

## Gene expression in Peyronie's disease

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Currently, surgical intervention is the only efficacious treatment for Peyronie's disease (PD), a fibromatosis of the tunica albuginea of the penis. Therapies based on the molecular pathways for this disease could provide alternatives to surgical treatment but only recently has the pathophysiology of the Peyronie's disease plaque been investigated at the molecular level. In this review, we examine the current knowledge of gene expression in the PD plaque and the relationship of PD with other fibrotic conditions such as Dupuytren's disease. TGF $\beta$ 1, along with other growth factors, pro-fibrotic genes, and collagen, are expressed in fibroblasts and myofibroblasts. Myofibroblasts are normally involved in wound contracture and largely eliminated via apoptosis during the late stages of wound remodeling. In the PD plaque, however, these cells persist and may play an important role in the PD plaque fibrosis. The expression levels of TGF $\beta$ 1 and pro- and anti-fibrotic gene products, along with the nitric oxide/reactive oxygen species (NO/ROS) ratio in the tunica albuginea, appear to be essential for the formation and progression of the PD plaque and effect the expression of multiple genes. This can be assessed with the recently developed DNA-based chip arrays and results with the PD plaque have been encouraging. OSF-1 (osteoblast recruitment), MCP-1 (macrophage recruitment), procollagenase IV (collagenase degradation), and other fibrotic genes have been identified as being possible candidate regulatory genes. Finally, possible therapeutic avenues for gene-based therapy in the treatment of PD are discussed that may eventually reduce the need for surgical intervention.

*International Journal of Impotence Research* (2002) 14, 361–374. doi:10.1038/sj.ijir.3900873

**Keywords:** fibrosis; DNA microarrays; gene expression; myofibroblast

### Pathophysiology of the Peyronie's fibrotic plaque

Peyronie's disease (PD) is a fibromatosis of the tunica albuginea (TA), the specialized lining of the corpora cavernosa of the penis.<sup>1–5</sup> Penile deformation (curved penis during erection), localized pain, and erectile dysfunction are frequent sequelae of this condition. Recent studies suggest that PD may affect about 2–3% of men, particularly those older than 50 y of age.<sup>6,7</sup> In the USA this would be translated into a prevalence of 3–4 million cases. Despite this prevalence, relatively very little scientific information is known about PD and this is reflected by the lack of any medical treatment that has been shown conclusively to alter the course or progression of the disorder. Therefore, novel forms of treatment for PD are needed and they may emerge

from an understanding of the pathophysiology of this disorder at the molecular level.

The initiating event in the development of the PD fibrotic plaque is believed to be some external stress to the TA of the penis, most likely in the erect state, usually during sexual activity. This may result in an injury to, or tear of, the TA. In its severest form it is called a penile fracture. In the detumesced state, the only indication of the disease is the palpation of a plaque or thickening within the TA which at times may even ossify. There is a growing consensus that the traumatic or microtraumatic events to the erect penis cause a wound that for unknown reasons undergoes an abnormal healing process.<sup>7–11</sup> Indeed, it is believed that the PD plaque is 'a scar that fails to remodel well'.<sup>11</sup> There may also be a genetic predisposition<sup>3</sup> since PD is associated with other fibrotic conditions such as Dupuytren's contracture (palmar fascia; 10–20% incidence or more in PD) and Ledeshore's disease (plantar fascia). Our current knowledge about the pathophysiology of PD is that it is characterized by localized disruption of the tunica albuginea, a local increase in microvascular permeability, persistent fibrin (deficient fibrinolysis) and collagen accumulation, perivascular inflammation, disorganization and loss of elastic fibers

