Elevation of transforming growth factor beta (TGFβ) and its downstream mediators in subcutaneous foreign body capsule tissue

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Abstract: Foreign body encapsulation represents a chronic fibrotic response and has been a major obstacle that reduces the useful life of implanted biomedical devices. The precise mechanism underlying such an encapsulation is still unknown. We hypothesized that, considering its central role in many other fibrotic conditions, transforming growth factor β (TGFβ) may play an important role during the formation of foreign body capsule (FBC). In the present study, we implanted mock sensors in rats subcutaneously and excised FBC samples at day 7, 21, and 48–55 postimplantation. The most abundant TGFβ isoform in all tissues was TGFβ1, which was expressed minimally in control tissue. The expression of both TGFβ1 RNA and protein was significantly increased in FBC tissues at all time points, with the highest level in day 7 FBC. The number of cells stained for phosphorylated Smad2, an indication of activated TGFβ signaling, paralleled the expression of TGFβ. A similar dynamic change was also observed in the numbers of FBC myofibroblasts, which in response to TGFβ, differentiate from quiescent fibroblasts and synthesize collagen. Type I collagen, the most prominent downstream target of TGFβ in fibrosis, was found in abundance in the FBC, especially during the latter time periods. We suggest that TGFβ plays an important role in the FBC formation. Inhibition of TGFβ signaling could be a promising strategy in the prevention of FBC formation, thereby extending the useful life of subcutaneous implants. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 82A: 498–508, 2007

Key words: implantation; foreign body capsule; transforming growth factor beta; collagen; Smad; biosensor

INTRODUCTION

Encapsulation of implanted devices is a well-described phenomenon.[1–3] In the setting of implanted biosensors, such encapsulation leads to progressive loss of function over time.[4,5] We have previously reported that subcutaneously-implanted amperometric glucose sensors are capable of measuring glucose accurately in rats,[6–8] rabbits,[9] and dogs.[10] There is a progressive decline of sensor sensitivity and a progressive increase in the lag time that it takes for a change in blood glucose to be recognized by the subcutaneous interstitial sensor.[8] The increased lag time, and the reduced permeation of analytes into the sensor, leads to a progressive loss of sensor accuracy. It would be very helpful to develop approaches that could prevent or reduce the encapsulation of subcutaneous implants, but the lack of understanding of the pathogenesis underlying the encapsulation has hindered this effort.

Pathologically, the encapsulation represents chronic fibrosis, and transforming growth factor β (TGFβ) has been thought to play a central role in other conditions in which fibrosis is present.[11–13] This background information led us to hypothesize that TGFβ plays an important role in foreign body encapsulation. TGFβ is a family of pluripotent cytokines that play pivotal roles in the maintenance of tissue homeostasis through regulating cell proliferation/differentiation, extracellular matrix (ECM) production and deposition, angiogenesis, and inflammation. Three TGFβ isoforms, including TGFβ1, 2, and 3, have been identified in mammals, with TGFβ1 expression being most abundant and present in virtually all

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tissues.[14] The three TGFβ isoforms share a high homology in DNA and amino acid sequences and behave similarly in many cellular effects. All TGFβ isoforms are secreted as latent forms, in which the N-terminal latency associated peptide remains noncovalently bound to the C-terminal mature TGFβ.[15] The latent forms require activation via proteolysis to release the mature TGFβ. TGFβ elicits its signaling via binding to a transmembrane serine/threonine kinase receptor complex comprised of TGFβ type I and type II receptors. Subsequently, the TGFβ signal is transduced through a group of intracellular mediators known as Smad proteins [for recent reviews, see Refs. 16–19]. The Smad proteins are divided into three groups: receptor-specific Smads (R-Smads), a common Smad (Co-Smad), and inhibitory Smads (I-Smads). To date, eight Smad proteins (Smad1–Smad8) have been identified in mammals. Among them, Smad2 and Smad3 function as R-Smads specifically responsible for TGFβ-mediated signaling.[16,20–23] Binding of TGFβ ligand to the type I–type II receptor complex phosphorylates Smad2 and Smad3, which in turn form oligomeric complexes with Smad4, the Co-Smad. Such Smad protein complexes then translocate into the nucleus where they transcriptionally regulate gene expression. The I-Smads, Smad6 and Smad7, inhibit the TGFβ signaling cascade by either blocking phosphorylation and subsequent nuclear translocation of signaling Smads [for reviews, see Refs. 24,25] or by degrading the receptors via specific ubiquitin-proteasome pathways.[26,27]

The fibrotic response is an injury-induced healing process characterized by fibroblast proliferation and ECM production and deposition in the mesenchymal compartment of a given tissue.[28] Under normal conditions, such a process can be ultimately shut down and minimized at later stages of tissue wound healing to prevent excessive scarring. However, excessive fibrotic responses may lead to tissue fibrosis under pathological conditions such as liver cirrhosis, hypertrophic scarring, and scleroderma, disorders in which TGFβ signaling is deregulated.[11,12,28,29]

In the present study, we asked whether TGFβ and its signaling pathways play a role in foreign body encapsulation of subcutaneously-implanted devices. Adult rats were implanted with mock biosensors and the FBC tissues were explanted at different time points after implantation. In these samples, TGFβ signaling was assessed by means of cellular and molecular approaches. In this study, we demonstrate that the level of TGFβ1 expression, its signaling transduction, and the expression of its downstream effector molecule, collagen I, are increased throughout the time course of FBC development when compared to normal subcutaneous tissues (SCs). Such an increase is very prominent in FBC tissues explanted at day 7 postimplantation and remains during later stages of FBC development. Our data, viewed in light of other studies that will be discussed, suggest that TGFβ signaling pathway is likely to play a causal role in the formation of FBC.

