Hyaluronan, TSG-6, and Inter-α-Inhibitor in Periprosthetic Breast Capsules: Reduced Levels of Free Hyaluronan and TSG-6 Expression in Contracted Capsules

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Abstract

Background: The exact mechanism of capsular contracture (CC) is still unknown. The covalent modification of hyaluronan (HA) with the heavy chains (HC) of inter-α-inhibitor (IαI) has been identified as an important pathway in inflammation and tissue remodeling, where HCHA formation is catalyzed by TSG-6 (the protein product of tumor necrosis factor stimulated gene-6).

Objective: The authors quantitatively assess the correlation between severity of CC (measured by Baker grade) and expression of HA, TSG-6, and IαI (ie, the polypeptides HC1, HC2, and bikunin) in periprosthetic breast capsules.

Methods: Immunofluorescent staining for HA, TSG-6, HC1, HC2, and bikunin was carried out on periprosthetic breast capsules (n = 7) of each Baker grade from four anatomical locations. Quantitative analysis was performed to identify differences in staining intensity. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to determine differences in TSG-6 gene expression levels.

Results: Severity of contracture was associated with reduced staining for both free HA (Pearson correlation coefficient, \( r = -0.645, P < .001 \)) and TSG-6 (\( r = -0.642, P = .002 \)). RT-qPCR showed a significant negative correlation between severity of contracture and TSG-6 gene expression levels (\( r = -0.750, P = .001 \)).

Conclusions: The negative correlation between TSG-6 expression levels and severity of CC suggests a possible protective role for TSG-6 in the context of CC formation, and this may have a clinically relevant role in prevention of breast CC.

Level of Evidence: 3

Keywords

breast prosthesis, periprosthetic capsule, capsular contracture, hyaluronan, TSG-6, inter-α-inhibitor

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Periprosthetic fibrous capsule formation is a normal phenomenon following insertion of breast prostheses for post-mastectomy reconstruction or breast augmentation. This occurs through transforming growth factor (TGF)-β-mediated production of collagen I, collagen III, fibronectin, and proteoglycans by fibroblasts during the resolution stage of the foreign body response. The most common reported complication of breast implant surgery is capsular contracture (CC), characterized by tightening of the periprosthetic capsule which causes distortion, pain, and discomfort. This complication may require further surgery, often involving capsulectomy and complete removal of the prosthesis. The most widely-applied classification system for CC is the Baker grading system, which grades CC by clinical severity. The exact mechanism underlying CC is currently unknown, although an association between chronic inflammation and this pathology has been suggested. One recent study reported elevated mRNA expression levels of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) and of cysteinyl leukotriene receptor-2 (cysLTR2) in contracted capsules (Baker grade III or IV).

The insertion of an implant can be seen as analogous to the formation of a three-dimensional wound, initiating tissue repair processes. Key features of tissue repair include inflammation and formation of a new extracellular matrix (ECM). Hyaluronan (HA), a high molecular weight glycosaminoglycan, is a major component of the ECM that plays important roles in cell migration and proliferation, angiogenesis, and collagen synthesis and is found in large amounts in damaged or growing tissues. Breast capsule biopsies have been shown to contain an increased amount of HA compared to normal breast tissue and a positive correlation has been reported between serum HA levels and Baker grade of CC. Despite this, there have been no reported studies on the existence of a correlation between HA content in capsule tissues and Baker grade of contracture.

TSG-6 (the product of tumor necrosis factor–stimulated gene-6) is a pluripotent protein that is upregulated in physiological and pathological conditions associated with inflammation and tissue remodeling. Its expression is induced by growth factors such as TGF-β, as well as by cytokines (including TNF-α and interleukin-1) in various cells such as fibroblasts, smooth muscle cells, monocytes, chondrocytes, and synoviocytes. TSG-6 has anti-inflammatory properties that likely relate to its ability to inhibit neutrophil migration and its role in downregulation of the protease network. The involvement of TSG-6 in HA cross-linking might also contribute to its anti-inflammatory effects, particularly through its catalysis of the covalent modification of HA with the heavy chains of the HA cross-linking enzyme, TSG-6-mediated HC·HA complex formation has been implicated in HA cross-linking through HC-HC interactions. TSG-6-/– mice are female infertile due to the absence of TSG-6-mediated HC·HA formation, where these complexes are essential for ECM formation of the cumulus around the oocyte during ovulation. Aside from ovulation (an inflammation-like process), TSG-6 upregulation and the presence of HC·HA complexes have been reported at various inflammatory sites, including the joints of rheumatoid arthritis patients and the lungs of asthmatics. The observation that HC·HA complexes could promote leukocyte adhesion suggested that they might be proinflammatory, but recent analysis of HC·HA purified from amniotic membranes revealed anti-inflammatory properties.