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Received July 30 2001

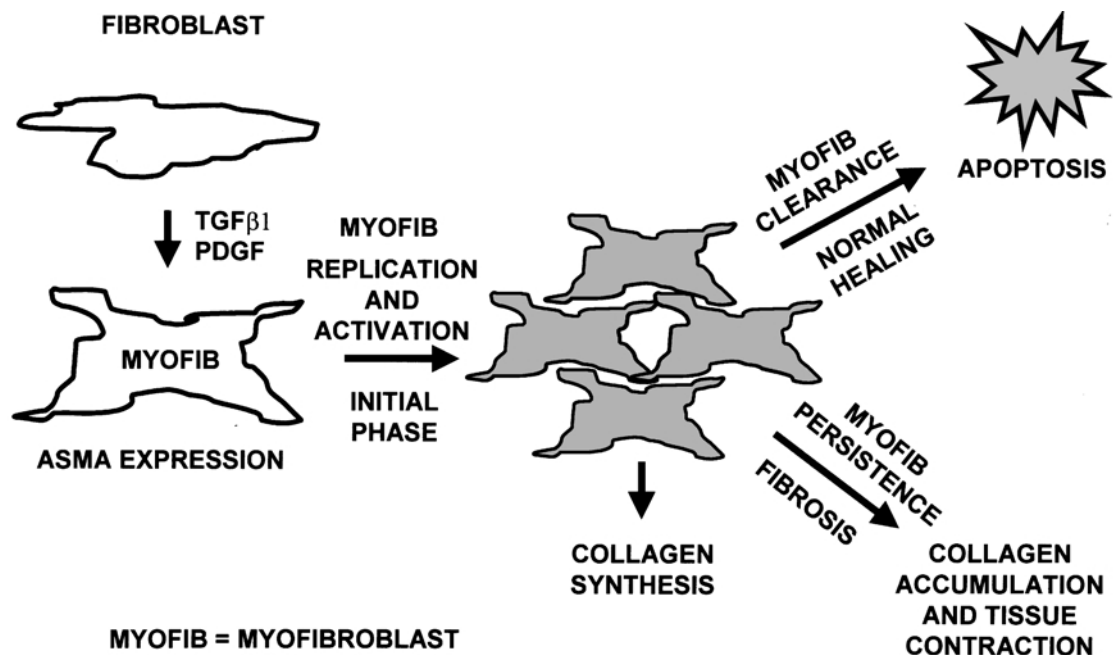
(release of elastase by macrophages), disorganized collagen bundles, an increase in transforming growth factor  $\beta 1$  (TGF $\beta 1$ ) synthesis, sustained cellular and collagen fiber-mediated contraction of the injured TA, and eventually calcification and ossification.<sup>1-5,8-13</sup>

Therefore, the focus of a unifying concept in the development of the PD plaque (and the Dupuytren's nodules) as an abnormal healing process is not only essential to understanding the molecular mechanisms causing these conditions, but at the same time PD constitutes by itself a valuable model for other types of hypertrophic scarring (eg keloids, burns)<sup>14-17</sup> and more diffuse organ fibrosis (eg benign prostatic hyperplasia, liver cirrhosis, myocardial hypertrophy, renovascular and glomerular fibrosis, etc.).<sup>15-18</sup> The value of PD as a model for studying basic mechanisms of fibrosis is due to the localized nature of the PD plaque, and the easy surgical availability of the affected tissue and the normal tunica. Additionally, an animal model for this disease has been developed by Lue's group,<sup>5,19,20</sup> based on the administration of a synthetic heptapeptide of the fibrogenetic agent TGF $\beta 1$  directly into the tunica albuginea of the rat. After 45 days, the TGF $\beta 1$ -injected animal develops histological alterations and collagen deposition resembling those observed in the human PD plaque and in the rat tunica after trauma.<sup>21-26</sup> The administration of the actual TGF $\beta 1$  in a single injection, with or without prior injury to the tunica albuginea, leads to a similar process in the rat,<sup>26-29</sup> and the chronic effects are probably due to the ability of TGF $\beta 1$  to trigger its own synthesis. This agrees with

the increase in TGF $\beta 1$  levels seen in the plaque of patients with PD,<sup>30</sup> as well in the TGF $\beta 1$ -injected rat model itself.<sup>19</sup>

### Cellular and molecular basis of fibrosis

In many fibrotic conditions, it is the inflammatory process, characterized by recruitment of macrophages, monocytes and related cells, that is the initial cellular event. Subsequently, the development of the fibrotic process *per se* becomes the major pathological end-point involving activated fibroblasts and myofibroblasts in most, if not all, the affected tissues. These key players, the fibroblasts and myofibroblasts, are not only important in normal wound healing<sup>31-34</sup> but they are also assumed to be responsible for the scarring and contracture in Dupuytren's<sup>35</sup> and PD,<sup>36-38</sup> and in fibrotic processes in general. The myofibroblasts, discovered by Gabbiani in 1971, are assumed to differentiate from fibroblasts under the autocrine or paracrine effects of growth factors released during inflammation,<sup>39</sup> particularly TGF $\beta$  and platelet-derived growth factor (PDGF). During this process, the differentiated fibroblasts acquire a phenotype intermediate between the progenitor cell and a smooth muscle cell, mainly recognized by the expression of  $\alpha$  smooth muscle actin (ASMA, or A). They replicate and are activated to produce collagen, in a process resembling the one also occurring for regular fibroblasts (Figure 1). The collagen fiber secretion and contractile forces devel-



**Figure 1** Myofibroblast role in normal wound healing and fibrosis.

oped by myofibroblasts help to close the wound during normal healing, and after fulfilling their role these cells disappear by programmed cell death (apoptosis). However, in Dupuytren's disease and other fibrotic conditions, the myofibroblast persists (no apoptosis) thereby resulting in pathological contracture and fibrosis. In Dupuytren's, the myofibroblast may ultimately disappear at late stages.