MATERIALS AND METHODS

Animals, implantation, and explantation

Each adult male Sprague-Dawley rat weighing 350–650 g was subcutaneously implanted (dorsal region) with two mock biosensors, defined as having the same biomaterials as functioning sensors but without the electronic circuitry (Fig. 1). The dimensions of the implant were 5.3 cm × 3.3 cm × 0.9 cm. In each mock sensor, four peripheral platinum electrodes and one central Ag/AgCl reference electrode were located underneath the polymer layer.[30] Isoflurane given by inhalation (2%) was used for surgical anesthesia. Prior to implantation, the sensors were coated with an outer membrane of polyurethane (Polymer Technology Group, Berkeley, CA) through which oxygen and glucose enter the sensor. The only material that directly contacts the tissue was this polyurethane. Animals were housed in the animal facility at the Legacy Clinical Research and Technology Center and all study procedures were approved by the Legacy Institutional Animal Care and Use Committee. NIH guidelines for the care and use of laboratory animals (NIH Publications #85-23 Rev. 1985) were observed.

Before being allowed to recover from isoflurane anesthesia, rats were euthanized by carbon dioxide narcosis at 7 days ($n = 3$), 21 days ($n = 3$), and 48–55 days ($n = 4$) after implantation. When the sensors were explanted, FBC tissues were collected from each of two locations (2.5 cm apart from one another, each of which was located directly above a sensing electrode) that contacted the face of the sensor. Normal-appearing SC was obtained from a point at least 2 cm away from the implant to serve as control tissue. As a secondary control (richer in collagen than SC), dermal tissue was obtained from a point at least 2 cm away from the implant. Each piece of tissue was cut into smaller samples and placed in either 10% zinc formalin (for later paraffin sectioning), in OCT media at –20°C (for later cryosectioning), or into liquid nitrogen tubes for later quantitative reverse transcription-polymerase chain reaction (qRT-PCR) experiments.

Histology, trichrome staining, immunohistochemistry, and immunofluorescence

Control and FBC tissue samples were fixed in 10% neutral-buffered formalin and then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis. Masson's Trichrome staining was performed on paraffin-embedded sections to stain collagen.[31] The levels of phosphorylated Smad2 (pSmad2) and TGFβ1 were examined by immunohistochemistry (IHC) on paraffin-embedded sections using a rabbit anti-pSmad2 antibody (Cell Signaling Technology, Danvers, MA) and a chicken anti-TGFβ1 antibody (R&D Systems, Minneapolis, MN) as previously described.[32] The antibody against pSmad2 may

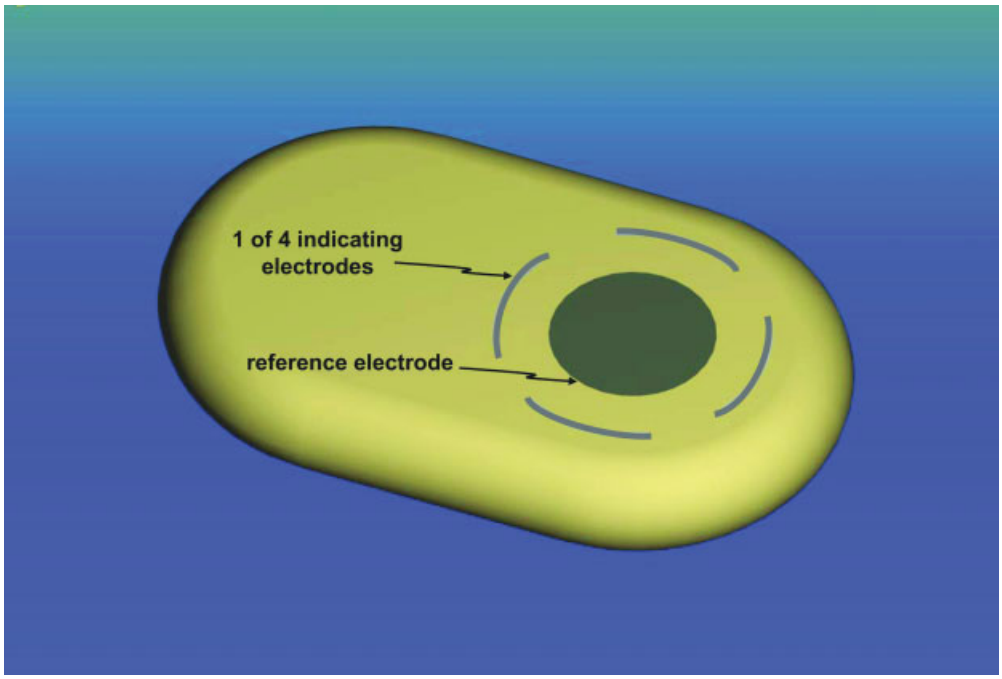

Figure 1. Diagram of the sensor implant. The dimensions of the sensor, which was implanted subcutaneously in the dorsal region of Sprague-Dawley rats, were 5.3 cm × 3.3 cm × 0.9 cm. The sensor was coated with a polyurethane multipolymer, as discussed in Methods. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

