Distribution of fibronectins and their integrin receptors in interface tissue from aseptic loosening of hip prostheses

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Abstract

Objectives

To assess the distribution of fibronectins (FNs) and their integrin (Int) receptors in synovial membrane-like interface tissue (SMLIT) from aseptic loosening of total hip replacement (THR), and potential role of FN-Int interaction in the loosening process.

Methods

The alkaline phosphatase anti-alkaline phosphatase (APAAP) method was used to detect the distribution of FNs and their Int receptors in SMLIT and control samples. Double immunofluorescence labeling was used to reveal the different co-localizations.

Results

Intensive FN staining appeared in the lining layers, sublining area, and vascular endothelium, while immunoreactivities for Int α4, α5, β1 subunits were detected in the lining and endothelial cells of SMLIT. Immunofluorescence labeling revealed Int α5 and collagenase-1/collagenase-3 double positive cells in lining layers and sublining area of SMLIT.

Conclusion

Increased expression of FNs, Int α4β1 and α5β1 appeared in SMLIT compared with that in OA synovial membrane. FN-Int interactions may play a role in local collagenase production.

Key words

Total hip replacement, aseptic loosening, interface tissue, fibronectin, integrin, collagenase.

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Introduction

Aseptic loosening is the most common complication of total hip replacement (THR). An important pathologic finding in aseptic loosening is the formation of a synovial membrane-like interface tissue (SMLIT) between the cement/prosthesis and the bone (1). Cellular mediators produced by constituent cells of SMLIT have been shown to play an important role in periprosthetic osteolysis (2). In contrast, little is known about the effect of the altered extracellular matrix (ECM) components on the loosening process.

Fibronectin (FN) is a high molecular weight glycoprotein found throughout the body. Two different forms of FN exist: the soluble form in biological fluids and the insoluble form in connective tissues. FNs contain the RGD binding site for cells, as well as binding sites for many biological substances (3). Several integrins (Int), including α4β1 and α5β1, can bind FN. FN-Int interactions results in signal transduction and the modulation of gene expression (4).

The production of some matrix metalloproteinases (MMPs), i.e. MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3), are increased in aseptic loosening of THR (5). In addition, upregulated expressions of FNs and their Int receptors are found in synovial inflammation (6). We intended to assess the distribution of FNs and their Int receptors in SMLIT, to shed light on the potential role of FN-Int interactions in aseptic loosening of THR.

Materials and methods

Patients and samples

Ten SMLIT samples were collected from the interface between the cement and bone in the osteolysis areas from patients undergoing revision operations due to aseptic loosening of THR. The indication for the primary THR was OA. The mean interval from the primary THR to revision was 11.7 years (range 5-22 yrs.). Pain was the main symptom of the loosening and had persisted for 2 to 30 months (average: 8 months). No patient used steroids, while 2 of them took non-steroid anti-inflammatory drugs (NSAID) for 2 years and 1 year, respectively, before the revision operation.

For comparison, 10 synovial samples were collected from patients undergoing primary THR due to OA. Pain again was the most common symptom, and had lasted from 1 to 7 years (average: 4 yrs.). Only 3 of the patients, however, had used NSAIDs intermittently and none of them had used steroids. All samples were frozen immediately and stored at -70°C.

Immunohistochemistry

Cryostat sections 6 μm thick were fixed in acetone for 5 minutes at +4°C. After washes with 20 mM Tris buffered saline (TBS), sections were incubated with the following reagents:

1) normal rabbit serum (1:50 in TBS containing 0.1% BSA) for 20 minutes;
2) the following mouse monoclonal antibodies (Mab) overnight at +4°C: (A) 52BF12 against total FN (1:10,000), (B) 52DH1 against ED-A domain of locally produced FN (1:50), (C) B5-G10 against Int α4 subunit (1:1000), (D) SAM-1 against Int α5 (1:500), (E) 102DF5 against Int β1 subunit (1:50).
3) rabbit anti-mouse IgG (1:25) for 30 minutes at +22°C.
4) APAAP-solution (1:25) for 30 minutes at +22°C.
5) 5% new fuchsin (NF)/8.3% naphtol AS-BI-phosphate (NABP) in alkaline phosphatase-buffer for 15 minutes in the dark.