In molecular terms, fibrosis and contracture are elicited and characterized by a concerted alteration in the expression of genes controlling related biochemical pathways. In a simplified categorization, fibrosis-related genes may be grouped as follows: (a) fibrogenic genes leading to a final endpoint, which is the accumulation of disorganized collagen fibers through either a stimulation of collagen gene expression at the transcriptional or translational levels, an altered post-translational modification, or a reduction in collagenolytic activity by down-regulation of the expression of metalloproteinases or up-regulation of their inhibitors; (b) genes controlling the proliferation, differentiation, and apoptosis of fibroblasts and myofibroblasts. Finally, we should also consider: (c) genes whose expression is essentially antifibrotic that counteract the fibrogenic factors or the pathways involved in the persistence of myofibroblasts. We will restrict our discussion to those genes which in our opinion may be specifically involved in PD.

### Pro-fibrotic genes

TGF $\beta$ 1<sup>18,39-41</sup> is perhaps the best characterized of the fibrotic factors and is hyper-expressed in many fibrotic conditions. Not only does it stimulate collagen synthesis by both fibroblasts and myofibroblasts, but it also induces the production of other potent fibrogenic factor, namely reactive oxygen species (ROS).<sup>42-45</sup> TGF $\beta$ 1 also inhibits fibrinolysis, and transcriptionally represses inducible nitric oxide synthase (iNOS),<sup>46,47</sup> which we postulate is an antifibrotic agent through the production of nitric oxide (NO) (see Anti-fibrotic genes section).

ROS are essentially hydroxyl radicals and superoxide anions that induce lipid peroxidation in cell membranes and increase vascular permeability and leakage of fibrinogen and other clotting factors.<sup>44-47</sup> ROS are quenched by the antioxidant effect of NO to form peroxynitrite, which is not a fibrogenic compound.<sup>46-48</sup> Additionally, ROS are eliminated by a series of antioxidant enzymes, mainly superoxide dismutases, catalase, hemoxygenase I, and xanthine oxidase.<sup>42,43</sup> Peroxynitrite induces apoptosis and nitrotyrosinylates proteins. The balance between NO and ROS is altered in the tissues with a decrease in NO and/or an increase in ROS in many fibrotic conditions, eg the liver (cirrhosis), kidney (obstructive nephropathy), heart (cardiac hypertro-

phy) and vascular tree (arterial medial hyperplasia).<sup>49-51</sup> Endotoxins, despite being potent iNOS inducers, can cause liver fibrosis presumably by activating NF $\kappa$ B-mediated cytokine synthesis and a subsequent increase in ROS that may alter the oxidative/nitrosative balance.<sup>44-52</sup> ROS generation during oxidative stress is accompanied by a considerable induction of hemoxygenase-1 (HO-1),<sup>53</sup> also known as heat shock protein 32 (HSP 32). The role of the NO/ROS balance in the fibrotic processes induced by myofibroblast activation is outlined in Figure 2A, and the interaction of different cell types in the production of NO and its subsequent reaction with ROS is represented, in Figure 2B.

Endothelin (ET) and angiotensin II are important mediators of renal and cardiac vascular fibrosis through the increase of TGF $\beta$ , in a process that appears to be counteracted by NO.<sup>54-60</sup> These conditions can be induced experimentally in rodents by blocking NOS activity and hence NO production (thus decreasing the NO/ROS ratio) by long-term administration of a NOS inhibitor such as L-Nar-Nitro-L-arginine methyl ester (L-NAME). The resulting fibrosis is independent from hypertension or haemodynamic alterations and can be counteracted by blocking the activity of endothelin 1. This was elegantly shown in a transgenic mouse model harboring the regulatory region of the collagen I- $\alpha$ 2 gene linked to two reporter genes, luciferase and  $\beta$ -galactosidase,<sup>56-59</sup> so that whenever collagen mRNA synthesis is stimulated the reporter proteins are expressed, allowing quantitation of promoter activity (and hence of mRNA synthesis) by immunohistochemistry or measurement in tissue extracts by luminometry. The nephroangio- and glomerulofibrosis induced by L-NAME administration was accompanied by a parallel expression of luciferase and an increased urinary excretion rate of endothelin. The blockade of the endothelin receptors with the selective ET antagonist bosentan reduced collagen deposition and abolished collagen I promoter activation. A similar situation was demonstrated for angiotensin II, by using AT1 antagonists (losartan) or ACE inhibitors (captopril or imidapril). NO therefore appears to down-regulate endothelin synthesis or activation and counteract AT2 fibrogenic effects.<sup>58-61</sup>

