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Immunohistochemical demonstration of TGF-ß and decorin in paracoccidioidal granulomas

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Abstract

Different patterns of granulomas have been observed in 6- to 8-week-old mice after *ip* inoculation with 5 x 10^6 yeast cells of *Paracoccidioides brasiliensis*. Transforming growth factor-ß (TGF-ß) is a cytokine that has been shown to participate in fibrosis and granuloma formation; its activities seem to be modulated by the small proteoglycan decorin. In the present study, TGF-ß and decorin expression in epiploon granulomas was assessed by immunohistochemistry in susceptible (B10.A) and resistant (A/J) mice after 15, 30, 120 and 150 days of *P. brasiliensis ip* infection. The epiploon was collected, fixed in Methacarn solution and embedded in paraffin, and 5-µm thick sections were used for immunohistochemical analysis employing the streptavidin-biotin-peroxidase technique. The former mouse strain developed fatal disease with many disseminated lesions increasing in size and number during the infection and the latter developed mild disease with the presence of encapsulated granulomas. In the epiploon, TGF-ß was present on macrophages, giant cells, lymphocytes and fibroblasts, and absent on neutrophils. It was also detected in areas of fibrosis and necrosis, as well as disperse in amorphous extracellular matrix, mostly in resistant mice. Decorin was present circumscribing macrophages and giant cells containing fungi, but absent on these cells. In both mouse strains, decorin was found at the periphery of the lesions, and markedly in milky spot granulomas. In resistant mice, positivity was found around fibrotic and necrotic areas of encapsulated and residual lesions containing lysed fungi. Decorin was found associated with thick fibers around encapsulated lesions. In susceptible mice, the size and number of lesions increased with the progression of the disease and were correlated with the weaker expression of decorin. We suggest an association of decorin with the fibrogenic process observed in paracoccidioidal granulomas.

Key words
- TGF-ß
- Decorin
- *Paracoccidioides brasiliensis*
- Granulomatous lesions
- Susceptibility and resistance

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Paracoccidioides brasiliensis is a thermally dimorphic fungus that causes the most prevalent deep mycosis in Latin America, named paracoccidioidomycosis (PCM). Chronic inflammation characterized by the granulomatous response is a common aspect of PCM infection. Using an isogenic murine model of PCM, distinct patterns of granulomas have been observed in susceptible and resistant mice during *P. brasiliensis ip* infection. In experimental infection with the highly virulent isolate Pb18, it was observed that susceptible mice develop many disseminated nonencapsulated lesions with large numbers

of yeast cells and scarce type III collagen, while resistant mice produce few encapsulated granulomas with type I collagen or residual lesions with degenerated fungi (1).

Regulatory mechanisms established in the host immune response and particularly in the development of granulomatous lesions have been attributed to both pathogen and host factors (cellular and extracellular components). In this last context, participation of cytokines in granuloma formation has been reported in several infectious diseases. Transforming growth factor-ß (TGF-ß) is a cytokine with anti-inflammatory and regulatory properties that has been shown to participe in granuloma formation and fibrosis (2,3). Synthesis and deposition of extracellular matrix (ECM) components in granulomatous lesions seem to be associated with the regulatory activities of some cytokines. Distinct modulatory effects of TGF-ß and tumor necrosis factor-α on gene expression of collagens type I and IV have been observed (4). Fibrosis and regulation of the expression of ECM proteins such as collagen, fibronectin and decorin have been correlated with the presence of TGF-ß in experimental granulomas (5,6). Conversely, regulation of cell growth and differentiation has been attributed to several ECM elements that bind to different growth factors, such as endothelial cell growth factor, epidermal growth factor and TGF-ß. Decorin is a small proteoglycan formed by one or more glycosaminoglycan chains containing dermatan or chondroitin sulfate and a core protein composed of repeated sequences of amino acids rich in leucine, that has been demonstrated to bind to TGF-ß (7), acting as a reservoir of this cytokine in ECM and at the same time playing a regulatory role in TGF-ß activity (8). On the other hand, it was shown that TGF-ß could be released from the complex, in which it is found together with decorin, to the ECM by action of matrix metalloproteinases, that are able to digest and degrade the proteoglycan, releasing TGF-ß and allowing it to carry out its biological functions (9). Both negative (10) and positive (11) modulatory effects of decorin on TGF-ß functions have been reported. The *in situ* expression of ECM components and cytokines in paracoccidioidal granulomas has been poorly investigated.