Here, we investigate the presence of HA, TSG-6, HC1, HC2, and bikunin in periprosthetic breast capsules and evaluate the existence of any correlation between the levels of TSG-6 or free HA and Baker grade of CC.

**METHODS**

**Periprosthetic Breast Capsule Tissue**

Periprosthetic breast capsule tissue samples (n = 7) were collected from six female Caucasian patients (ages 27-61 years) during capsulectomy or capsulotomy procedures at the University Hospital of South Manchester and Countess of Chester Hospital. Samples were collected from four different anatomical sites within the breast capsule: superior, inferior, anterior, and posterior. Bilateral capsules were collected from one patient who had bilateral capsulectomies. In total, four contracted capsules (Baker grades III and IV) and three noncontracted capsules (Baker grades I and II) were collected. This study received ethical approval from the Bolton Research Ethics Committee (Reference number: 07/Q1409/38).

**Histological Sections**

Tissue samples were fixed in 10% (v/v) neutral buffered formalin for 48 hours and processed with a Microm Spin Tissue Processor 120 (Microm International, Walldorf, Germany). After paraffin embedding, tissues were cut into serial 4-µm-thick sections with a microtome and adhered onto Vectabond-treated (Vector Laboratories, Burlingame, California) Polysine glass slides (Menzel-Glaser, Braunschweig, Germany).

**Hematoxylin and Eosin Staining**

Tissue sections were stained with Harris hematoxylin (Surgipath, Richmond, Illinois) and 0.1% (v/v) eosin (Surgipath) before being analyzed by a histopathologist to determine cellular composition, capsule thickness, and the presence of synovia-like metaplasia (which was defined as...
a layer of epithelial-like cells in contact with the implant containing basally located nuclei).22

**Immunofluorescent Staining**

Heat-induced epitope retrieval (HIER) was carried out in a sodium citrate buffer (10 mM sodium citrate, 0.05% [v/v] Tween 20, pH 6.0) with an 800-W microwave oven (full power, 10 minutes) prior to immunofluorescent staining.

An indirect, two-step immunofluorescent staining protocol was employed. Sections were blocked with phosphate-buffered saline (PBS)/2% (v/v) fetal bovine serum (FBS), followed by the Streptavidin/Biotin Blocking Kit (Vector Laboratories) at room temperature. Tissue sections were incubated in a humidified chamber with a primary antiserum (rabbit antihuman TSG-6, HC1, HC2, or bikunin) and biotinylated HA-binding protein (Seikagaku, Tokyo, Japan) in PBS/2% (v/v) FBS. Negative control sections were treated with the relevant preimmune serum (1:1000) in PBS/2% (v/v) FBS. Tissue sections were then incubated with Alexa Fluor 488-streptavidin (Invitrogen, Carlsbad, California) and Alexa Fluor 555-antirabbit IgG (Invitrogen). Samples were mounted with Vectashield containing DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories) to allow visualization of cell nuclei.

**Fluorescent Microscopy**

Images were collected on an Olympus BX51 upright microscope (10×, 20×, and 40× Plan Fln objective) and captured with a CoolSnap HQ camera (Photometrics, Tucson, Arizona) and Metavue Imaging System software (Molecular Devices, Sunnyvale, California). Specific band pass filter sets for DAPI, Alexa Fluor 488, and Alexa Fluor 555 were applied to prevent bleed-through from one channel to the next. Image processing and quantitative analysis were carried out with WCIF ImageJ v1.40 software (http://rsb.info.nih.gov/ij). Staining intensity was measured by mean gray values generated from monochrome images of single-antigen fluorescence in serial breast capsule sections. All data were collected on standard microscopy settings.

**Real-Time Quantitative Polymerase Chain Reaction**

Following RNA extraction with the RNeasy kit (Qiagen, Hilden, Germany) and complementary DNA (cDNA) synthesis with the SuperScript II Reverse Transcriptase kit (Invitrogen), real-time quantitative polymerase chain reaction (RT-qPCR) was carried out with a LightCycler 480 platform (Roche Diagnostics GmbH, Basel, Switzerland), and TSG-6 gene expression (forward primer, GGCCATCTCGCAACTTACA; reverse primer, CAGCACAGACATGAAATCCAA) was determined relative to RPL32 and GAPDH reference genes. As two copies of amplicons were made during each cycle, \(2^{-\Delta\Delta CT}\) was calculated to represent relative expression levels.