slightly crossreact with pSmad3 but not with nonphosphorylated Smad proteins. The biotinylated secondary antibodies were used in conjunction with VECTASTAIN® streptavidin-peroxidase immunodetection kit (Vector Laboratories, Burlingame, CA). The immune complex was visualized using a diaminobenzidine substrate chromogen system (DakoCytomation, Carpinteria, CA). Double immunofluorescence (IF) was performed on OCT-embedded frozen sections using a rabbit antibody against type I collagen (Chemicon, Temicula, CA) and a mouse antibody against α smooth muscle actin (αSMA) (Sigma-Aldrich, St Louis, MO) as previously described.[33] Alexa Fluor® 594 (red) or 488 (green) conjugated secondary antibodies (Invitrogen, Carlsbad, CA) were used to visualize antigens. All light and fluorescent microscopic images were acquired under an EC600 Nikon microscope (Nikon USA, Farmingdale, NY) and analyzed using a MetaMorph™ software (version 6.1) (Molecular Devices, Downingtown, PA). Although quantification of angiogenesis was not the primary goal in this study, we did evaluate vascular structures in FBC tissues using H&E. Our previous experience with H&E and IHC[30] suggested that H&E fails less often than IHC to detect capillaries even with the use of antigen retrieval techniques during IHC. We do acknowledge, however, that IHC is more specific than H&E. The thickness of the FBC capsule was not quantitatively measured in this study.

RNA extraction and qRT-PCR

Total RNA was isolated from control SCs and FBC using RNAzol B (Tel-Test, Friendswood, TX) as previously described[32] and further purified using RNeasy Mini kit (Qiagen, Valencia, CA). The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was achieved by combining *in vitro* reverse transcription (RT) with quantitative PCR (qPCR), which was performed in a Stratagene Mx3000P thermal cycler (Stratagene, La Jolla, CA). Briefly, 5 μg of RNA from each sample were treated with Turbo™ DNase (Ambion, Austin, TX) and then subject to a RT reaction using avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, MN). The resultant cDNA products were then used as templates in qPCR, which was carried out using Taqman® Assays-on-Demand™ probes (Applied Biosystems, Foster City, CA) to test expression of TGFβ1 and type I collagen [COL1(A)I] on a Stratagene Mx3000P qPCR system (Stratagene) as previously described.[32]

A β-2 microglobulin (B2M) probe was used as internal control and the data were analyzed using MxPro™ qPCR Software (Stratagene). The relative RNA expression levels were calculated by using the Comparative C_T Method.[32] To compare the relative gene expression levels between experimental groups, the C_T value of one of control tissues was set as an arbitrary value of "1." For data presentation, the results averaged from three samples taken from three individual rats are shown (mean ± SD).

Assays of TGFβ1 protein levels by ELISA

Protein extraction was performed as we have previously described[34] with modifications. Briefly, tissue samples were disrupted in liquid nitrogen and then homogenized in a lysis buffer [20 m*M* Tris-HCl (pH 7.5), 140 m*M* NaCl, 1 m*M*

EDTA-Na_2 (pH 8.0), 1 m*M* EGTA, 1% Triton X-100, 2.5 m*M* sodium pyrophosphate, 1 m*M* glycerophosphate, 1 m*M* Na_3VO_4, 1 m*M* NaF][35] with protease inhibitors (Sigma-Aldrich, St. Louis, MO) added freshly. The supernatants were collected after centrifugation and used for assays to determine the concentration of TGFβ1 protein using an ELISA kit specific for rat TGFβ1 (R&D Systems). The protein sample with and without acidification was used to detect the active form of TGFβ1 and the total TGFβ1, respectively. The difference between the total TGFβ1 and the active TGFβ1 is the amount of latent TGFβ1 in the sample. The TGFβ1 protein levels (pg/mg protein) were normalized to the protein concentration of each sample, as measured by the Bradford technique.[36] Results were averaged from three samples isolated from three different rats.

Statistics

Comparisons between the values obtained in each assay on samples from control and FBC tissues were determined using a two-tailed Student's *t* tests. A *p* value of 0.05 or less was taken as being significant.

RESULTS

Increased collagen and fibroblast number throughout the time course of the FBC

To examine histological changes and collagen deposition during FBC development, sections of control (SC and dermis) and FBC tissues taken at different time points post implantation were examined. The control tissues harvested at different time points postimplantation were indistinguishable among each other such that only one control image is shown in all figures. Masson's Trichrome staining revealed only sparse collagenous fibers (blue) in control SCs (Fig. 2). In contrast, all FBC samples at different time points postimplantation appeared to be fibrotic tissues characterized by numerous fibroblasts and varying amounts of collagen.

At day 7, FBC tissue contained many activated fibroblasts characterized by pleiomorphic morphology, numerous inflammatory cells, and prominent neovascularization. At this stage, collagen in FBC tissue has begun to increase as compared to control tissues but was still interspersed with cellular contents (Fig. 2). At day 21, typical spindle-shaped fibroblasts were present and bundles of parallel collagen were present, especially in the area nearest to the implant (Fig. 2). Day 48–55 FBC tissues contained less prominent cellular contents but collagenous bundles were denser. At this late time point, consistent with our previous studies,[1] almost no visible vascular structure was observed in the FBC region near the implant. Throughout the three time points we analyzed, fibroblasts and collagen were more abundant than that in normal SCs. Interestingly, we observed a dynamic trend during FBC development, in which the collagen content increases as the cellular content declines over the time.