The fifth reaction was stopped with 20 mM EDTA in TBS for 5 minutes at +22° C. Monoclonal mouse IgG1 with irrelevant specificity was used at the same concentration as, and instead of, the primary antibodies as a staining control. Staining intensity scores were designated values as follows: no staining (0 points), very weak staining (1 point), weak staining (2 points), moderate staining (3 points), and strong staining (4 points). The staining extent was measured with a Leitz Diaplan lens system and scores were designated values as follows: no staining (0 points), < 10% area (1 point), 10-30% area (2 points), 31-50% area (3 points), > 50% area (4 points). The rank sum test was used for statistical analysis.

Double immunofluorescence labeling

After fixation in cold acetone for 20 minutes at -20°C, sections were incubated

with normal goat serum (Vector, diluted 1:20 in PBS containing 1.25% BSA) for 30 minutes at +22°C followed by blotting of the excess normal serum. Then the sections were incubated with following primary and conjugated antibodies at +22°C:

1) mouse anti- Int α5 IgG1 (diluted 1: 500 in PBS containing 1.25% BSA) for 60 min.
2) FITC-conjugated goat anti mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, diluted in 1:100 in PBS containing 12.5% BSA) for 30 min.
3) rabbit anti-human MMP-1 IgG (Chemicon International Inc, Temecula, CA, diluted 1:1000 in PBS containing 1.25% BSA) for 60 min.
4) TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, diluted 1:100 in PBS containing 12.5% BSA) for 30 min.

Human IgG (0.8g/L) was added to the conjugated antibody solutions to reduce non-specific staining. Slides were air-dried and mounted with Vectashield (Vector). Similar procedures were used for the Int α5 subunit and MMP-8/ MMP-13 double straining.

For FN and macrophage/fibroblast marker double labeling, the section was first incubated mouse anti- locally produced FN (52DH1), followed by incubation with FITC conjugated goat anti-mouse Fab fragments. The sections were blocked with monovalent Fab fragments. After blocking, CD 68 (the macrophage marker) and 5B5 (the fibroblast marker) were applied, respectively, followed by incubation with TRITC-conjugated goat anti-mouse IgG. A similar protocol was used for Int α4/α5 and β1 subunit double staining.

Results

Histologic evaluation of SMLIT

Synovial lining layers, usually 1 to 3 layers thick, were found in all the SMLIT samples. The stroma of SMLIT was characterized by a foreign body reaction. It can be roughly divided into two different morphologic regions: a cell-rich area containing macrophages and multi-nucleate giant cells, and a fibrotic area consisting of collagenous fibers and elongated fibroblasts. The vascularity was irregular. Some of the samples displayed poor vascularity, while others were highly vascularized.

Distribution of FNs

The distribution pattern was similar for total FN and locally produced FN. All samples of SMLIT showed immunoreactive FNs. Staining was strong in the lining layers and vascular endothelium, while in the stroma it was weak and irregular (Fig. 1A). Immunoreactivity was invariably detected in the areas with macrophage infiltration (Fig. 1B, 1C), while it was hardly discernible in the fibrosis area (Fig. 1D, 1E). The FN staining pattern in OA specimens was similar to that in SMLIT, but relatively weak and less extensive. In the stroma of OA samples, the immunoreactivity was hardly detectable (Fig. 1F). Statistical analysis of the staining scores for both intensity and extent disclosed significant differences between the SMLIT and OA samples.

Distribution of Int subunits

Double immunofluorescence revealed frequent co-localization of Int α4 and α5 with the β1 subunit (data not shown). The distribution pattern of Int α4 and α5 subunits was similar. Here, immunoreactivity for the Int α5 subunit is described as an example. Moderate to strong staining appeared in the lining layers and sublining area of SMLIT (Fig. 2A), while it was comparatively weak and less extensive in OA samples (Fig. 2B). Moderate reactivity was found in the macrophage-like cells (Fig. 2C), while it was weak in the fibroblast-like cells (Fig. 2D). The vascular endothelium usually displayed strong reactivity (Fig. 2E).