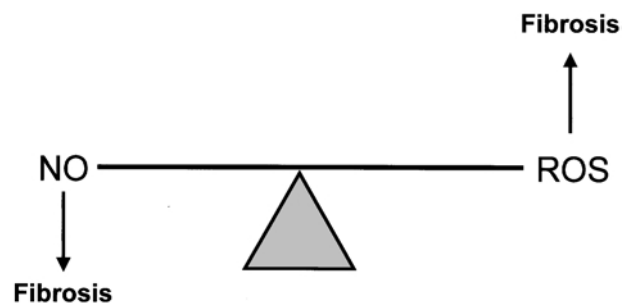


Figure 2a Role of NO and ROS in fibrosis. NO : ROS interaction.

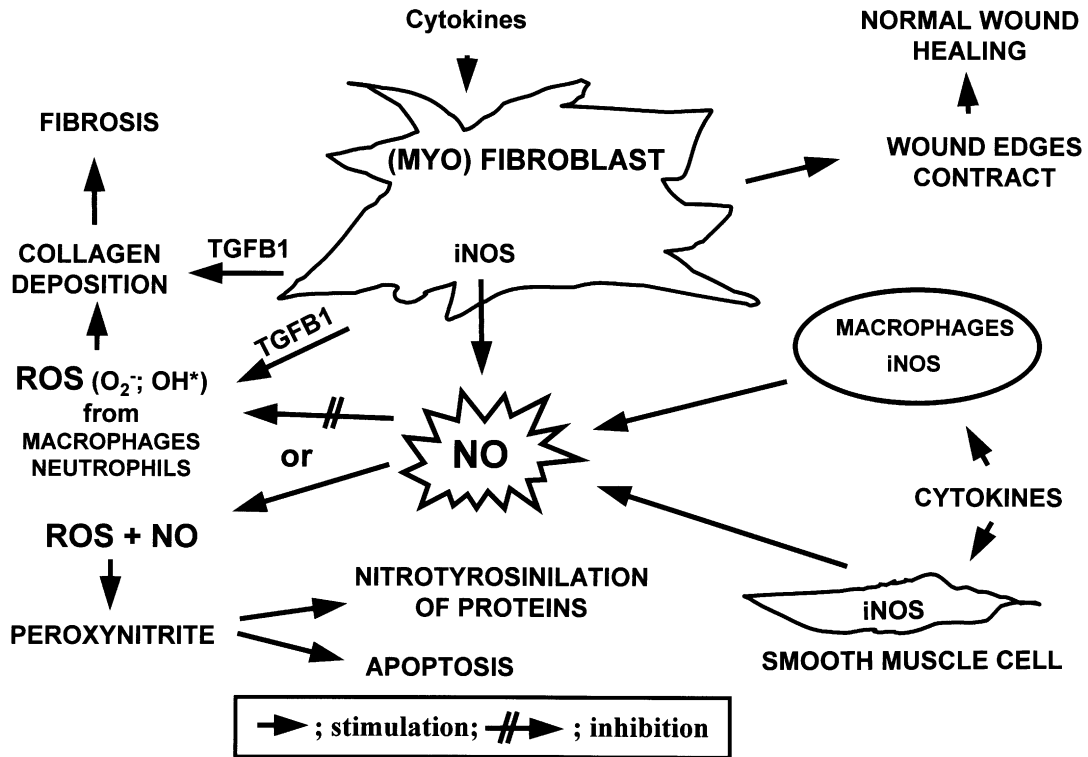


Figure 2b Molecular pathways of NO and ROS.