In the present study, we investigated the immunolocalization of TGF-ß and decorin expression in granulomatous lesions of epiploon in mice susceptible (B10.A) and resistant (A/J) to *P. brasiliensis* infection (12) and inoculated with the highly virulent isolate Pb18 (13). We correlated the distribution of these components with the kinetics of granuloma formation and with the lesion patterns, as well as with their role in the immune response to PCM infection.

Six- to eight-week-old female mice of the B10.A and A/J strains were inoculated *ip* with 5 x 10^6 yeast cells/ml and sacrificed after 15, 30, 120 and 150 days of infection. The epiploon was collected, fixed in Methacarn solution, embedded in paraffin and 5-µm thick sections were used for immunohistochemical analysis of TGF-ß and decorin expression. Briefly, sections were deparaffinized and rehydrated, and then incubated with 30% hydrogen peroxide for 30 min at room temperature in a shaker to block endogenous peroxidase. Next, nonspecific sites were blocked with normal serum/10% bovine serum albumin (1:1 dilution) for 30 min at room temperature. Blocking solution in Tris-buffered saline (Pierce Chemical Co., Rockford, IL, USA) was then added to block endogenous biotin in tissues for 2 h. Slides were incubated with an appropriate volume of rabbit anti-TGF-ß pan-specific polyclonal antibody (1/30) (R&D Systems, Inc., Minneapolis, MN, USA) or with rabbit polyclonal antibody to mouse decorin (1/2000) (LF-113 clone, generously donated by Dr. Larry W. Fisher, National Institutes of Health, Bethesda, MD, USA), overnight at 4ºC, diluted in PBS/0.3% Tween 20. After incubation with primary antibody, biotinylated goat anti-

rabbit IgG antibody (1/1000; Rockland, Gilbertsville, PA, USA) was applied to the tissues for 1 h at room temperature, followed by incubation with streptavidin-peroxidase (1/50) (Pharmingen, San Diego, CA, USA). Finally, 0.05% 3,3' diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) added to 3 µl 30% hydrogen peroxide was applied to the slides and the reaction was stopped after visualization of a brown color on tissue sections. The sections were then counterstained with Mayer's hematoxylin and mounted slides were observed with a light microscope (Hund Wetzlar, Wetzlar, Germany) at 40, 100, 250 and 400X magnification. Image capture was used for microscopic analysis employing a video camera (Kodo, Tokyo, Japan) and a Microsoft Video Capture software.

In the epiploon, TGF-ß was present on macrophages, multinucleated giant cells, lymphocytes and fibroblasts, and absent on neutrophils in granulomatous lesions observed in both resistant and susceptible mice infected with Pb18. It was also detected in areas of fibrosis and necrosis, as well as disperse in amorphous extracellular matrix, mostly in resistant mice that developed residual lesions containing TGF-ß-positive pseudoxanthomatous macrophages in the later phase of infection (shown in Figure 1A-H). Decorin was present circumscribing macrophages and giant cells containing fungi, but absent on these cells. In both mouse strains, decorin was found at the periphery of the lesions and/or surrounding macrophages and multinucleated giant cells around yeast cells, forming granulomatous structures resembling fibrillar cocoons especially in concentric lesions localized in milky spot granulomas. In resistant mice, positivity was found around fibrotic and necrotic areas of encapsulated and residual lesions containing lysed fungi (shown in Figure 2A-J).

