Further Evidence for the Role of Fibrosis in the Pathobiology of Rhinophyma

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Recent evidence suggests that fibrosis may play an important role in the pathobiology of rhinophyma. The fibrogenic cytokine transforming growth factor (TGF)-β_2 has been reported to be upregulated in rhinophyma tissue. Of the three common isoforms of TGF-β, TGF-β_1 and TGF-β_2 are considered fibrogenic, whereas TGF-β_3 has antiscarring properties. To provide further evidence for the role of fibrosis in the pathobiology of rhinophyma, specimens from 8 patients with rhinophyma were compared with nine specimens of normal nasal skin. Immunohistochemistry was used to compare intensity levels of TGFβ_1 and TGFβ_3 proteins, and quantitative reverse transcription–polymerase chain reaction was used to determine messenger ribonucleic acid (mRNA) expression levels of TGFβ_1 and TGFβ_3. TGF-β_1 was elevated significantly in rhinophyma tissue ($p < 0.001$), whereas TGF-β_3 was no different in the rhinophyma specimens compared with normal nasal skin ($p = 0.06$). TGFβ_1 mRNA expression was five-fold higher in rhinophyma tissue compared with normal skin ($p < 0.001$). The mRNA expression of TGF-β_3 was the same for both pathological and normal tissue ($p < 0.09$). These data, together with previously published observations, present further evidence that fibrosis mediated by the fibrogenic cytokines TGFβ_1 and TGFβ_2 play a role in the pathobiology of rhinophyma and suggest a means of treatment by neutralizing or down-regulating these cytokines.

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The pathobiology of rhinophyma remains unclear. Although rhinophyma has been considered a form of end-stage acne rosacea of the nasal skin, and proliferation of sebaceous glands has been reported to be the main mechanism in its pathology,[1,2] recent evidence suggests that fibrosis may play a prominent role in rhinophyma.[1,3,4] Several physical characteristics of the disease are consistent with proliferative scarring.[3]

Medical therapy aimed at treating stimulating factors for acne rosacea have not been particularly successful except for treatment of early rhinophyma with vitamin A derivatives.[5] Therefore, various surgical modalities have been the mainstay of treatment.[6]

Recent work by Pu and colleagues[1] demonstrated increased levels of the fibrogenic cytokine transforming growth factor-beta 2 (TGF-β_2) and the TGF-β type II receptor in rhinophyma tissue compared with normal nasal skin. They suggested that if further evidence showed that fibrosis played a role in the pathobiology of rhinophyma, medical therapies could be devised using antifibrotic regimens that have been suggested for other fibrotic diseases such as burn hypertrophic scar, keloid, periprosthetic breast capsules, or Dupuytren's contracture.

The dense fibrosis, increased collagen production, and proliferative scarring seen in rhinophyma may be mediated by the persistent overexpression or dysregulated activation of TGF-β, which has been related to overabundant scar formation. Of the isoforms of TGF-β, TGF-β_1 and TGF-β_2 have been associated with fibrotic conditions, whereas TGF-β_3 tends to decrease fibrosis and scarring.[7–10]

Although TGF-β_2 has been the most fibrogenic isoform,[11–13] knowledge of TGFβ_1 and TGFβ_3 would be useful before outlining a potential medical treatment for rhinophyma. To provide this, the current study investigated the expression of TGF-β_1, and TGF-β_3 from specimens of patients

with rhinophyma and compared the levels with that of normal nasal skin.

Materials and Methods

Tissue Biopsy Collections

Rhinophyma biopsy samples were obtained from discarded tissues from 8 white male patients undergoing elective excisions (patient mean age, 68.5 years). Seven additional white male patients (mean age, 67 years) who underwent simple excisions of nasal skin benign lesions were chosen as control subjects. Normal nasal skin biopsies adjacent to the lesions were obtained from these patients. These control subjects were chosen because it was not possible to obtain normal control nasal skin from the patients with rhinophyma lesions. The study was approved by the institutional review board at the Bay Pines VA Medical Center. Each specimen was divided into two parts. The first portion was snap-frozen in liquid nitrogen and stored at −70°C for ribonucleic acid (RNA) isolation. The remaining portion was embedded immediately in Histo Prep (Fisher Scientific, Pittsburgh, PA) and snap-frozen in liquid nitrogen. The tissue was cut in 8-μm sections on a cryostat at −20°C and mounted on polysialic acid glass slides in preparation for immunohistochemical staining of TGF-β_1 and TGF-β_3.

Immunohistochemistry

Cryosections were incubated in phosphate-buffered saline (PBS) for 30 minutes at room temperature. The sections were then fixed in 4% paraformaldehyde for 20 minutes.