### Genes affecting myofibroblasts

Myofibroblasts express large bundles of actin filaments (actin, myosin, and associated proteins: 'stress fibers'), with a fibrillar space material named the 'fibronexus', composed of fibronectin. The fibronexus links the extracellular fibronectin fibrils with the intracellular actin myofilaments, and it is proposed to be the structure transducing the intracellular contractile force to the extracellular matrix. Gap junctions further link these cells among themselves and the smooth muscle.<sup>31,35</sup> Myofibroblasts can be identified by the detection of both ASMA (A) (absent in skeletal muscle cells and fibroblasts), and vimentin (V) (fibroblast marker absent in skeletal fibers and smooth muscle cells).<sup>31</sup> These cells are classified within the 'VAD' or the 'VA' types, according to whether they express the additional smooth muscle marker desmin (D) or lack it, respectively. In some cases, myofibroblasts can be A negative, and only electron microscopy can distinguish them from undifferentiated fibroblasts.

It is proposed that myofibroblasts originate from fibroblasts, although smooth muscle cells and so far unidentified neuroepithelial stem cells from the neural crest, are also candidates.<sup>31</sup> In addition to TGFβ1 and PDGF mentioned earlier, endothelin, insulin-like growth factor 2 (IGF-2), stem cell factor, and other agents, stimulate differentiation in culture of fibroblasts into 'activated' myofibroblasts and can

be further transformed into the non-proliferating 'stellate' form by cAMP and prostaglandin E2 (PGE2).<sup>31-34</sup> The stellate cell can be dedifferentiated to fibroblasts by INFγ. The activated myofibroblast is able to secrete cytokines, TGFβ1 and other growth factors, inflammatory mediators, NO and ROS, and produces matrix proteins involved in wound repair and fibrosis, such as collagens I, III, IV, VI, and XVIII, laminins, proteoglycans, adhesion molecules and matrix modifying proteins. These cells also have receptors for TGFβ1 (TGFβR1 and RII), PDGF, basic fibroblast growth factor (bFGF), endothelin, angiotensin, prostaglandins, adhesion proteins, and other fibrosis-related factors.<sup>31</sup>

### Anti-fibrotic genes

Among the several regulators of collagen deposition and wound healing that have been defined in experimental studies in animals and cell cultures, NO may be a potential key inhibitor of penile fibrosis, because of the fundamental role NO plays in the penis as the mediator of erection.<sup>62,63</sup> In general terms, the participation of NO in wound healing is well established *in vivo* and *in vitro*.<sup>64-69</sup> NO acts as a positive modulator during the early phases of normal wound healing, via a mechanism different from the one inhibiting fibrosis. For instance, the impaired skin wound healing seen in

iNOS deficient mice was reversed by topical administration of an iNOS cDNA construct,<sup>69</sup> and the same effect was achieved in rats with subcutaneous sponges containing this construct.<sup>68</sup> In addition, the blockade of TGF $\beta$ 1 translation by antisense therapy impairs wound healing,<sup>70</sup> indicating that a certain level of both NO and the growth factor may be required initially for normal collagen deposition. NO, and the fibrotic compounds, ROS and TGF $\beta$ 1, are all 'swords of two edges', where relative concentrations will determine their effects on collagen accumulation and cellular apoptosis.

However, in the phases of wound healing following the inflammatory response phase, and in generalized organ fibrosis, NO is clearly antifibrotic, since the preservation of NOS activity is believed to be fundamental in avoiding excessive collagen deposition. NO donors and the NOS substrate, L-arginine, inhibit collagen fiber accumulation<sup>60,64–66,71,72</sup> fibrin deposition<sup>73,74</sup> and TGF $\beta$ 1 synthesis<sup>75</sup> in various *in vivo* and *in vitro* systems. The most compelling evidence arises, as stated previously, from studies on the experimental decrease of NO synthesis by blocking NOS activity with L-NAME (an inhibitor of all NOS isoforms). This inhibition of NO production leads to fibrosis as seen in myocardial hypertrophy, coronary vascular remodeling and obstructive nephropathy.<sup>54–61,76–82</sup> Reduced endogenous NO synthesis may be also responsible for the impaired wound healing seen in diabetics.<sup>67</sup>