Expression of TGFβ1 is elevated throughout the development of FBC

In addition to examining the histology of fibrotic responses in FBC tissues, we also examined expression levels of TGFβ1, which is the isoform most implicated in fibrosis of other organs.[11,37] We investigated both mRNA and protein levels of TGFβ1 in control and FBC samples. The qRT-PCR results showed a ~10 fold, 5–6 fold, and 3–4 fold increase of TGFβ1 transcripts at day 7, day 21, and day 48–55 FBC tissues, respectively, as compared to subcutaneous and dermal control tissues [Fig. 3(A)].

At each of the three time points, levels of TGFβ1 transcript were significantly higher in FBC tissues than in subcutaneous control tissues (day 7: 9.4 ± 1.0 vs. 1.0 ± 0.18, $p < 0.01$; day 21: 5.7 ± 0.80 vs. 1.2 ± 0.21, $p < 0.01$; day 48–55: 3.2 ± 0.90 vs. 1.1 ± 0.17, $p < 0.01$, $n = 3$ at each point). Levels of the TGFβ1 transcript in SC and dermis at all three time points were similar (day 7: 1.0 ± 0.18 vs. 1.3 ± 0.21; day 21: 1.2 ± 0.21 vs. 1.5 ± 0.18; and day 48–55: 1.1 ± 0.17 vs. 1.2 ± 0.19).

We then carried out ELISA assays to determine the TGFβ1 protein levels in FBC tissues. The total TGFβ1 protein in day 7, day 21, and day 48–55 FBC tissues was ~20, ~13, and ~10 times as much as in subcutaneous control tissue, respectively (Table I). The active and latent TGFβ1 showed a similar increase in FBC tissues as compared to control tissues (Table I). These data indicate that TGFβ1 expression is elevated throughout FBC development, and on day 7 both TGFβ1 mRNA and protein levels are significantly higher than those of the other two points ($p < 0.05$).

To further identify the cell types that expressed TGFβ1, we performed IHC on control and FBC tissues using an antibody against the active form of TGFβ1. We found that while control tissue exhibited a weak staining, FBC tissue revealed a strong positive TGFβ1 staining [Fig. 3(B)]. The cells expressing TGFβ1 included primarily activated fibroblasts and to a lesser extent, inflammatory cells (both polymorphonuclear and mononuclear cells), especially in day 7 FBC [Fig. 3(B)].

TGFβ signaling (activated Smad expression) remains activated throughout FBC formation

As FBC tissues showed a significant upregulation of TGFβ1 at both mRNA and protein levels, it is rea-

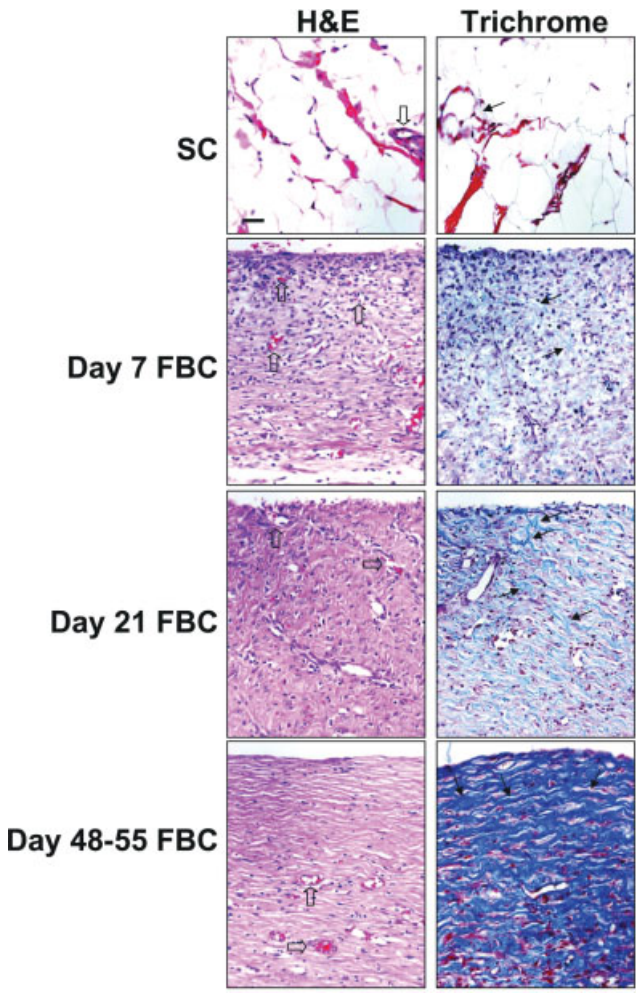

Figure 2. Histology (left panels) and Masson's Trichrome staining (right panels) on control subcutaneous tissues (SC) and foreign body capsule (FBC) tissues at different time points. Open arrows point to blood vessels. Black arrows denote collagenous fibers (blue). Note that elastic fibers and nuclei, red blood cells and cytoplasm are stained black and red, respectively. The top of each micrograph of FBC tissues bordered the implant. The bar in the first panel represents 75 μm for all sections.

sonable to evaluate the level of phosphorylated Smad2 (pSmad2) in FBC. It is well known that pSmad2 is an indicator of activation of TGFβ signaling.[12,16] We carried out IHC on control and FBC tissues using an antibody recognizing rat pSmad2. While only scattered staining was shown in control tissues, an increased number of cells in FBC tissues were stained positively for pSmad2 (Fig. 4). Day 7 FBC tissues demonstrated very prominent pSmad2 staining and nearly all fibroblasts throughout the FBC tissue were positively stained (Fig. 4). In contrast, day 48–55 FBC tissues showed positive staining primarily in FBC tissue somewhat removed from the tissue–implant interface (Fig. 4). In a manner similar to its TGFβ1 expression level, FBC tissue from day 21 was intermediate in terms of pSmad2 staining in comparison with day 7 and day 48–55. Our data confirm that TGFβ signaling remains active throughout FBC formation. Interestingly, fibroblasts may become quiescent during maturation and stabilization of chronic FBC tissue as shown in day 48–55 FBC samples.

