New Features of Invasive Candidiasis in Humans: Amyloid Formation by Fungi and Deposition of Serum Amyloid P Component by the Host

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(See the editorial commentary by Pepys, on pages 1339–41.)

Background. Invasive candidiasis occurs in the gastrointestinal tract, especially in neutropenic patients. We were interested in determining whether invasive fungi formed amyloid in humans as they are known to do in vitro. We also sought to characterize the consequence(s) of such amyloid formation.

Methods. Tissue from 25 autopsy patients with invasive candidiasis of the gastrointestinal tract was stained with amyloidophilic dyes and for the presence of serum amyloid P component (SAP). Confirmation of the interaction of SAP and Candida was demonstrated using Candida albicans and mutants for amyloid formation.

Results. Amyloid was present on the cellular surface of fungi invading gut tissue. Moreover, SAP bound to the fungal cell walls, confirming the presence of amyloid. In vitro observations showed SAP bound avidly to fungi when amyloid formed in fungal cell walls. An unexpected result was the lack of host neutrophils in response to the invading fungi, not only in neutropenic patients but also in patients with normal or increased white blood counts.

Conclusions. We report the first demonstration of functional fungal amyloid in human tissue and the binding of SAP to invading fungi. It is postulated that fungal amyloid, SAP, or a complex of the proteins may inhibit the neutrophil response.
The role of SAP in infections is not fully understood, and it has not been previously studied in candidiasis. Serum amyloid P component is a homopentamer with the protomers arranged with cyclic pentameric symmetry in a disc-like conformation [12, 13]. Each protomer has a mass of 25,462 Da, and the total mass of the protein is 127,310 Da. Serum amyloid P component binds to amyloid wherever it occurs in the body [14]. Aside from binding to amyloid, another function of SAP is believed to be binding to DNA when exposed during cellular apoptosis or necrosis, thus protecting host DNA from degradation and digestion and averting loss of self-tolerance [15–18].

We found amyloid present on the surface of yeasts and filamentous forms of Candida but no evidence of amyloid AA. Serum amyloid P component, however, decorated the cell surface of all fungi in tissue. In vitro experiments demonstrated binding of SAP to Candida functional amyloid. We also found that the host neutrophil response to invasive fungi was minimal in almost all patients, including those with normal and elevated white blood cell counts. This is the first demonstration of candidal amyloid in human tissue and the first demonstration of SAP interacting with fungi in human disease. The results suggest that fungal amyloid and/or SAP may inhibit the host neutrophil response in invasive candidiasis.

MATERIALS AND METHODS

Human Tissue

Stored autopsy specimens from 25 patients with histological evidence of invasive candidiasis of the gastrointestinal tract were examined. Five of the patients died with evidence of postmortem candidemia [19]. Tissue blocks were cut thinly, deparaffinized, and stained with hematoxylin-eosin (H-E). Slides found to be positive for Candida species (presence of yeasts, pseudohyphae, and/or hyphae) were then stained with Gomori methamine silver (GMS), a polyclonal antibody to Candida (Biocare Medical, Concord, CA), and thioflavin T stains for amyloid [20] were performed on the tissue as well. Multiple H-E stained slides of infected material were carefully examined for the presence of neutrophils and other inflammatory response cells in tissue adjacent to yeasts and filamentous forms. They were designated as minimal (none to almost none seen), moderate, or brisk (too numerous to count neutrophils). The microscopist was blinded to the patients’ autopsy findings or peripheral white blood cell count. Thioflavin T fluorescence images were acquired on a Nikon Eclipse 90i confocal microscope with 408-nm excitation and 450-nm emission filters.

Fungi

Saccharomyces cerevisiae W303–1B with or without the wild-type ALS5 gene or a nonamyloid ALSV326N allele and C. albicans DAY286 were grown overnight in liquid media and washed extensively with phosphate-buffered saline [21]. Images of yeast in vitro were acquired using an Olympus BX51 fluorescence microscope.

SAP Interaction With Fungi

Fungal cells were also observed for binding of the SAP antibody or fungal cell binding of purified SAP. Yeast cells, $1 \times 10^8$, were washed 3 times with 3 ml tris-buffered saline, pH 7.4, with 4% BSA and 2 mM CaCl$_2$. The cells were pelleted by centrifugation at 4000 RPM for 3 min and supernatants discarded. The cells were resuspended in 1 ml of the same buffer supplemented with SAP, 25 µg/ml (EMD Biosciences, San Diego, CA), and incubated for 1 hour at room temperature. The cells were washed twice with the above buffer. The cells were then incubated overnight with antibody to SAP (BioCare Medical, Concord, CA), washed with above buffer, and incubated with secondary antibody at 1:500 dilutions for 1.5 hours. Cells were washed 3× with the above buffer and wet mounts were visualized by microscopy.