Physiologically, the reduction of NO levels may contribute to fibrosis through a number of mechanisms. These may involve transcriptional blockade<sup>46,47</sup> of the spontaneous iNOS induction observed with aging in organs such as testis and penis<sup>83–85</sup> or in inflammation<sup>47,48</sup> the down-regulation of eNOS or nNOS levels,<sup>85</sup> or by endogenous inhibitors of NOS activity, such as asymmetric dimethyl arginine (ADMA) or advanced glycation-end products (AGE).<sup>86,87</sup> There is no clear experimental evidence of a decrease in NOS expression in fibrosis, other than a possible one in pulmonary fibrosis where NO is decreased in exhaled air,<sup>82</sup> but this is unlikely to be a general mechanism since the role of NO is probably to inhibit fibrosis already underway rather than to prevent it. Therefore, a decrease of NO may not be a triggering factor, but instead the inability to produce sufficient NO to counteract the fibrogenic factors would be what would exacerbate fibrosis. This interpretation is supported by the intensification of interstitial fibrosis after experimental unilateral ureteral obstruction in the kidneys of mice lacking the iNOS gene.<sup>88</sup> The putative beneficial effect of NO in opposing fibrosis contrasts with the association of aging-related spontaneous iNOS induction and apoptosis in the brain.<sup>89,90</sup>

The antifibrotic effects of NO are exerted not only in reducing ROS levels, but also in certain organs through more specific pathways, such as the

angiotensin II/endothelin interaction discussed above. In addition, NO may also stimulate both the activity and the expression of metalloproteinases, although these effects may differ according to the system studied.<sup>91–94</sup> In turn, the expression of antioxidant genes, such as hemoxygenase I, glutathione, or mitochondrial superoxide dismutase, is also an effective mechanism to regulate the tissue redox state and avoid the accumulation of the profibrotic ROS.<sup>42,43,53</sup> ROS induce TGF $\beta$ 1 expression and the subsequent stimulation of collagen production *in vivo* and in myofibroblasts.<sup>95–98</sup> In the same fashion that ROS, and therefore TGF $\beta$ 1 synthesis, are neutralized by the expression of antioxidant genes, other gene products help to reduce TGF $\beta$ 1 levels, such as NO itself<sup>75,94</sup> or bind TGF $\beta$ 1, such as the small proteoglycan decorin,<sup>99</sup> and block its interaction with its receptor, particularly the type II receptor. Decorin is expressed in fibrotic tissues and is being extensively studied for its possible use as an anti-scarring and antifibrotic agent.<sup>100</sup> In contrast, genes such as SMAD7 mediate TGF $\beta$ 1 effects and are potentially profibrotic.<sup>101,102</sup> A series of other proteins produced by fibroblasts, such as hepatocyte growth factor (HGF), can counteract fibrosis by increasing collagenase activity.<sup>54</sup>

## Gene expression in the Peyronie's disease plaque

### *Studies with assays for individual gene expression*

In contrast to the evidence accumulated in other fibrotic conditions, the patterns and interconnections of gene expression in PD are only now beginning to be investigated, which is a reflection of the lack of an experimental animal model until Lue's published report of the TGF $\beta$ 1 model in 1997.<sup>15</sup> Most of the gene expression data in this model, as well as in human PD plaques, have been obtained following the expression of individual genes utilizing conventional procedures (immunohistochemistry, histochemistry, RT/PCR, and Western blots), or recently with more elaborate assays (promoter activation), but no results with Northern blots or *in situ* hybridization have been published.

**Collagen.** The first gene product explored has been collagen itself. However, human studies have been mostly limited to a qualitative estimation of an apparent increase in collagen staining with the Masson Trichrome,<sup>13,104</sup> or specific antibodies against collagen I or II<sup>103</sup> and with a more quantitative assessment based on hydroxyproline determination in tissue homogenates from the plaque as compared with the normal tunica.<sup>29</sup> The image

analysis applied to corpus cavernosum tissue to estimate collagen content<sup>107</sup> has not yet been used for PD. Since the intensity of the Masson staining is affected by the disorganization of collagen fibers in the human PD plaque, the hydroxyproline estimation should be more reliable. Earlier reports indicated an increase of collagen III over I in the PD plaque,<sup>105,106</sup> but other studies have claimed opposite results.<sup>103</sup> The levels of collagen I mRNA as determined by RT/PCR are consistently higher in the plaque than in the TA.<sup>29</sup> In the TGF $\beta$ 1-induced animal model of PD, the Masson staining is more quantitative than in the human specimens and shows the expected increase in collagen.<sup>19–21,26–29</sup> This appears to be due to an intensification of collagen I transcriptional activation,<sup>29</sup> although translational and post-translational up-regulation may also occur as well as a decrease in collagenase activity.