Increased expression of collagen I in FBC

Because collagen I is the major collagen type in SC and a well-known TGFβ downstream target during fibrosis,[12] we decided to measure the mRNA and

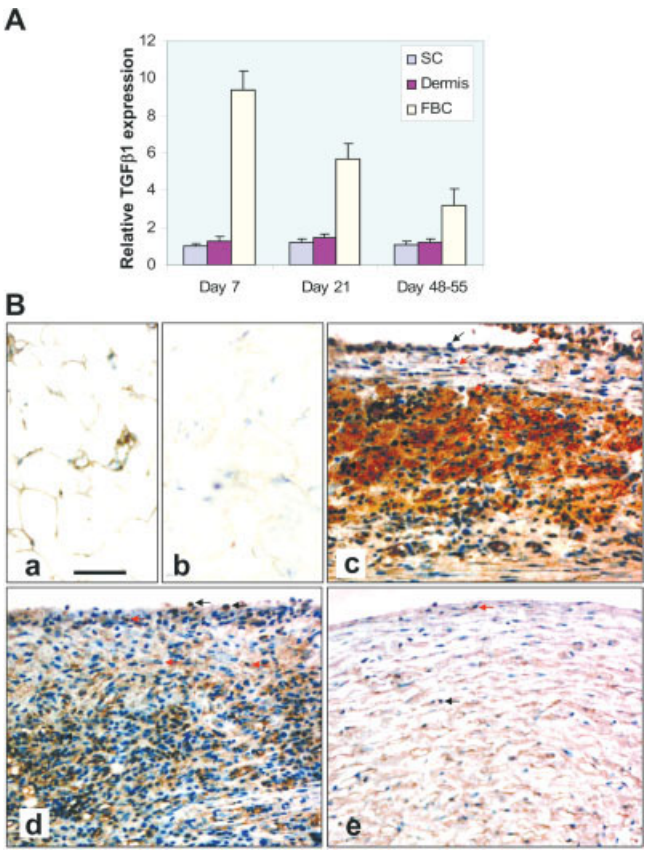

Figure 3. Upregulated TGFβ1 in FBC tissues throughout three time points. A: Quantitative RT-PCR (qRT-PCR) results of TGFβ1 mRNA levels. The TGFβ1 mRNA levels in FBC tissues were expressed as fold changes (relative expression) as compared to the control subcutaneous (SC) from day 7, whose comparative C_T value was set as an arbitrary value of "1." Graphed results were averaged from three samples in subcutaneous control tissue (SC), dermis control tissue, and FBC tissues at day 7, day 21, and day 48–55. Note that compared to low levels in both SCs and dermis, TGFβ1 transcript is significantly elevated in FBC tissues throughout all three time points. B: Immunohistochemistry of TGFβ1 on (a) control SC, (b) control dermis, (c) day 7 FBC, (d) day 21 FBC, and (e) day 48–55 FBC tissues. Hematoxylin was used as a counterstain. Representative fibroblasts (red arrows) and leukocytes (black arrows) stained positive for TGFβ1 antibody are denoted. The top of each micrograph of FBC tissues bordered the implant. The bar in the first panel represents 100 μm for all sections.

TABLE I
A Comparison of Protein Concentrations (ELISA) of Total TGFβ1, Active TGFβ1, and Latent TGFβ1 in Foreign Body Capsule (FBC) Tissue vs. Undisturbed Control Subcutaneous Tissue (SC)

		TGFβ1 Protein Levels (pg/mg protein)		
Days Post Implantation	Groups	Total	Active	Latent
7	control	125.3 ± 13.5	66.7 ± 9.1	58.6 ± 4.6
	FBC	2508.3 ± 168.8	1181.7 ± 123.6	1326.7 ± 108.9
21	control	136.3 ± 14.3	59.0 ± 4.0	77.0 ± 10.1
	FBC	1877.7 ± 96.0	960.1 ± 54.5	917.5 ± 53.6
45–55	control	137.33 ± 8.5	59.3 ± 5.3	78.0 ± 7.5
	FBC	1343.3 ± 85.7	592.1 ± 34.8	751.2 ± 56.2

See text for description of assay methods. At each time point, there was an increase in the concentration of TGFβ1 (total, active, and latent) in the FBC tissue vs. the control tissue.

protein expression of collagen I by qRT-PCR and IF, respectively. Day 7, 21, and 48–55 FBC tissues revealed a ~10, ~30, and ~5-fold increase in collagen I mRNA level versus subcutaneous control tissue shown by qRT-PCR results [Fig. 5(A)]. This suggests that the highest activity of collagen synthesis by activated fibroblasts in the FBC occurs during intermediate stages of FBC formation. We also performed double IF on subcutaneous control and FBC tissues using antibodies against collagen I, and α-smooth muscle actin (αSMA), a marker for activated fibroblasts (myofibroblasts), and a downstream TGFβ target molecule.[38] As shown in Figure 5(B), αSMA staining was most prominent at day 7 FBC as compared to the other two time points, which coincided with highest TGFβ1 level.