RESULTS

Tissue from the gastrointestinal tract of 25 patients with invasive candidiasis stained with H-E and GMS demonstrated a mixture of yeasts and filamentous forms of fungi invading intestinal tissue (Figure 1A and 1B). Host response was often minimal to the fungi. This would be an expected finding in the case of neutropenic patients, but, surprisingly, patients with normal or increased white blood cell counts often had minimal or no infiltrating neutrophils. Eighteen patients had sufficient fungi observable on H-E stain to assess host response accurately on multiple sections. Eight patients were neutropenic at death, and of those, 7 had minimal and 1 had moderate numbers of neutrophils in proximity to the fungi. Ten patients died with normal or increased numbers of peripheral neutrophils: 3 of those patients had a brisk neutrophil response to the presence of fungi, 1 patient had a moderate response, and 6 patients had a minimal response to the presence of fungi, similar to the neutropenic patients (Table 1). The difference between patients who were neutropenic and those with normal or increased white blood cell counts was not significant ($P = .21$ by $\chi^2$ analysis). There was no evidence of neutrophils phagocytosing yeasts or attacking filamentous forms of the fungi as they are known to do in vitro [22].

All specimens showed heavy SAP deposition on fungal surfaces (Figure 1C) consistent with its binding to Candida functional amyloid. Staining for SAP was equal to that of GMS for highlighting fungi from the surrounding host tissue (compare Figure 1B and 1C). In vitro experiments showed that fungi with cell surface amyloid bound SAP. When yeasts were preincubated with SAP, the anti-SAP antibody bound weakly to C. albicans or to S. cerevisiae expressing the C. albicans adhesin Als5p. When functional amyloid formation was induced by...
aggregation (11), SAP bound extensively to C. albicans and S. cerevisiae expressing the adhesive protein Als5p (Figure 2) but not to S. cerevisiae not expressing Als5p (Figure 2A) or a nonamyloid mutant AlsrpV326N [21] (data not shown). Figure 2 shows the results of binding of SAP to yeast cells in vitro. SAP associated with C. albicans cells and with Als5p-expressing S. cerevisiae cells. There was extensive aggregation of the cells (Figure 2D, 2E, and 2H), and SAP binding to individual cells in the cellular aggregates (Figure 2B and 2F) in annular pattern, as expected for surface binding. Some of the cells were fluorescent and others were not. In contrast, control S. cerevisiae cells not expressing the C. albicans adhesin were not stained (Figure 2I and 2J).

Within large cellular aggregates of C. albicans and Als5p-expressing S. cerevisiae, there were also brightly stained precipitates, apparently aggregates of SAP (Figure 2C and 2G). These aggregates were both rare and small in preparations with nonexpressing control cells (Figure 2F and 2K). These results were consistent with SAP binding specifically to Als5p, accompanied by aggregation of the SAP itself. Saccharomyces cerevisiae expressing Als5p behaves phenotypically identical to C. albicans [23].

Thioflavin T staining demonstrated strong fluorescence on all fungi in the tissue (Figure 3), which is consistent with extensive amyloid formation on the fungal cell surfaces. The same pattern occurred with thioflavin S and Congo red (Figure 4A–C). Congo red, an excellent inhibitor of amyloid-dependent fungal cell aggregation [11], clearly bound to fungi in tissue, demonstrating apple green fluorescence with polarized light characteristic of amyloid (Figure 4D). A monoclonal antibody to amyloid AA did not detect any evidence of amyloid AA on the surface of the fungi.

Table 1. Neutrophil Response to Invasive Candidiasis in Infected Gastrointestinal Tissue

<table>
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<th>Minimal Response</th>
<th>Moderate Response</th>
<th>Brisk Response</th>
<th>Total</th>
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<tr>
<td>Neutropenic patients</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>8</td>
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<tr>
<td>Patients with normal or increased numbers of white blood cells</td>
<td>6</td>
<td>1</td>
<td>3</td>
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Eighteen patients had observable fungi on hematoxylin-eosin stain of the tissue with multiple sections available to judge the host response to the fungi. Neutropenia: <1500 neutrophils and band forms/μL, normal white blood cell count: 3400–10,400 cells/μL. There was no significant difference between the 2 groups (P = .21 by χ² analysis).

Figure 1. Invasion of gastrointestinal tissue by Candida. A, Hematoxylin-eosin stain of intestinal tissue (×200). At the top of the figure, yeasts and filamentous forms of fungi staining reddish-blue (arrows) are present in an ulcerating lesion of the gastrointestinal mucosa with little inflammatory response. B, Gomori methenamine silver stain of intestinal tissue (×200). Yeast and filamentous forms of Candida stain black (arrows) and are seen within tissue, on the ulcerated mucosal tissue, and in the lumen of the gastrointestinal tract. C, Submucosal tissue of the gastrointestinal tract with invasive candidiasis stained for serum amyloid P component (×200). The protein is present on yeasts and filamentous forms of fungi, staining a reddish-brown (arrows).
DISCUSSION

This investigation demonstrates 3 novel findings with potential importance for the pathogenesis of invasive candidiasis. They are the presence of functional amyloid on fungal cell surface proteins in human disease, the binding of host SAP to invading fungi, and the possible inhibition of the host neutrophil response by the fungal amyloid–SAP complex.