**Statistical Analysis**

Statistical analysis was carried out with SPSS 14.0 (SPSS, Inc., Chicago, Illinois). Pearson’s correlation coefficient was measured to identify significant linear correlations between continuous variables. The independent samples t-test was performed to test for significant differences in staining intensity. One-way analysis of variance (ANOVA) was carried out to determine significant differences in staining intensity and relative gene expression levels at different anatomical sites. Statistical significance was defined as \(P < .05\).

**RESULTS**

**Light Microscopy**

Breast capsule sections from superior, inferior, anterior, and posterior anatomical locations showed no gross differences in histological morphology. The typical appearance was of dense collagen bundles with sparsely distributed fibroblasts interspersed between the bundles. However, there was a clear difference in the architecture of the capsule tissue from the inner aspect (in contact with the implant) compared with the outer aspect (Figure 1). A gradient of increasing thickness and density of collagen bundles from the inner to outer aspects was apparent.
whereas cellularity was maximal in the region of contact with the implant and became more sparse in the outer layers. Blood vessels lined by endothelial cells were visible in some samples.

Synovia-like metaplasia (Figure 2) was observed in two of the five capsules surrounding textured implants. This feature was not seen in the smooth implant capsules. Overall mean capsule thickness across all seven samples was 0.84 mm (range, 0.5-1.4 mm). Mean capsular thickness within each sample was found to decrease with increased duration of implant insertion (Pearson correlation coefficient, $r = -0.505$, $P = .06$). However, there were no significant differences in mean capsular thickness with different Baker grades or anatomical locations.

**Immunohistolocalization of HA, TSG-6, HC1, HC2, and Bikunin**

All breast capsule sections stained positive for HA, TSG-6, HC1, HC2, and bikunin, as exemplified in Figure 3A-D. Staining intensities were significantly above background (ie, negative control sections; Figure 3E-H) in all cases (independent samples $t$ test: HA, $P < .001$; TSG-6, $P = .002$; HC1, $P < .001$; HC2, $P < .001$; bikunin, $P < .001$).

Extensive extracellular HA staining was seen in the capsule tissues, closely associated with and parallel to the collagen bundles. Staining was most intense in the zone of capsule tissue in close contact with the implant. HA staining intensity showed a negative correlation with Baker grade of contracture ($r = -0.645$, $P < .001$; Figure 4A) and implant age ($r = -0.662$, $P < .001$).

TSG-6 staining was localized predominantly to areas surrounding the DAPI-positive nuclei of fibroblasts and endothelial cells, with reduced staining in the immediate vicinity of the inner aspect (Figure 3A). HC1 and HC2 staining (Figure 3B,C) was also associated with fibroblasts but was less extensive than the TSG-6 staining. Bikunin staining associated with fibroblasts appeared more intense than for HC1 or HC2 and was both cell associated and colocalizing with HA (Figure 3D). Increasing Baker grade of contracture ($r = -0.642$, $P = .002$; Figure 4B) and implant age ($r = -0.619$, $P = .004$) were both associated with decreased TSG-6 staining. However, there were no significant correlations between HC1, HC2, or bikunin staining intensities with implant age or Baker grade. Positive staining was also detected for HA, TSG-6, HC1, HC2, and bikunin in synovia-like cells in areas of synovia-like metaplasia seen in two breast capsule sections (Figure 5). There were no significant associations between anatomical site and staining intensity for any of the molecules studied.

**TSG-6 Gene Expression**

RT-qPCR revealed the presence of TSG-6 mRNA in periprosthetic breast capsules. There was a significant negative correlation ($r = -0.750$, $P = .001$) between relative TSG-6 gene expression levels and Baker grade (Figure 4C) and between relative TSG-6 gene expression levels and implant age ($r = -0.662$, $P = .004$). However, there were no significant differences in relative TSG-6 gene expression levels between anatomical sites.

**DISCUSSION**

This study has identified the presence of TSG-6 and the IαI polypeptides HC1, HC2, and bikunin within the fibrous capsule tissue surrounding silicone breast implants. The association of immunofluorescent staining for TSG-6 with breast capsule fibroblasts is indicative of local expression of TSG-6 by these cells and this was supported by the detection of TSG-6 mRNA by RT-qPCR. The expression of TSG-6 by fibroblasts has been shown previously to be upregulated by IL-1 and TNF (Figure 6); this is consistent with the detection of elevated levels of TNF mRNA in contracted capsules. In this study, we found that TSG-6 localization and gene expression were reduced in contracted capsules compared to non-contracted capsules.

