Immunohistochemical Evidence of Nerve Growth Factor in Dupuytren's Diseased Palmar Fascia

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Purpose: Histologically, the pathognomic feature of Dupuytren's contracture (DC) is the myofibroblast. Its occurrence in this disease has been associated with local production of transforming growth factor (TGF)-B. However, nerve growth factor (NGF) is a recognized growth factor involved in wound healing and has been shown to induce the myofibroblast phenotype in cultured fibroblasts. We hypothesized that NGF would be abundant in this disease entity.

Methods: Immunohistochemistry was used to examine for the presence of NGF in 25 surgical specimens from patients with DC and in surgical specimens from 5 other, unrelated procedures. Patient demographics showed that nearly all patients were men, with a mean age of 61 years (range 36–77). Serial sections were probed with antibodies, stained, and then digitally photomicrographed. Disease staging was also performed. Image analysis was then used to measure the percentage of area stained. In addition, representative sections were probed for TrkA, the high-affinity receptor for NGF, and alpha–smooth muscle actin, a cytoskeletal marker of the myofibroblast phenotype. These alternate steps were used to infer functional dependence and the association of NGF with myofibroblast populations.

Results: Histologically, all patients had either stage II or III disease. Biopsy results showed an abundance of NGF—over double that of controls. The highest levels of NGF occurred in hypercellular stage II disease. In addition, we confirmed that NGF is linked to the expression of both TrkA receptors and alpha–smooth muscle actin.

Conclusions: Tissue levels of NGF are elevated in Dupuytren's disease. This tissue is competent to respond to NGF and manifests an abundance of myofibroblasts in areas of NGF expression. Nerve growth factor is most apparent in the proliferative (hyperplastic) stage of the disease. These data infer that NGF is linked to the pathologic process. (J Hand Surg 2007; 32A:337–342. Copyright © 2007 by the American Society for Surgery of the Hand.)

Key words: Nerve growth factor, Dupuytren's contracture.

Dupuytren's contracture (DC) involves hyperplasia of the palmar fascia, giving rise to fibromatosis. Despite extensive, detailed knowledge of DC spanning centuries, the disease and its treatment are not fully understood. Dupuytren's contracture is linked to northern European or Scandinavian ancestry, with disease manifestation between the fifth and sixth decades.[1–3] Smoking represents a consensus risk factor, especially as it relates to impaired perfusion.[1–6] Alcohol consumption, diabetes, and seizure disorders play far less conclusive roles and are controversial.[1,5,7] Trauma, repetitive manual labor, and chronic vibration may also contribute to the disease process,[3,8] but others[1,4,5,9] have refuted this.

Clinically, treatment is not always successful. Although not every contracture recurs, recurrence of the disease somewhere in the hand is almost certain if the patient lives long enough. In the hand, DC most often affects the pretendinous bands and the natatory ligament, whereas in the finger, Grayson's ligament and the lateral digital sheet of Gosset are affected by the disease. For some reason, the superficial transverse metacarpal ligament in the palm and Cleland's ligament on the finger are typically spared. The pathophysiology of Dupuytren's disease may be di-

vided into 3 overlapping stages: the spectrum of cell proliferation, matrix production, and residual fibrous cords.[9,10] Nonsurgical treatment alternatives include nonsteroidal anti-inflammatory drugs and steroid injection, neither of which are curative. Triamcinolone injections have been shown to retard but not eradicate the disease process.[11] A promising third option is to rupture the cords of the disease by an injection of the enzyme collagenase, a procedure that awaits the completion of clinical trials.[12] When a flexion contracture becomes so severe as to limit a patient's ability to shake hands, retrieve objects from his/her pocket, and perform daily personal hygiene, surgery becomes the treatment of choice. As with any surgical procedure, there are attendant risks, including nerve or vascular injury, hematoma, infection, flare, wound dehiscence, development of a pseudoaneurysm or formation of inclusion cyst, and recurrence.[13] With respect to the last of these, the recurrence rate has been estimated to be as high as 40%.[14] Overall, improving treatment efficacy necessitates advances in understanding the biology of the disease.