Patients in this study died with invasive gastrointestinal candidiasis found at autopsy. Five of the patients had Candida species present in heart blood removed at postmortem [19], and it is possible that some of the intestinal lesions were the source of the candidemia. The H-E and GMS stains (Figure 1A and 1B) demonstrated fungi coursing through intestinal tissue with numerous yeasts and filamentous forms in close proximity to one another. There was little or no host reaction to the fungi, even among most of the patients with normal or elevated white blood cell counts (Table 1). There was no evidence of host cells directly attacking the fungi, and the lack of host cells did not appear to be related to the numbers of circulating neutrophils noted prior to death. Invasive candidiasis is often associated with neutropenia and a subsequent anatomical breach of normal host defenses [24, 25]. Indeed, gastrointestinal

Figure 2. Binding of serum amyloid P (SAP) component to yeast in vitro. SAP, 25 µg/ml, was incubated with (A–D) C. albicans; (E–H) S. cerevisiae expressing Als5p on its surface; or (I–L) S. cerevisiae transformed with empty vector only. The cells were washed and incubated with fluorescent antibody to SAP component. A, D, E, H, I, and L are brightfield images, and B, C, F, G, J, and K are the respective corresponding fluorescence images. There was no fluorescence in preparations of C. albicans or Als5p-expressing S. cerevisiae when SAP or primary antibody was omitted (data not shown).

Figure 3. Thioflavin T staining of tissue samples. Infected gut tissue (A) and uninfected spleen sample (B) were stained with 100 nM thioflavin T and imaged via confocal microscopy. Lower panels are bright light without stain.
candidiasis in neutropenic patients has little or no acute inflammatory response [26], although neutrophils seem to predominate among host response cells in variable numbers depending upon the organ of the body infected [27]. Whereas neutropenia seems to predispose to invasive candidiasis, the role of innate immunity (other than neutrophils) is difficult to characterize. Many innate immune proteins interact with *C. albicans* and may serve a protective role against disease [28]. Serum amyloid P component has not heretofore been studied in candidiasis.

*Candida albicans* binds to peptides. This step leads to fungal cell aggregation [29] and culminates in the formation of amyloid in adhesin proteins on the cell surface [5]. Cellular aggregation is the preferred social mode of *C. albicans* [29] and an important step in the formation of mature biofilms that are rich in amyloid [21]. The adhesive proteins that form functional amyloid are expressed on all morphological forms of *C. albicans* [30]. Our work demonstrates the presence of amyloid on the surface of invasive yeasts, hyphae, and pseudohyphae by staining with Congo red, thioflavin S, or thioflavin T. Slifkin and Cumbie reported Congo red staining of *Candida albicans* in human tissue in 1988 but did not relate the finding to fungal amyloid [31], and more recently, Axelson et al were unable to show fungal amyloid in conjunction with cutaneous candidiasis [32].

Amyloid deposits in the human body are coated with SAP [33], and our work shows that SAP precipitated from its fluid phase in the circulation onto a nucleus of fungal amyloid. Microscopic sections show SAP coating yeasts and filamentous forms of *Candida* (Figure 1C). The process of fungal cellular aggregation puts tension on the cell surface adhesive proteins, leading to the spontaneous formation of amyloid [6, 8, 21]. Serum amyloid P component then deposits on the fungal functional amyloid (Figure 2). Other *Candida* species and fungi possess amyloidogenic sequences on their cell surface and have the potential to form amyloid as well [6].

Serum amyloid P component was found in some animal studies to inhibit the inflammatory response. For example, SAP bound to *Streptococcus pyogenes* and rough strains of *E. coli*, but it inhibited phagocytosis and reduced murine survival after intravenous challenge [34]. Similar to our findings of diminished inflammation in the presence of fungi, SAP was shown to inhibit macrophage function and reduce the inflammatory response to *Aspergillus* conidia in the lungs [35]. Furthermore, it is interesting to note that amyloid by itself is characteristically “inert” and does not provoke a neutrophil reaction. For example, there is little or no host response to mature plaques (amyloid β) of Alzheimer’s disease [36] or to infiltrating cardiac amyloid. It is possible that the amyloid formed by the fungi may inhibit the normal host neutrophil response. Or perhaps a complex of functional *Candida* amyloid and SAP may inhibit or retard the host neutrophil response. This may be a contributing factor in some patients with candidemia that is difficult to eradicate in spite of normal or increased numbers of peripheral neutrophils.

In conclusion, this report demonstrates the formation of amyloid by *Candida* species in human tissue. The microbial amyloid was associated with precipitation of SAP on the fungal cell surface. The presence of functional *Candida* amyloid and/or SAP may inhibit the host response to the fungi, perhaps by coating antigens that would be expected to elicit a strong host response.

**Note**

*Potential conflicts of interest.* All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**