Endogenous peroxidase was blocked by a 30-minute incubation in 1% hydrogen peroxide in PBS. After PBS washing, sections were incubated with diluted normal goat serum (1:10) to block nonspecific protein binding. Sections were then incubated for 2 hours at room temperature in a humidified chamber with rabbit antibodies specific for TGF-β_1 and TGF-β_3 (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:50 in 1% bovine serum albumin in PBS. Unbound antibody was removed by washing with PBS. Bound antibody was localized with biotinylated goat antibody. After applying avidin–biotin–complex solution (Vector Labs, St. Louis, MO), 3,3-diaminobenzidine with hydrogen peroxide was added as a substrate. Control staining was performed using PBS in place of the primary antibody and in place of the secondary antibody separately. At least two sections from each specimen were immunostained for TGF-β_1 and TGF-β_3.

Quantification of Immunohistochemical Staining

Sections of immunohistochemical staining were photographed using a Leitz Dialux microscope (Ernst Leitz, Wetzlar, Germany) corrected for tungsten light. The photographs were then digitized using a standard desktop flatbed scanner. The staining was changed from brown to black on a white background. The intensity of staining was measured by a blinded observer (XW) in 10 noncontiguous and no overlapping squares of each section chosen by computer randomization using Sigma Scan (Jandel Scientific, Corte Madeira, CA). The intensity was measured between 0 and 255 (with 0 being no light intensity or black and 255 being the greatest intensity or pure white). Because the staining was displayed as black on white, we subtracted the intensity measurement from 255 to depict greater staining with a higher value. The mean values between the rhinophyma and control groups were compared using an unpaired Student's *t*-test. Significant differences were considered if p was less than 0.05.

RNA Isolation

Total RNA was isolated from rhinophyma and normal skin specimens as described previously.[14] Tissue samples were homogenized in 1 ml TRIZOL (Life Technologies) reagent using a glass–Teflon homogenizer. The homogenized samples were then incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. After the addition of 200 μl chloroform to the sample tubes, the tubes were shaken, put at room temperature for 3 minutes and then centrifuged at 12,000 g for 15 minutes at 4°C. The RNA-containing aqueous phase was transferred to a sterile tube to which 500 μl isopropyl alcohol was added, and they were placed on ice for 10 minutes. The RNA was precipitated by centrifugation at 12,000 g for 10 minutes at 4°C. After the supernatant was discarded, the RNA pellet was

PCR Primer Sequences Used in the Study

Cytokine	Primers	Template (bp)	mRNA (bp)
TGF-β_1	5′ CAAGCAGAGTAC ACACACAGCA	337	442
	3′ GATGCTGGGCCC TCTCCAGC		
TGF-β_3	5′ ATTACCTCCAAG GTTTTCCG	337	541
	3′ GCCCGCTTCTTC CTCTGACC		

bp = base pair; mRNA = messenger ribonucleic acid.

resuspended in 20 μl DEPC water, incubated for 10 minutes at 60°C, and the 2 μl of RNAse inhibitor (10 U per microliter) was added to each tube. One microliter of RNA solution was taken and diluted in 100 μl with DEPC water for spectrophotometric verification of RNA presence.

Competition-Based Quantitative Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Complementary deoxyribonucleic acid was synthesized in 50-μl volumes that contained 2 μg total RNA, serial dilutions of competitive human template complementary RNAs (8.84×10^3–8.84×10^{-2} copies per cell) for TGF-β_1, 0.5 μg oligo (dT), 0.01 mol per liter dithiothreitol, 200 μM of each dNTP (Promega, Madison, WI), and 299 U per microgram RNA Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD). The reaction was incubated at 25°C for 10 minutes, 37°C for 60 minutes, and 92°C for 5 minutes.[15] DNA amplification was performed in a 50-μl reaction volume containing 5-μl RT reaction, 25 μl of the 2× Taq Ready Mix (Sigma), and 50 pmol of each 3' and 5' PCR primer (Table). Amplification reactions were carried out in 40 sequential cycles of 94°C for 1.5 minutes, 58°C for 2 minutes, and 72°C for 3 minutes in a GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT). A previous study demonstrated colinear amplification of template and message targets during this condition.[15]

Detection and Quantitation of PCR Products

PCR products were separated on 2% agarose gel containing 0.1 μg per microliter ethidium bromide and were photographed using Polaroid-type 667 black-and-white film (Sigma). Photographs were scanned using a Hewlett–Packard flat scanner (Scanjet 3C; Hewlett–Packard, Palo Alto, CA) and stored as .BMP files using Paint Shop Pro 5 (Jasc Software, Eden Prairie, MN) and Scion Image (Scion Corporation, Frederick, MD). The band intensity values were normalized to their molecular weight. The log of the ratio of the band intensities within each lane was plotted against the copy number of the template added per reaction. The quantity of target messages was determined when the ratio of template and target band intensities was equal to 1.[15] Messenger RNA (mRNA) copy numbers were determined using the widely accepted value of 26 pg total RNA per average fibroblast cell.[16] Data were analyzed by Student's *t*-test. Significant differences were determined by $p < 0.05$.