Previous studies have shown increased levels of several cytokines associated with either nodule formation or collagen production. These include transforming growth factor alpha and beta isoforms,[15–17] platelet-derived growth factor,[18,19] basic fibroblast growth factor,[15,20,21] and epidermal growth factor[22] or its receptor.[17] Pathogenesis involves not only fibroblast proliferation but also morphogenesis into the myofibroblast phenotype. The appearance of this fibroblast subpopulation represents a pathognomic feature of the disease. A similar pattern of cytokine expression and myofibroblast transformation is a hallmark of wound healing. An underappreciated constituent of the generalized wound-healing milieu is nerve growth factor (NGF). Because of this, we hypothesized that NGF is detectable and abundant in this disease entity, whereas little or no NGF would be present in undiseased palmar fascia. Furthermore, NGF localization should correspond to populations of myofibroblasts.

Materials and Methods

Control tissue from otherwise undiseased palmar fascia was collected during surgery from routine carpal tunnel and trigger finger releases performed between 1999 to 2006. Before the advent of Health Information Portability and Accountability Act (HIPAA) in 2003, material was collected without patient consent as an institutional review board–approved study exemption to U.S. Food and Drug Association requirements (no patient identifiers or traceable information). Thereafter, consent was obtained along with patient demographic information. In all cases, sample acquisition complied with the 1975 Declaration of Helsinki. Tissue samples were snap frozen in liquid nitrogen and stored at −80°C until processing. The 30 specimens included 25 derived from contractures and 5 controls derived from other hand procedures. For the 16 patients for whom demographic data were obtained, all had a diagnosis of Dupuytren's disease, all but 1 were male, and the average age was 61 years (range, 36–77 y). Because control tissue was collected without patient identifiers, demographic data for these individuals could not be retrieved.

Frozen specimens were cut into 10-μm sections in a cryostat, fixed and permeabolized in cold acetone for 10 minutes, washed in phosphate-buffered saline (PBS), and quenched of endogenous peroxidase (1% peroxide in PBS). After a wash step, sections were blocked with either 3% horse or goat serum in PBS overnight at 4°C.

Slides were probed with 1.5 μg/mL of rabbit antihuman NGF immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% horse or goat serum/PBS for 90 minutes in a 25°C humidity chamber. After a PBS wash, a biotin-labeled secondary antibody and streptavidin–peroxidase conjugate were used to label primary binding sites. These reagents were either used in kit form (RTU; Vector Laboratories, Burlingame, CA) or were purchased separately for the purpose. If the latter, biotinylated goat antirabbit IgG (Sigma Chemical, St. Louis, MO), 1:800 in PBS was used. Thirty-minute incubation steps were used, and recommended wash steps were implemented with 0.2% polyethylenesorbitan monolaurate (Tween 20, Sigma Chemical) in PBS. Aminoethylcarbazole Vector NovaRed™ (Vector Laboratories) was used as the chromogen with hematoxylin as a counterstain. Slides were then rinsed in deionized (DI) water and mounted with either crystal mount (Biomeda Corp., Foster City, CA) or permount (Fisher Scientific, Fair Lawn, NJ). Primary antibody specificity was assessed in select slides by substituting naive rabbit or IgG (Sigma-Aldrich Chemical, St. Louis, MO) for the primary antibody. As a part of microscopic examination of the tissue, disease pathology was staged according to the rating system of Chiu and McFarlane.[10] In addition, digital photomicrographs of the images were obtained and imported into an image analysis software program (Scion Image for Windows, version 4.0.2; Scion Corp., Frederick, MD) to permit quantification of the