TGF-ß has been associated with the anti-inflammatory response and synthesis of ECM components through activation of fibroblasts

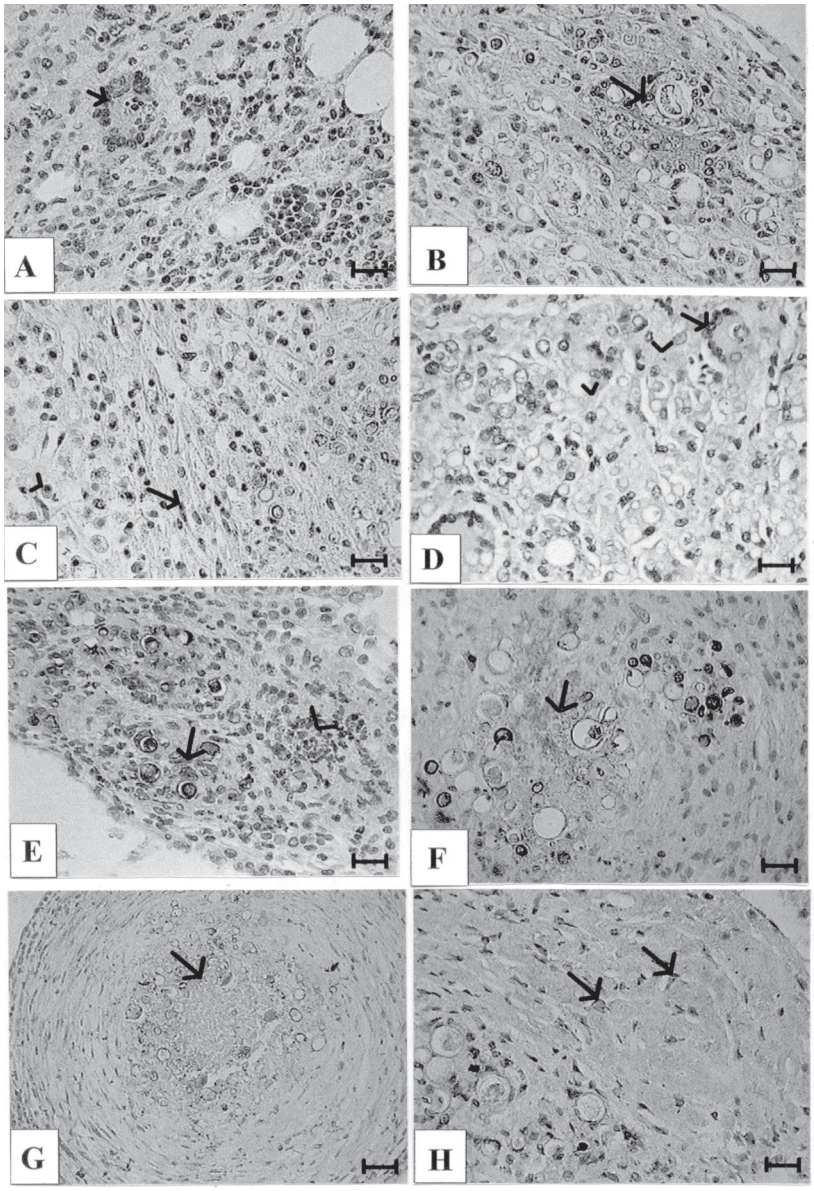

Figure 1. Immunohistochemical analysis of transforming growth factor-ß (TGF-ß) in granulomatous lesions. *A*, Positive reaction of macrophages and multinucleated giant (arrow) cells surrouding fungal cells in susceptible mice after 15 days of infection (bar = 25 µm). *B*, TGF-ß positive macrophages (arrow) in susceptible mice after 120 days of infection (bar = 25 µm). *C*, Fibroblasts (arrow) and plasma cells (arrowhead) positively labeled in susceptible mice after 120 days of infection (bar = 25 µm). *D*, Both multinucleated giant cells (arrow) and stained tissue (arrowheads) surrounding yeast cells in susceptible mice after 150 days of infection (bar = 25 µm). *E*, Pb18 surrounded by positive macrophages (arrow) and presence of TGF-ß-negative polymorphonuclear neutrophils (arrowhead) in resistant mice after 15 days of infection (bar = 25 µm). *F*, Staining of the initial area of fibrosis (arrow) in a lesion of resistant mice after 120 days of infection (bar = 25 µm). *G*, Positive reaction in the central necrosis (arrow) of a concentric lesion in resistant mice after 150 days of infection (bar = 31.25 µm). *H*, Pseudoxanthomatous macrophages (arrows) in a residual lesion in resistant mice after 150 days of infection (bar = 25 µm). Streptavidin-biotin-peroxidase reaction; original magnifications: 250X (G); 400X (A, B, C, D, E, F and H).