*Genes involved in the NO/ROS balance.* TGF $\beta$ 1 is the most relevant pro-fibrotic gene, and as expected both its mRNA and protein are up-regulated in both the human PD plaque,<sup>30</sup> and in the PD-like plaque of the rat model.<sup>19</sup> TGF $\beta$ 1, in addition to directly inhibiting collagenase and promoting collagen synthesis, increases ROS levels and in certain cell types inhibits iNOS transcriptional induction, thus decreasing NO synthesis. Therefore, the hyper-expression of TGF $\beta$ 1 should lead to a substantial decrease of the NO/ROS ratio. Whether this is the case in PD was investigated in the human tunical plaque and in the PD-like lesion induced by TGF $\beta$  in the rat model. By a combination of procedures (immunohistochemistry/image analysis, Western blot, RT/PCR), it was shown that ROS, as measured by its marker, hemoxygenase I, was indeed increased, but the NOS isoform not normally expressed, iNOS, was induced in parallel in the human plaque.<sup>29,38</sup> A similar situation occurred in the animal model.<sup>27–29,38</sup> This should cause a higher ROS level as expected, but accompanied by more NO synthesis, resulting in an intensification of the NO–ROS reaction that produces peroxynitrite as evidenced by an increase in the nitrotyrosinylation of proteins, an indicator of peroxynitrite formation.

These changes in the absolute concentrations of ROS and NO, and in the balance between both compounds, are accompanied in the TGF $\beta$ 1-induced PD-like plaque by a decrease in eNOS, and an increase in arginase, the enzyme that degrades L-arginine, the substrate of the NOS-catalyzed synthesis of NO.<sup>28</sup> Hellstrom and his colleagues proposed that the down-regulation of eNOS and the putative increased degradation of L-arginine by arginase would lead to a reduction in the rate of NO formation by eNOS and presumably nNOS. They proposed that this may be responsible

for the erectile dysfunction (ED) observed in the animal model of PD.<sup>27,28</sup> However, the ED seen in this animal model may be due to the fibrosis induced by TGF $\beta$ 1 injection that impairs the rigidity of the tunica albuginea and to a certain extent the compliance of the underlying cavernosal smooth muscle.

The role of iNOS induction and subsequent increase in NO synthesis in the PD fibrotic plaque was clarified in the TGF $\beta$ 1 animal model by using L-iminoethyl-L-Lysine (L-NIL) to inhibit specifically iNOS activity during the long-term formation of the plaque as evidenced by the reduction of nitrotyrosine formation.<sup>29,38</sup> A marked intensification of collagen expression primarily around the site of TGF $\beta$ 1 injection was observed, in a process restricted to the penis since no fibrosis was detected in heart, lung, kidney and liver. This was due, at least in part, to the transcriptional activation of collagen synthesis. As expected, there was stimulation of hemoxygenase I staining, suggesting that iNOS expression is a compensatory response to the induction of fibrosis by TGF $\beta$ 1 in the TA. This compensatory response would then lead to the elevation of the NO/ROS ratio which results in a reduction in collagen synthesis.

Endothelin and the EA1 and angiotensin II receptors are expressed in the penis,<sup>108–110</sup> and endothelin may fulfill an important role in penile erection by counteracting NO-dependent cavernosal relaxation.<sup>111</sup> Although both receptors have been detected in the smooth muscle but not in the TA, it is possible that endothelin-mediated processes initiated elsewhere can trigger fibrotic cascades in the tunical myofibroblasts. Therefore, it is possible that the inhibition of NO in the TA controls the pro-fibrotic effects of the endothelin/angiotensin II system, a process that is also seen in the heart and vascular system of the kidney (see earlier).

*Myofibroblast-related genes.* Studies of individual gene expression in the human and rat PD plaque have also confirmed that myofibroblast accumulation in the lesion correlates with collagen deposition. ASMA is increased in the fibrotic lesion as compared to the normal TA, particularly in relation to the vimentin + fibroblasts, as shown by immunohistochemistry, Western blot, and RT/PCR, and also in myofibroblasts cultured from both normal tunica and plaque.<sup>38,112</sup> Accordingly, the inhibition of iNOS activity by L-NIL leads to a further increase in the ASMA/vimentin ratio, thus implying either additional myofibroblast differentiation or a reduction in their apoptosis rate. This is also reflected in the expression in the human and rat PD plaque of a novel growth and differentiation factor, myostatin (GDF8),<sup>113–117</sup> which is virtually absent in the normal tunica albuginea.<sup>38</sup> This gene until recently has been found exclusively in skeletal muscle and

its function appeared to be restricted to the negative regulation of muscle growth. However, there is new evidence suggesting that myostatin may drive the differentiation of fibroblasts and pluripotent stem cells into myofibroblasts during tissue damage and repair.<sup>118</sup>

