1990). Either way, once the coagulation factors are activated as a serin protease, activated prothrombin (alpha-thrombin) and activated factor XIII (factor XIIIa) finally convert fibrinogen to stabilized fibrin (Bain, 1986).

In this study, as shown in Table 2, the majority of coagulation factors were found to be restricted to the GC, being especially marked in the light zone. Some of them were also found in the mantle zone. In contrast, factor VIII and fibrinogen were never found in the GC, being localized only in the vascular walls and/or lumen. Active alpha-thrombin (EST4) was hardly ever expressed, although inactive alpha-thrombin (EST1) and prothrombin were found.
Coagulation factors in GCs of human PPs

concurrently in the light zone of the GC. On the other hand, both factor XIIb and factor XIIa were found in the light zone and mantle zone. Factor XIIb is a factor released from factor XIII as a result of the thrombin-catalyzed cleavage of factor XIIIa (Muszbek et al., 1985), functioning to regulate factor XIIa and contact activation of blood coagulation (Halkier and Magnusson, 1988). Furthermore, hematoxylin-eosin staining of the GC showed it to be free of fibrin deposits (unpublished data). These results indicate that coagulation activation in the GC is not completed to its final stage of stable fibrin formation, although some blood coagulation factors are activated. It is evident that immune complexes induce the activation of the extrinsic pathway of coagulation (Rothberger et al., 1977), and that immune complexes bearing complement C3, which are trapped and retained by FDCs, are abundant in the GC (Klaus and Humphrey, 1977; Tew and Mandel, 1978; Imai et al., 1986; Kasajima et al., 1987). These immune complexes in the GCs may contribute to activation of the coagulation cascade.

In this study, it has been demonstrated that most of the positive immunostaining had a lace-like pattern, similar to that shown by a monoclonal antibody R4/23 specific for FDCs in the GC. By electron microscopy, factors X and XIIa were observed on the surface of GC cells, FDCs, and/or in the intercellular spaces. Although it was not clear that these positive reactions were on the cell surface, for example through receptors, or in the intercellular spaces, some of them were detected distinctly on the cell surfaces and particularly marked on the surface of reticular-like cytoplasmic extensions of FDCs, which are essential for FDC function (Imai et al., 1986; Kasajima et al., 1987). These results indicate that some coagulation factors have a close topographic relationship with FDCs.

It is well known that the complement cascade system is not independent of the coagulation system. Factor XII is able to activate the classical pathway of complement (Ghebrehiwet et al., 1981). Alpha-thrombin converts complement fragment C3 to hemolytically-inactive C3b (Hugli, 1977). These evidences suggest that coagulation factors have the ability to initiate complement activation adjacent to sites of ongoing coagulation or thrombin activation. Furthermore, vitronectin-S-protein, which binds the terminal complement C5b-7 complex and prevents membrolysis (Podak and Muller-Eberhard, 1979), associates with the thrombin-antithrombin III complex (Jenne et al., 1985; Preissner et al., 1985). Recently, it has been demonstrated that vitronectin-S-protein is localized on FDC in GCs of human lymphoid tissue (Halstensen et al., 1988). In the present study EST1, which recognizes only thrombin-antithrombin III, was observed with lace-like pattern in GCs.

From our results it is concluded that, the majority of coagulation factors, such as factor XII, HMWK, kalilkrein, factors X, IX, VII, V, XIIa and XIIib, prothrombin, inactive alpha-thrombin (EST1) and ATIII are localized concurrently in the GC of human PPs, and that they may be closely associated with complement components and FDC function.

References


Coagulation factors in GCs of human PPs


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