Results

TGF-β_1 and TGF-β_3 Immunohistochemistry

TGF-β_1 immunostaining results showed that compared with normal nasal skin specimens, the protein levels of TGF-β_1 in rhinophyma specimens were significantly higher ($p < 0.001$; Fig 1). Strong expressions of TGF-β_1 protein could be found in the superficial and deep dermal regions of the rhinophyma specimens (Fig 2). There was no significant difference in TGF-β_3 protein expression between rhinophyma tissue and normal nasal skin ($p = 0.06$; Fig 3).

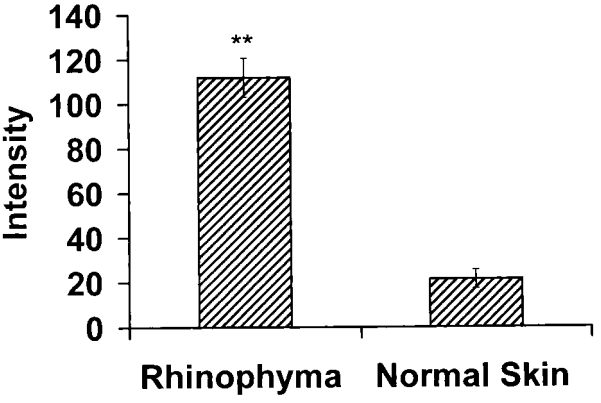

*Fig 1. Immunostaining intensity for transforming growth factor (TGF)-β_1 protein in rhinophyma and normal nasal skin specimens shows a five-fold higher level in the rhinophyma tissue (**p < 0.001).*

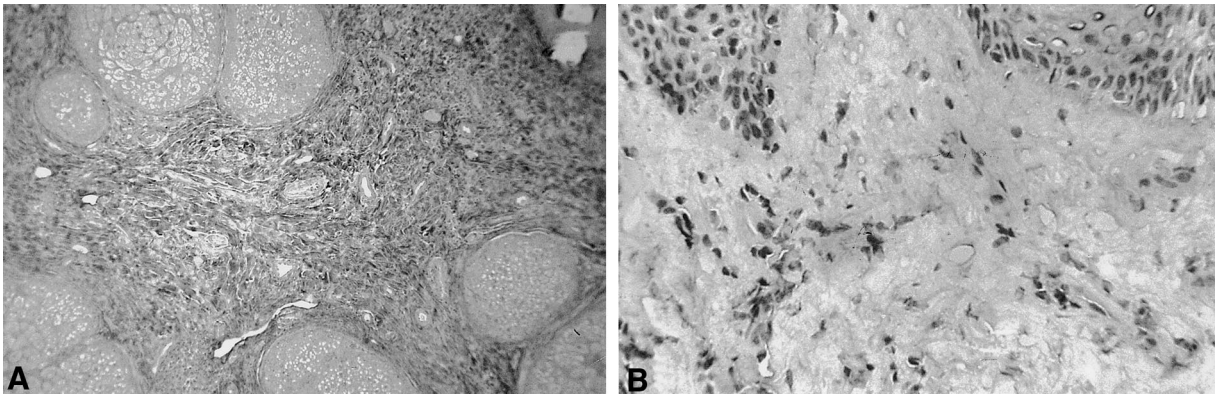

*Fig 2. (*A, B*) Immunohistochemical staining demonstrates a greater intensity of transforming growth factor beta-1 expression (as shown with the more darkly stained patchy areas) in the dermis of rhinophyma tissue (*A*) compared with the normal nasal skin (*B*). H&E, original magnification ×400 before reduction.*

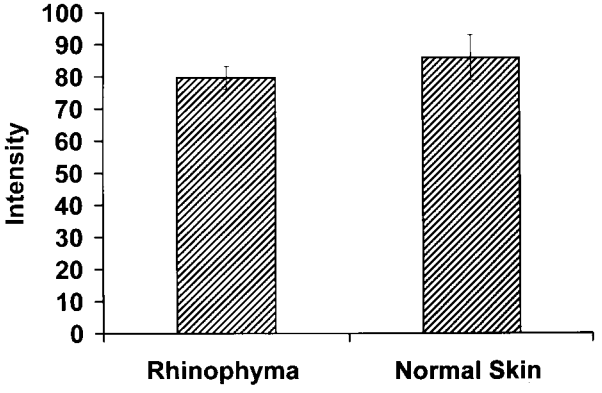

Fig 3. There is no significant difference in the levels of intensity of immunostaining for transforming growth factor β_3 protein in specimens from rhinophyma or normal nasal skin.