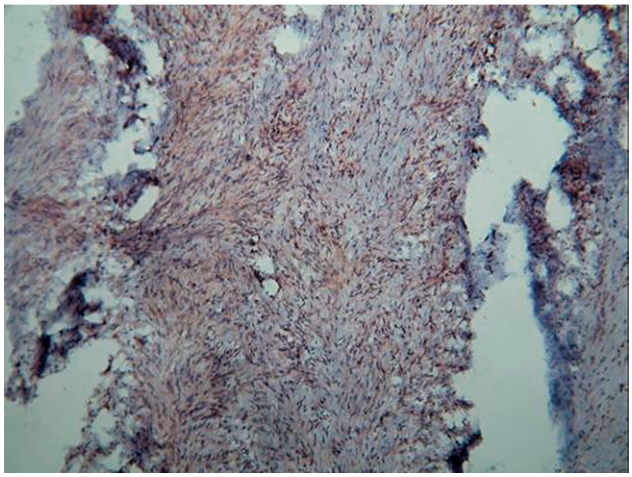

Figure 1. Staining of diseased fascia (aminoethylcarbazole (AEC)/hematoxylin; magnification, ×100).

staining result. Overall areal staining was based on a grand mean derived from the averages of at least 3 regions within—as well as between—sections. Analyzed areas included the extremes of the staining reaction and represented the majority of the tissue present on the slide. Analysis was conducted in an unblinded manner.

To establish the relationship between NGF localization and the myofibroblast phenotype, we obtained representative serial sections of both diseased and undiseased tissue to probe for alpha–smooth muscle actin (α-SMA), a myofibroblast phenotypic marker, or the NGF high-affinity receptor TrkA. In either case, sections were probed as described earlier using mouse antihuman α-SMA IgG (Sigma-Aldrich) (1:400) or rabbit antihuman TrkA (Sigma-Aldrich Chemical) (1:200) in 1% goat/PBS. Diaminobenzidine (Vector Laboratories) was used as the substrate with hematoxylin as the counterstain. Slides were then viewed and assessed for positive staining in a qualitative manner; image analysis was not performed.

Given that we were unaware of any histologic nonneurologic studies of NGF in this disease entity, the parameters used to establish a power analysis were unavailable, and a power analysis could not be performed. Alternatively, a sequential research design[23] was used wherein we periodically assessed differences between tissue sources (diseased vs control). Independent *t* tests were used to assess differences in areal staining between groups; differences by disease stage were analyzed by analysis of variance with Bonferoni-corrected *post hoc* comparisons. By convention, 0.05 was used as the threshold for statistical significance. Database management and statistical procedures were managed with statistical software (SPSS version 12.0 for Windows; SPSS, Chicago, IL).

Results

Nerve growth factor was most abundant and widespread in diseased (fibromatosis) tissue (Fig. 1), accounting for a mean of 17% of the area (range, 3%–49%). In many instances, NGF could be discretely localized to the pericellular region of the fibroblasts (Fig. 2). Staining was not merely confined to the perivascular region or nerve receptor endings, sites that are known to express NGF.[24,25] Undiseased samples (Fig. 3) manifested significantly lower amounts of staining (mean, 7%; range, 3%–12%; $p = 0.049$). Overall, staining intensity was widespread and heterogeneous between and within sections but highly abundant in hypercellular diseased tissue. As the cellularity decreased with the stage of disease, the amount of NGF also declined (Table 1). In particular, stage II specimens showed significantly greater amounts of NGF than those of more advanced disease stages ($p \leq .015$).

Nerve growth factor localization was associated with areas of smooth muscle actin, an unambiguous marker of the myofibroblast phenotype (Fig. 4). Smooth muscle actin staining consistently occurred in perivascular areas, as anticipated. It also was evident in fibroblasts associated with hyperplastic regions. That these fibroblasts were competent to respond to NGF was inferred by the presence of high-affinity receptor, TrkA (Fig. 5). Staining for either α-SMA or NGF high-affinity receptors did not occur in acellular specimens (data not shown).