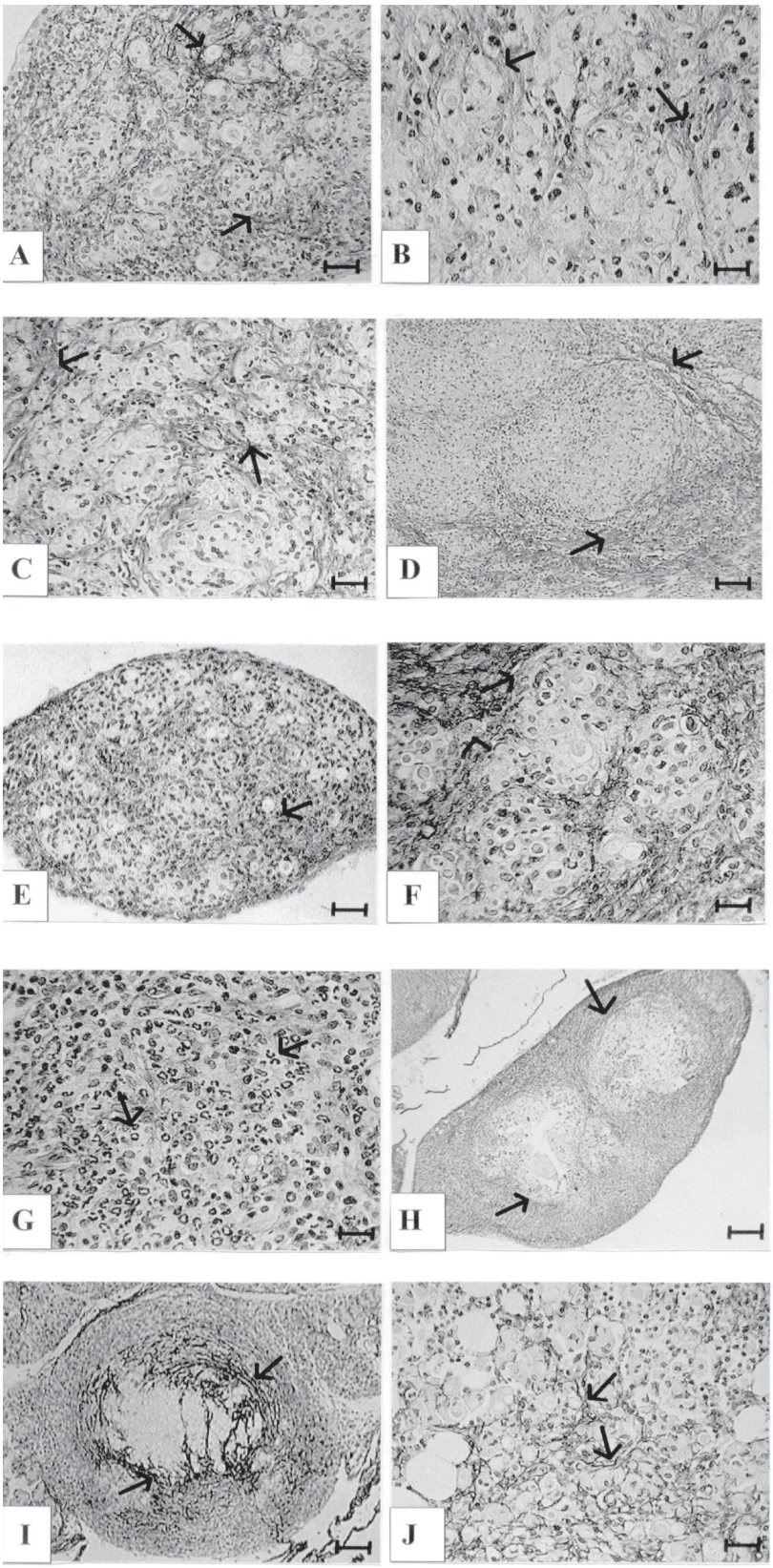

Figure 2. Immunohistochemical analysis of decorin in granulomatous lesions. *A*, Positive reaction in the extracellular matrix (ECM) around small granulomatous foci (arrows) in susceptible mice at 15 days after infection (bar = 31.25 µm). *B*, Positive loose lesion (arrows) in susceptible mice at 30 days after infection (bar = 25 µm). *C*, Decorin staining around small granulomas (arrows) in susceptible mice at 120 days after infection (bar = 31.25 µm). *D*, Extensive lesions with lesser deposition of decorin (arrows) around various granulomatous foci in susceptible mice at 150 days after infection (bar = 50 µm). *E*, Milky spot (arrow) stained in resistant mice at 15 days after infection (bar = 31.25 µm). *F*, Macrophages (arrowhead) and giant cells (arrow) surrounded by positively stained ECM in resistant mice at 30 days after infection (bar = 25 µm). *G*, Negative reaction in the cellular components (arrows) in resistant mice at 30 days after infection (bar = 25 µm). *H*, Necrotic area (arrows) in resistant mice at 120 days after infection (bar = 125 µm). *I*, Encapsulated lesion with a fibrotic area (arrows) enclosed by decorin in resistant mice at 150 days after infection (bar = 50 µm). *J*, Positivity around pseudoxanthomatous macrophages (arrows) in a residual lesion in resistant mice at 150 days after infection (bar = 31.25 µm). Streptavidin-biotin-peroxidase reaction; original magnifications: 40X (H); 100X (D and I); 250X (A, C, E and J); 400X (B, F and G).

and other cells, inducing a fibrotic reaction. The expression of TGF-ß in several cell populations, such as macrophages, giant cells and fibroblasts and/or linked to ECM observed here suggests an active role of this cytokine in the pattern of tissue response developed during granuloma formation and interfering with the type of lesions. By this interaction with cells and ECM, TGF-ß may influence the evolution or the control of PCM infection. In the present study, the presence of decorin forming fibrillar cocoons in the lesions suggests an effort to control *P. brasiliensis* dissemination by interactions of immune and inflammatory cells and ECM components against yeast cells. In an experimental infection of athymic and euthymic mice with *P. brasiliensis*, lesions with formation of fibrillar cocoons surrounding groups of fungal cells, surrounded or not by macrophages and giant cells were observed (14). In the literature, decorin has been shown to have a role in assembling and stabilizing the collagen fibrils; our results suggest the