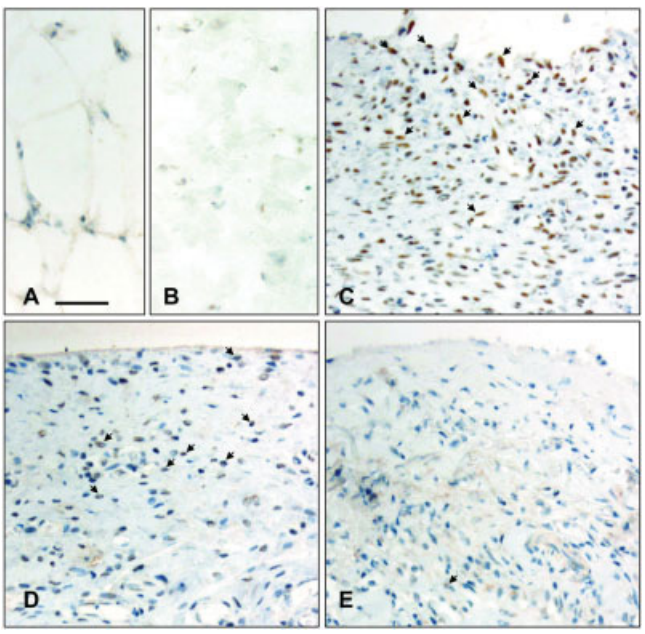

Figure 4. Immunohistochemistry of phosphorylated Smad2 (pSmad2) of SC (A), control dermis (B), and FBC tissues at different time points (C: day 7, D: day 21, E: day 48–55). Representative positive cells (nuclear staining) are denoted by arrows. The top of each micrograph of FBC tissues bordered the implant. Hematoxylin was used as a counterstain. The bar in the first panel represents 75 μm for all sections. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

At day 48–55, immunofluorescent staining for collagen I protein was the highest of all stages, consistent with the finding of the most intense staining of collagen fibers at the same stage using Masson's Trichrome (Fig. 2). The collagen I staining was least prominent at day 7 and was intermediate at day 21. In contrast, both with trichrome (Fig. 2) and with IF [Fig. 5(B)], subcutaneous control tissues demonstrated only sparse collagenous fibers at all stages. The scattered αSMA-positive structures in subcutaneous control tissues vessels are primarily blood vessels (myoepithelial cells) and a few fibroblasts. These data suggest that myofibroblasts are the predominant cell type during FBC formation and that they rapidly transdifferentiate from quiescent fibroblasts and produce collagen I in response to elevated TGFβ signaling.

DISCUSSION

FBC formation is a major factor in limiting the useful life of subcutaneous implants such as glucose biosensors or other interventional medical devices.[1–3] For this reason, it is important to gain a better understanding of mechanisms underlying the formation of FBC. In the present study, we analyzed FBC tissues harvested from day 7, day 21, and day 48–55 after subcutaneous implantation.

The day 48–55 FBC tissues are characterized by very high collagen content with fewer myofibroblasts and fewer vascular structures than at earlier time points. This chronic condition represents the mature and stable fibrotic tissue that we have observed previously to reduce sensor accuracy.[8] In contrast, the day 7 FBC tissues showed numerous myofibroblasts and inflammatory cells, and collagen content that was substantially higher than the control, but much

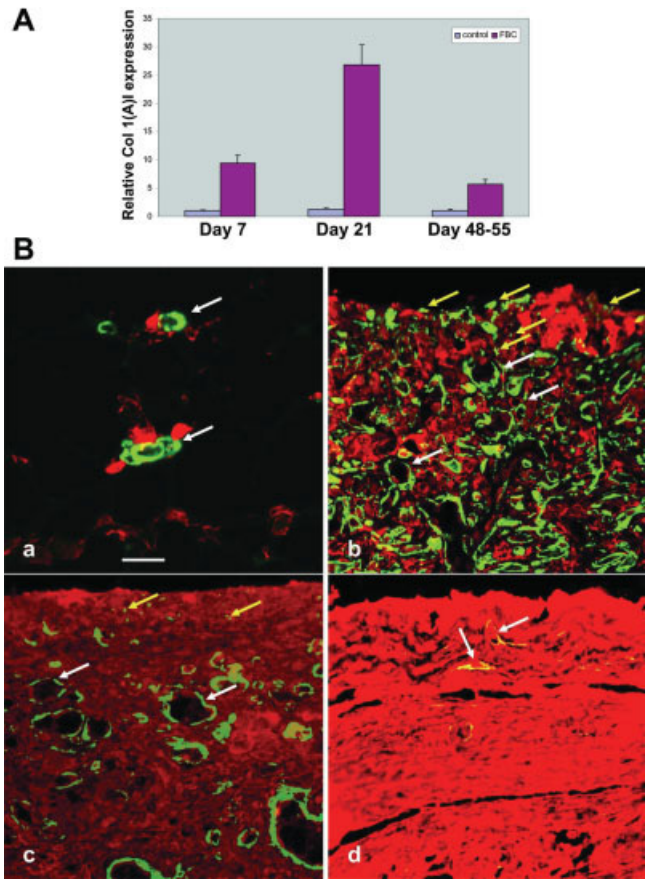

Figure 5. Collagen I is upregulated during FBC development. A: Quantitative RT-PCR (qRT-PCR) results of collagen I [COL 1(A)I] mRNA levels. The results were averaged from three samples of subcutaneous control and FBC groups at day 7 (1.0 ± 0.2 vs. 9.5 ± 1.3, $n = 3$, $p < 0.01$), day 21 (1.3 ± 0.23 vs. 26.8 ± 3.5, $n = 3$, $p < 0.01$), and day 48–55 (1.1 ± 0.18 vs. 5.7 ± 0.8, $n = 3$, $p < 0.01$). B: Double immunofluorescence of collagen I (red) and α smooth muscle actin (αSMA) (green) on control subcutaneous (a) and FBC tissues at different time points (b: day 7, c: day 21, d: day 48–55). White and yellow arrows point to typical vascular structures and myofibroblasts that are stained positive for αSMA, respectively. The top of each micrograph of FBC tissues bordered the implant. The bar in the first panel represents 100 μm for all sections.