Little nonspecific staining occurred. Use of rabbit

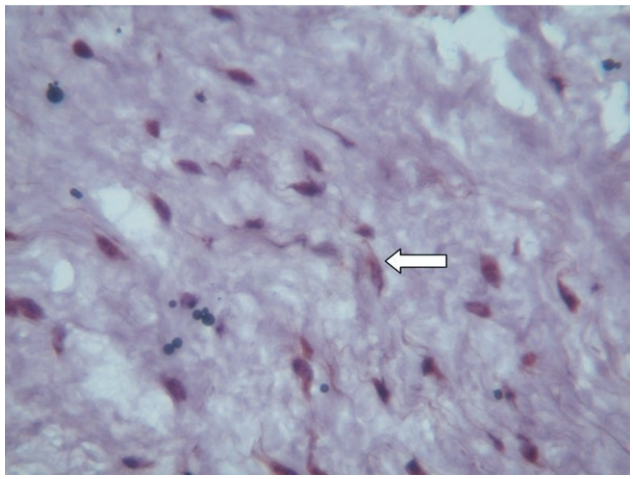

Figure 2. Pericellular localized NGF staining (arrow) (Vector Red [Vector Laboratories]/hematoxylin; magnification, ×400).

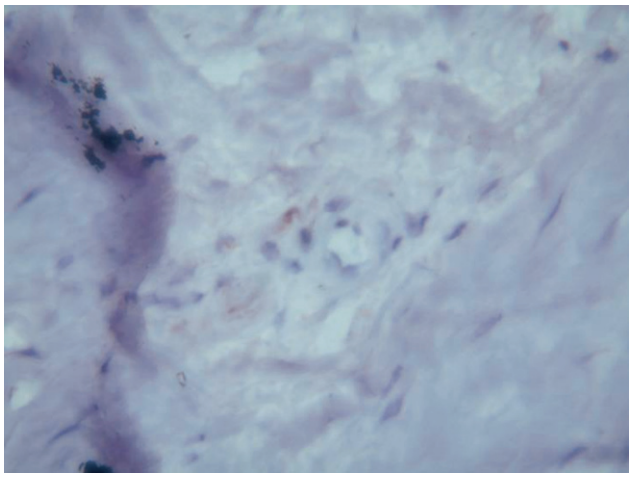

Figure 3. Staining of undiseased tissue (Vector Red [Vector Laboratories]/hematoxylin; magnification, ×400).

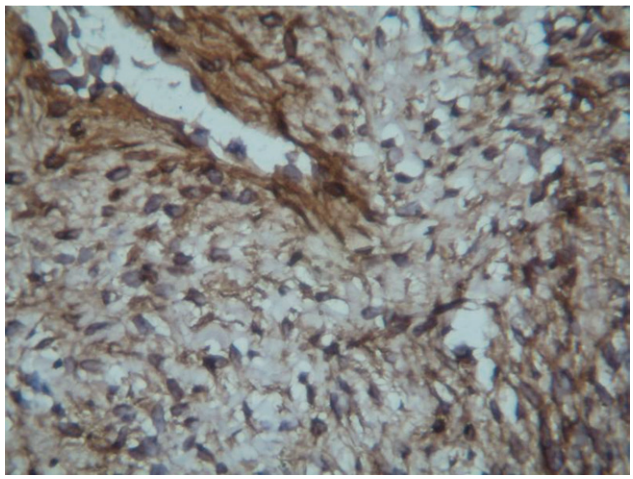

Figure 4. Alpha–smooth muscle actin staining, diseased tissue (diaminobenzidine (DAB)/hematoxylin; magnification, ×400).

or mouse serum nearly ablated the staining response (data not shown). Overall, nonspecific staining amounted to 3% of the area.

Discussion

Tissue specimens derived from patients with stage II through stage III disease had increased amounts of NGF compared with undiseased tissue. As suspected, NGF levels decline with more advanced disease, although substantial amounts remained detectable in the most mature form. That NGF is associated with the myofibroblast phenotype is suggested by the presence of α-SMA in hyperplastic areas of the tissue samples. The localization of TrkA receptors to this same region infers cellular competence to respond to NGF. This, in turn, suggests that NGF exerts either a paracrine and/or autocrine effect.