participation of decorin in cocoon formation in an attempt to enclose *P. brasiliensis* cells, and a lesser deposition of decorin was correlated with extense loose granulomatous lesions observed mainly in susceptible mice.

The present investigation of paracoccidioidal granulomas focusing on the expression of TGF-ß and decorin indicated differences between susceptible and resistant mouse strains. In the earlier phases of infection, resistant mice showed marked TGF-ß positivity in various cell populations and the presence of both nonencapsulated and encapsulated granulomas. In the later phases, encapsulated granulomas predominated, with decorin-positive thick fibers delimiting inner necrosis areas and TGF-ß-positivity around fibrotic areas and within necrosis areas, associated with amorphous ECM and lysed *P. brasiliensis* in the center of the lesions. Polymorphonuclear neutrophils were present during the earlier stages of the infection, but were negative for TGF-ß, while positive giant cells were observed until the later phases of infection. In susceptible mice, compact lesions could be observed in the initial phases of infection, but loose granulomas predominated in later phases. Positivity for TGF-ß was ubiquitous, being found in cells, predominantly in giant cells, from the onset of the infection and in both amorphous and fibrillar ECM. A positive reaction for decorin was more associated with encapsulated lesions and with later stages of the disease. Since these lesions were less frequent, a weaker expression of decorin was observed.

We described here for the first time the presence of decorin as one of the ECM components of the fibrous capsule of PCM lesions. An association of this proteoglycan with the fibrogenic process detected mainly in lesions from resistant mice is suggested. The interactions between different cellular and extracellular host components and *P. brasiliensis* yeast cells present in granulomas are extremely complex and are important in the development of control or dissemination of PCM infection.

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