A strong Int β1 subunit reactivity was detected around cells in the lining layers and sublining area of SMLIT (Fig. 3A). Although staining in vascular endothelial cells was usually very strong (Fig. 3A), a weak reaction was occasionally found in some blood vessel walls (Fig. 3B). Similar but comparatively weak and less extensive staining was found in OA samples. However, immunoreactivity seemed quite strong in the newly formed blood vessel walls (Fig. 3C). Negative staining controls verified the specificity of the method (Fig. 3D). Statistical analysis of the staining scores of these subunits disclosed a significant difference between SMLIT and OA samples. Int β1 subunit staining was usually

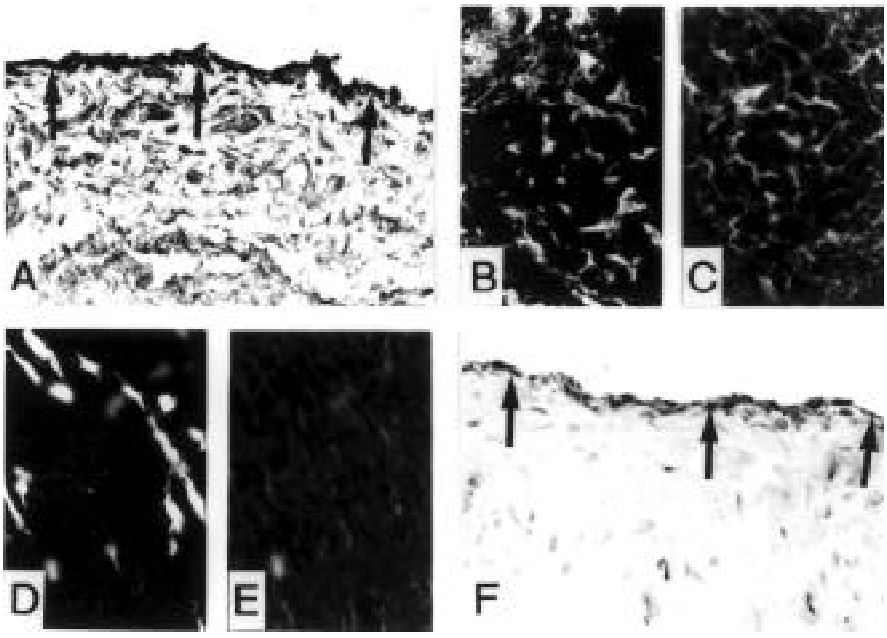

Fig. 1. Distribution of locally produced FN. (**A**) strong staining in the lining layers (**arrows**) and weak staining in the stroma of SMLIT (x 250). **B** and **C**: Double immunofluorescence labeling of FN and CD 68 in a macrophage-rich area (**B**) and FN in the same area as B (**C**) (x 426). **D** and **E**: double labeling of FN and 5B5 (x 426) showing (**D**) fibroblasts in a fibrotic area and (**E**) FN hardly detectable in the same area as D. (**F**) Relatively weak staining in the lining layer of the OA sample (x 250).