IαI is known to be synthesized in the liver and distributed to various tissues via the serum, where it is present in high concentrations. However, local synthesis of HC1, HC2, and bikunin in various tissues has been reported. The association of HC1, HC2 and bikunin...
Figure 3. Immunofluorescent staining for hyaluronan (HA) together with TSG-6, HC1, HC2, or bikunin in periprosthetic breast capsules (green: HA; blue: cell nuclei; red: TSG-6, HC1, HC2, or bikunin). (A-D) Positive and (E-H) negative controls are shown in each case. Scale bars: 100 µm. Yellow asterisks indicate inner aspects. The tissue sections shown correspond to the inner aspect of the capsule from one patient; similar results were obtained for sections from all patients tested and all locations within the capsule.
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staining with fibroblasts suggests that there may be local expression of these polypeptides within breast capsules. Colocalization of TSG-6, Iα, and HA is known to result in TSG-6-mediated HC·HA formation. This broadly similar distributions of HA, TSG-6, and Iα polypeptides in our data suggest that TSG-6-mediated HC·HA formation may be occurring in periprosthetic breast capsules.

Some of the breast capsule sections from this study showed evidence of synovia-like metaplasia, defined as the formation of synovia-like tissue over the smooth gliding surfaces surrounding various implants, including joint, tendon, and breast prostheses. This is thought to represent a unique repair process driven by the mechanical stresses encountered over these surfaces and has been described most frequently in association with textured silicone breast implants. The detection of TSG-6, HC1, HC2 and bikunin together with HA in areas of synovia-like metaplasia from the breast capsules studied here might be indicative of remodeling and inflammation in these tissues. It should also be noted that TSG-6 was identified as one of a very small number of genes upregulated (approximately four-fold) in human arterial smooth muscle cells in response to mechanical strain. These same conditions were seen to differentially modulate the synthesis of vascular proteoglycans and give rise to increased aggregation of versican with HA. This suggests a possible role for TSG-6 in ECM reorganization following a biomechanical stimulus.

Previous studies have shown a positive correlation between serum HA concentrations and Baker grade of CC. Here, we observed a paradoxical decrease in tissue HA staining in breast capsules in contracted capsules. This could signify that elevated levels of circulating HA are not indicative of increased HA synthesis in contracted breast capsules. However, it should be noted that the biotinylated HA binding protein used here is specific to free HA and not HA associated with matrix proteoglycans (eg, versican).

A number of previous studies have reported correlations between CC and an increased inflammatory response, possibly directed toward silicone droplets or due to subclinical infections. The anti-inflammatory effects of TSG-6 (eg, inhibition of neutrophil migration and downregulation of the protease network) may therefore play a protective role in preventing capsule contracture formation; this is supported by the negative correlation between TSG-6 staining intensity and relative gene expression levels with Baker grade of contracture. Furthermore, the negative correlation between staining for TSG-6 and implant duration suggests that this protein might be particularly important in the early stages of inflammation and tissue remodeling following implant insertion. HC·HA has been identified at various sites of inflammation. The recent observation that HC·HA complexes can mediate anti-inflammatory and antiscarring effects is consistent with the negative correlations between TSG-6 protein staining/mRNA expression and severity of CC reported here.

**CONCLUSIONS**

The results from this study indicate that TSG-6-mediated HA cross-linking occurs in periprosthetic breast capsules.
Figure 5. Immunofluorescent staining for hyaluronan (HA) together with TSG-6, HC1, HC2, or bikunin in periprosthetic breast capsule tissue showing synovia-like metaplasia (white arrows) (green: HA; blue: cell nuclei; red: TSG-6, HC1, HC2, or bikunin). (A-D) Positive and (E-H) negative controls are shown in each case. Scale bars: 100 µm. Yellow asterisks indicate inner aspects. The tissue sections shown correspond to the inner aspect of the capsule from one patient; similar results were obtained for all the sections exhibiting synovia-like metaplasia that were tested.
The higher TSG-6 expression in noncontracted capsules suggests that TSG-6 may play a protective role against CC, which may in part be due to its anti-inflammatory effects. This may be a clinically important finding, but further work is needed to determine the potential clinical applicability of TSG-6 and TSG-6-mediated HA cross-linking in CC.

**Disclosures**

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