There is some precedence for our findings. Several studies have shown tissue elevation of NGF levels, or its receptors, for a variety of hyperplastic conditions. Erhmantant et al[25] demonstrated its association with enlarged pacinian corpuscles in Dupuytren's tissue. p75 or TrkA receptor expression has been immunohistochemically shown in tumors of both neurogenic and nonneurogenic origin, including nerve sheath tumors,[26] von Recklinghausen's neurofibromatosis,[27] invasive carcinomas,[28] and dermatofibrosarcomas.[29] In general, NGF is associated with the development of neoplasms or conditions involving cell hyperplasia.

Nerve growth factor also appears to be a constituent of the inflammatory and wound healing processes. In murine models of cutaneous wound repair, exogenous administration of NGF accelerated the rate of healing.[30,31] Substantial elevations in both NGF and TrkA transcription and translation have been observed in chronic bowel inflammatory disease.[32] *In vitro*, skin and lung fibroblasts exposed to NGF became positive for α-SMA and manifested

Table 1. Nerve Growth Factor Abundance by Disease Stage

Stage	n	% Area Stained (min, max)
2	10	25 (9, 49)
2/3*	8	12 (6, 22)
3	7	11 (3, 22)

*Transitional between stage II and III disease pathology.

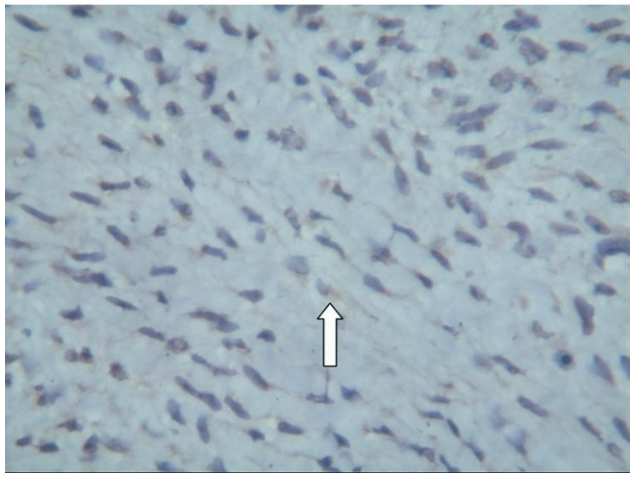

Figure 5. TrkA staining, diseased tissue (DAB/hematoxylin; magnification, ×400). Arrow shows area of receptor localization.

both chemotaxis and contractile properties.[33] Biopsies of areas of skin wounds showed increased NGF and alpha–smooth muscle messenger RNA production in a diversity of cell types, including hypodermal fibroblasts and myofibroblasts.[34] Therefore, nerve growth factor production appears linked to an inflammatory response. Although immunoreactive NGF has been observed in undamaged dermis,[34] its abundance in the undiseased palmar fascia used in this study was impressive and not strictly confined to vascular endothelium. In this regard, NGF levels in control samples cannot be construed as normal (constitutive expression); tissue trauma may have induced increases in NGF in these samples.

The present study has limitations, however. Proof of demographic comparability of controls to test samples is absent. This reflected a flawed, pre-HIPAA approach to the procurement of surgical samples. Further, these controls exhibited substantial levels of NGF. Likely this reflected a chronic inflammatory condition associated with the biopsy material. In addition, we note substantial variability in the data, even within the same sample. We specifically attempted to address this by analyzing the extremes of the staining reactions and providing an average. Nonetheless, this points to the overall limitation of any biopsy-based study, because samples may reflect substantial histologic heterogeneity, dependent on the site of excised tissue. Finally, analysis was not conducted in a blinded manner.

Our study does suggest, however, that NGF is involved in the pathogenesis of DC. Pharmacologic-based strategies to impede NGF production, then, may provide a nonsurgical avenue to arrest or control disease progression.

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