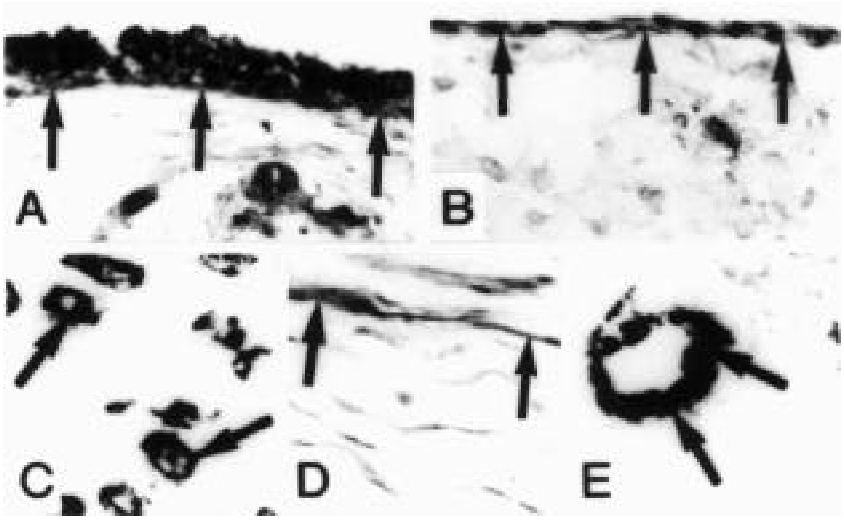

Fig. 2. Distribution of the Int α5 subunit. (**A**) Strong staining in the lining layers of SMLIT (x 312); (**B**) weak and less extensive staining in the OA sample (x 312); (**C**) moderate staining around macrophage-like cells in the cell-rich area of SMLIT (x 400); (**D**) weak staining around fibroblast-like cells (x 400) in a fibrotic region of SMLIT; (**E**) moderate staining in vascular endothelial cells in the SMLIT stroma (x 400).

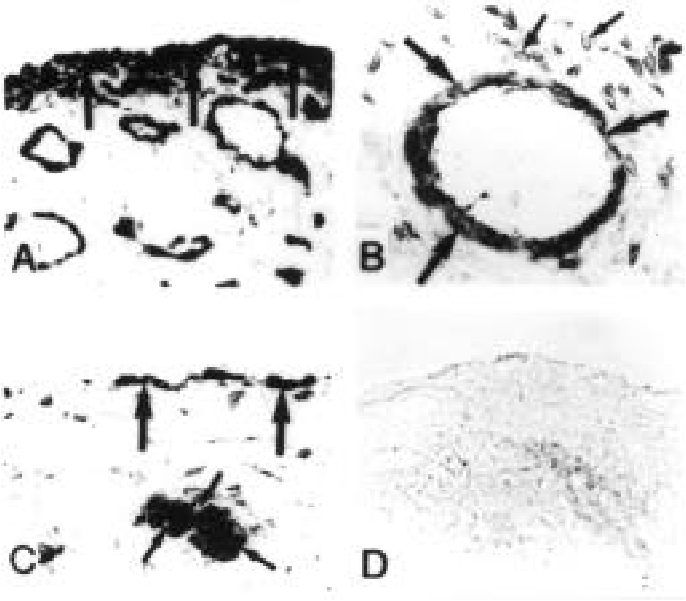

Fig. 3. Distribution of the Int β1 subunit. (**A**) Strong staining of cells in the lining layers, sublining area, and vascular endothelium of SMLIT (x 250); (**B**) weak to moderate staining of vascular endothelial cells in this SMLIT sample (x 400); (**C**) moderate, less extensive staining in the OA sample (x 250); (**D**) staining control demonstrates the specificity of the method (x 250).