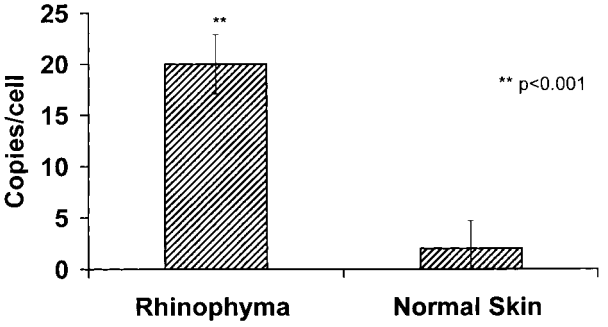

*Fig 4. Quantitative reverse transcription–polymerase chain reaction for transforming growth factor β_1 messenger ribonucleic acid expression demonstrated markedly higher expression in rhinophyma tissue compared with normal nasal skin (**p < 0.001).*

TGF-β_1 and TGF-β_3 mRNA Comparisons

Strong TGF-β_1 mRNA expression was detected in all rhinophyma specimens. In contrast, most of the normal nasal skin specimens demonstrated no TGF-β_1 mRNA expression (Fig 4). Quantitative analyses of RT-PCR products revealed the expression for TGF-β_1 in rhinophyma was 20 ± 3.1 copies per cell vs. 2.7 ± 2.7 copies per cell in normal nasal skin ($p < 0.001$; see Fig 4). TGF-β_3 mRNA was expressed in both rhinophyma and normal nasal skin (Fig 5). However, the quantitative levels were no different in the pathological and normal specimens (5 ± 0.7 copies per cell for rhinophyma vs. 6 ± 1.4 copies per cell for normal nasal skin; $p = 0.09$; Fig 5).

Discussion

It is obvious from this study and the previous one reported by Pu and colleagues[1] that the fibrogenic cytokines TGF-β_1 and TGF-β_2 are up-regulated in rhinophyma tissue. Conversely, TGF-β_3, the antiscarring isoform of TGF-β, is not up-regulated. This suggests that fibrosis may play a role in the pathobiology of this condition. Although, Tope and Sanqueza[4] reported that there might be a fibrous variant of the rhinophyma condition, our data suggest that fibrosis and scarring is an integral part of all rhinophymas. Our patients had the typical exuberant, hyperplastic lesions pathognomonic of the condition. It is the coincident interstitial fibrosis and inflammation seen in histological sections of the specimens that may be the result of overexpression or dysregulated activity of TGF-β_1 and TGF-β_2.

Although both the message for TGF-β_3 and the TGF-β_3 protein are present in rhinophyma, they are not elevated proportionately compared with levels in normal skin. This is distinctly different from the fibrogenic isoforms TGF-β_1 and TGF-β_2,

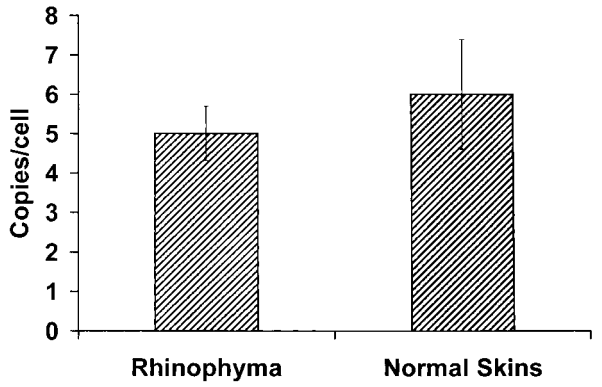

Fig 5. The expression for transforming growth factor β_3 is not significantly different in rhinophyma and normal nasal skin specimens.

which are elevated markedly compared with respective levels in normal skin. The level of TGF-β_3 may not be sufficient to abate the fibrotic overdrive function of TGF-β_1 and TGF-β_2. This would be similar to the hypothesis proposed by Lee and associates[17] for keloids.

The fact that TGF-β_1 and TGF-β_2 have high levels during this condition, whereas TGF-β_3 does not, adds evidence to the hypothesis by Pu and colleagues[1] that perhaps decreasing the production of, or neutralizing, the fibrogenic cytokines could be a possible medical therapy for rhinophyma.

Conclusion

The three most common isoforms of TGF-β include the fibrogenic proteins TGF-β_1 and TGF-β_2, and the antiscarring isoform TGF-β_3. The fact that rhinophyma tissue shows increased levels of TGF-β_1 and TGF-β_2 compared with normal nasal skin and that TGF-β_3 is not up-regulated gives further evidence that fibrosis is an integral part of the pathobiology of rhinophyma. Neutralization or down-regulation of TGF-β_1 and/or TGF-β_2 may provide a novel approach to the treatment of rhinophyma.

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