less than at day 48–55. These early findings thus represent an acute phase of granulation tissue formation.[28] Day 21 FBC tissues appeared to be in transition in terms of the number of myofibroblasts (declining) and the amount of collagen fibers (increasing) in the ECM. Our data suggest that day 7, 21, and 48–55 reflects early, intermediate, and late stages of the FBC development in response to the implant used in the present study. We believe that a staging system like this one is helpful in understanding mechanisms underlying the process of FBC formation. Using this staging system, we demonstrated that the FBC development represents a series of fibrotic events in which TGFβ1 and its signaling remains increased. In particular, TGFβ1 expression and its primary signaling mediator, pSmad2, were highest at day 7. In addition, the day 7 FBC showed the highest number of myofibroblasts, cells that differentiate from quiescent fibroblasts in the presence of increased TGFβ1.[12,39] The highest mRNA level of collagen I was shown at day 21 and the highest amount of collagen I protein deposition was found at day 48–55.

Others have also investigated cytokines that participate in the foreign body response. Brodbeck et al. from the Case Western Reserve University examined the expression of cytokine transcripts that developed in cellular material adherent to subcutaneously-implanted cages coated with various biomaterials. The Brodbeck study did not directly compare the expression of TGFβ in the foreign body response to normal SC. Unlike our study, their study examined the response to foreign materials (all of their animals were implanted with an uncoated steel cage or cages coated with hydrophobic polymer, hydrophilic polymer, or other coating). Therefore, even though they found TGFβ expression in coated cages to be roughly similar to the control state (uncoated steel cages), their results cannot be directly compared to ours.[40] We measured TGFβ expression directly from foreign body capsule (FBC) tissue surrounding a foreign subcutaneous implant and compared it to undisturbed SC (and undisturbed dermal tissue) in an attempt to understand the role of TGFβ in FBC development.

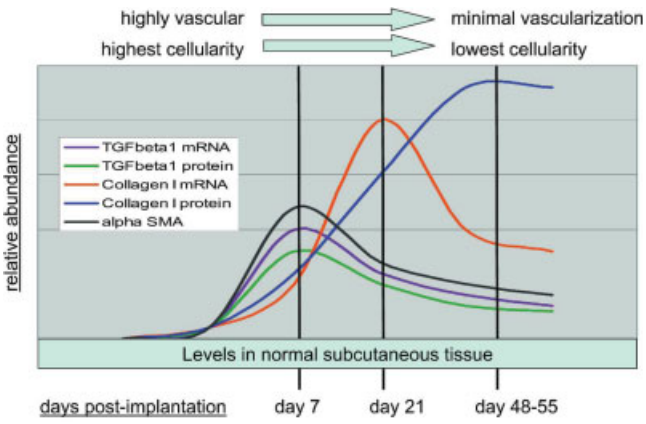

Figure 6. Depiction of the dynamics of biological events during FBC formation. The relative abundance of each molecule in the FBC is roughly plotted against the baseline level in control tissues to depict their dynamic trends during FBC development. This figure is not intended to be quantitatively accurate but is intended to depict trends. The vascularization and cellularity in the FBC appears highest at day 7 but lowest at day 48–55 postimplantation. TGFβ1 mRNA, TGFβ1 protein, and αSMA+ cells showed most abundant levels at day 7 postimplantation but then declined as FBC develops. Note that the collagen I mRNA and collagenous fibers appeared most prominent at day 21 and day 48–55 postimplantation, respectively.

Robitaille et al. also investigated the role of TGF during the foreign body response. They found an increased expression of TGFβ in peritoneal washings in animals that were implanted with intraperitoneal alginate capsules but not in animals that received saline.[41] Although the overexpression was found in washings rather than directly from fibrotic tissue, their finding of increased TGFβ expression in response to a peritoneal foreign body is analogous to our finding in SC. The same group also found increased expression of TGFβ surrounding alginate capsules that were implanted into rat epididymal fat pads.[42]

Our data depict a sequential course of biological events during the foreign body response to a subcutaneous implant, including rapid TGFβ1 production and signaling via Smad proteins, myofibroblast differentiation, and ECM production and deposition. Figure 6 depicts these trends (note that this figure is intended to show general trends rather than specific quantitation of cytokines and events). We acknowledge that our study of three time points limits our ability to make conclusions about other time points, such as days 0–2.