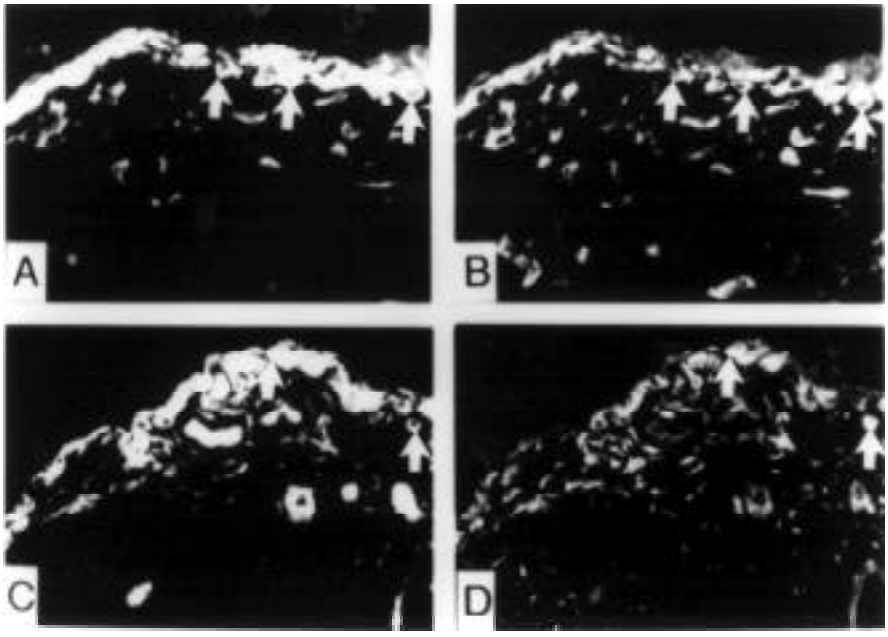

Fig. 4. Double immunofluorescence labeling of the Intα5 subunit and MMP-1/MMP-13 in SMLIT (x 533). (**A**) Intα5 subunit positive cells (**arrows**); (**B**) MMP-1 positive cells in the same field as A (**arrows**). Note that there were some Intα5 subunit/MMP-1 double positive cells. (**C**) Int α5 subunit positive cells (**arrows**); (**D**) MMP-13 positive cells in the same field as C (**arrows**). Note that there were some Int α5 subunit/MMP-13 double positive cells.

strong in the vascular endothelium of both SMLIT and OA samples, although weak reactivity occasionally appeared in some blood vessel walls. We did not, however, analyze the differences in staining intensity in the vascular endothelium between the SMLIT and OA samples separately.

Co-localization of the Int α5 subunit with MMPs

In the lining layers and sublining areas of SMLIT, there were many cells labeled both by the Int α5 subunit and MMP-1. Colocalization of the Int α5 subunit and MMP-1 occasionally appeared in vascular endothelial cells and macrophage-like cells in the stroma (Fig. 4A, 4B). The Int α5 subunit and MMP-13 double positive cells were detected in similar areas (Fig. 4C, 4D). In most of the samples, there was no MMP-8 staining colocalizing with Int α5 subunit positive cells.

Discussion

Our results demonstrated that local FN production is increased in SMLIT. A series of bioactive substances can modulate FN production. Interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-α), and some growth factors can enhance FN biosynthesis (7-9). The expression of these cytokines is upregulated in aseptic loosening of THR (10), which may contribute to the local accumulation of FN in SMLIT.

Integrins are active participants in the communication between cells and the surrounding environment. In addition to cell migration and adhesion, they mediate a wide variety of cellular processes, including cytoskeletal organization, cell proliferation, the prevention of apoptosis, and cell differentiation. Int α5β1 is known as a classic FN receptor, although FN can also bind to Int α3β1, α4β1, α4β7, αvβ1, αvβ3, αvβ6 and αIIbβ3 (11). Expression of Int subunits can be regulated by single cytokines or cytokine combinations. Expression of Int α4 subunit is upregulated by the combinations of TNF-α/IFN-γ and IL-1α/IFN-γ, whereas expression of Int α5 subunit can be modulated by TNF-α/IFN-γ, IL-1α/IFN-γ, IL-1β/TGF-β, IL-1β/TNF-α and GM-CSF (12). In aseptic loosening of THR, production of aforementioned cytokines is increased (13), which may in part explain the elevated expression of these Int subunits in SMLIT.

FN-Int interactions have profound effects on many physiological processes such as embryogenesis, wound healing, homeostasis, thrombosis and inflammation. Cooperative signaling by Int α5β1 and α4β1 regulates MMP gene expression in response to FN (14). In this study, we found the Int α5 subunit and MMP-1/MMP-13 double positive cells in the lining layers of SMLIT. Although colocalization only implies an associative and not necessarily a causative relationship, this might at least in part explain the increased expression of collagenases in SMLIT from aseptic loosening of THR (5).

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