TGFβ1 has been thought to be an important cytokine in mediating fibrotic responses during normal wound healing in many tissues.[11,13,43] Upon injury, TGFβ1 is rapidly released from platelets to initiate the healing process and subsequently produced by tissue cells and inflammatory cells (especially macrophages) in the wounds.[37,44] In response to increased TGFβ1, fibroblasts rapidly become activated and differentiate to myofibroblasts, which are characterized by a high proliferative capacity and high capacity for collagen synthesis. Under normal condition, such a fibrotic response will ultimately shut down to minimize the scarring.[11,28] Constitutive activation of TGFβ signaling due to either a persistent increase of TGFβ ligands in the tissue (e.g., hypertrophic scarring) or a defect in the inhibitory regulation of TGFβ signal transduction (e.g., lack of Smad7 in scleroderma fibroblasts) has been implicated in human diseases and animal models.[12,13,28,43,45,46] Transgenic mice that overexpress TGFβ1 under the control by an albumin promoter and thus carry high levels of TGFβ1 in circulation spontaneously develop renal fibrosis as a result of accumulation ECM proteins in the kidney.[47] Similarly, epidermal keratinocyte-derived TGFβ1 overexpression causes excessive ECM protein deposition in the dermis under several conditions.[48–50]

It is instructive to view our results in light of the earlier findings of Shah et al. who studied mice that constitutively overexpressed TGFβ. They found that subcutaneously-implanted polyvinyl alcohol sponges in these animals demonstrated increased collagen content compared to controls.[51] Their results clearly show that a primary abnormality of TGFβ (overexpression) is capable of leading to increased ECM collagen content in subcutaneously-implanted devices.

TGFβ is one of a network of cytokines that participate in wound healing and in the foreign body response. In addition to TGFβ, other cytokines such as platelet-derived growth factor, insulin-like growth factors, and connective tissue growth factor may also play a part in fibrosis as they are known to stimulate fibroblast proliferation.[52] Interestingly, these cytokines are considered to be downstream targets of TGFβ signaling and can be induced in the presence of excessive TGFβ1 in the tissue.[33,53–55] It should also be mentioned that a role of TGFβ in causing foreign body encapsulation does not rule out roles of other cytokines. While it now appears that TGFβ is at least one important mediator of this response, it may well not be the only mediator, given the well-studied redundancy in many biochemical pathways.

The interaction of TGFβ with other cytokines, such as MMPs, can be complex. For example, it has been shown that MMP-2 and MMP-9 are capable of activating latent TGFβ.[56] In addition, the treatment of fibroblasts with TGFβ antisense oligonucleotides resulted decreased the expression of MMP-1 and MMP-9.[57] Thus, in this complex interplay of compounds, a particular compound can be both upstream and downstream of the compound under study.

Thrombospondin-1 (TSP-1) is probably the most well-recognized stimulator of the activation of latent TGFβ.[58] In fact, one could theorize that the finding of increased TGFβ in FBC tissue might be an "innocent bystander" effect, that is, the direct cause of foreign body encapsulation might be TSP-1 and the increased TGFβ could be an epiphenomenon. However, several lines of evidence oppose the idea that the profibrotic action of TGFβ is secondary. First, it has been shown that in rodents, blockade of TGFβ with a neutralizing antibody successfully reduced skin fibrosis.[59] Second, it was found recently that an inhibitor of the ALK5 TGFβ receptor reduced the profibrotic signaling effects of TGFβ in dermal fibroblasts.[60] Thus, when one takes the current findings together with earlier published reports, it appears that TGFβ is likely to have an important role (and quite possibly, a primary role) in the generation of subcutaneous foreign body fibrosis.

There are many methods that one might consider to block the profibrotic effect of TGFβ. Two such methods include blockade of the ALK5 pathway or the use of neutralizing antibodies directed against TGFβ as mentioned earlier. In addition, one might consider blockade of the effect of TSP-1 to reduce activation of TGFβ, or blockade of Sp1, a TGFβ-dependent transcription factor necessary for collagen transcription.[61] It may also be possible to block the Smad signaling pathways. Mice lacking Smad3, the

major intracellular mediator of TGFβ signaling, show reduced fibrosis in response to radiation-induced.[62] and bleomycin-induced skin injury.[63] It should also be mentioned that if a cell adhesion signal can be engineered to occupy a biomaterial's surface, a more stable implant–tissue interface might result,[64] leading to less of a need to inhibit TGFβ-induced fibrosis.

Another method that has been used to assess the normal role of TGFβ is experimentation with TGFβ knockout mouse. Although mice who do not express TGFβ1 die within 4–6 weeks of life, there have been studies in which very young knockout animals have been used to evaluate the role of TGFβ1 deficiency. TGFβ1 knockout animals, as compared to controls, were found to have less wound granulation tissue and less collagen.[65,66] While this finding is consistent with a causal role of TGFβ1 to promote ECM collagen deposition, it should also be mentioned that these animals become undernourished before their death and the reduction in collagen could be in part due to this nutritional deficit. Perhaps a better model to address the effects of TGFβ deficiency is the administration of anti-TGFβ neutralizing antibodies. Such a treatment given to rodents led to reduced cutaneous scarring.[67]

Taken together, the studies of TGFβ overexpression, TGFβ blockade, and our study of FBC biology strongly suggest that TGFβ increases collagen deposition in the FBC that forms around a subcutaneous implant. When all these types of studies are considered, there is strong support for a causal rather than a secondary role of TGFβ to promote foreign body encapsulation and subcutaneous collagen deposition.

In conclusion, we present evidence that TGFβ1 mRNA and the TGFβ1 protein are increased throughout the time course of subcutaneous foreign body encapsulation and that such stimuli lead to increased collagen production by myofibroblasts. Taken in light of other studies, it appears that such a role is likely to be causative, rather than secondary. Inhibition of TGFβ signaling could be a promising strategy in the prevention of FBC formation, thereby extending the useful life of subcutaneous implants.

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