**Genes affecting ancillary pathways.** Fibrin deposition in the PD plaque has been detected by immunohistochemistry and Western blot in 95% of patients, but it was absent in normal or scarred tunica from control patients.<sup>12,13</sup> It has been proposed that the persistence of pro-fibrotic fibrin originating from the original trauma is due to the inhibition or existing fibrinolytic enzymes by extravasated plasma proteins such as 2-antiplasmin and  $\alpha 2$  macroglobulin, and/or a deficiency of fibrinolytic enzymes in the lesion, or their inhibition by TGF $\beta$ 1. To our knowledge, no studies on the level of these gene products or their inhibitors in the PD plaque have been conducted.

One consequence of fibrin deposition is macrophage recruitment, and these cells, that actively produce elastase,<sup>119</sup> may account for the disruption and decrease of elastic fibers detected immunohistochemically in the same patient population examined for fibrin deposition. However, no studies are available with individual gene assays on the levels of elastin, elastase, collagenase, or metalloproteinase inhibitors in the PD plaque.

*Studies with DNA expression microarrays*

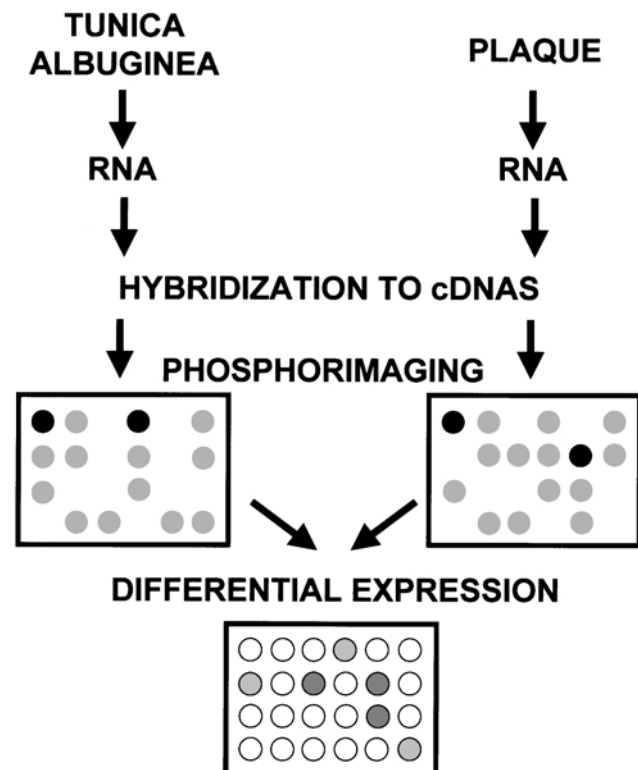
The advent of novel procedures for analyzing simultaneously the expression of multiple (up to 20 000) genes allows the establishment of ‘disease-related profiles’, an achievement that was impossible until recently. Such findings may provide insight into novel approaches to combat tissue fibrosis, and specifically in the progression of the PD plaque. Traditional procedures are limited in that only a few genes can be examined at any one time. This necessitates multiple experiments and the knowledge of which cDNAs are potential candidates to examine in a given disorder.

The DNA microarrays are designed to detect variations in gene expression between two specimens of total or polyA + RNA, by spotting multiple deoxyoligoribonucleotides or cDNA sequences from a catalog of specific genes or ESTs (expressed sequence tags belonging to genes without assigned function), onto membranes or glass chips in an array format. For instance, the Affymetrix Gene-Chip system, using glass chips, can analyze the fluorescent emission levels in one specimen and express them relative to another sample and display the results in an easily visualized fashion, for over 7000 genes.<sup>123–125</sup> The mRNA extracted from the tissue or

cell is labeled with either a radioisotope or fluorescent tag (known as the target) and is hybridized to the probe array. Binding is determined using a phosphorimager or fluorimeter and relative intensity at each spot is a quantitative indication of gene expression. Membrane based arrays are generally less informative (1000 or less spotted cDNAs), due to the limited number of cDNAs that can be spotted onto a membrane (Clontech),<sup>126,127</sup> grouped in families of functional pathways.

See Figure 3 for a diagrammatic representation of both procedures, where the intensity of each spot in one specimen is compared to the other and is represented by colored dots denoting the relative up- or down-regulation.

As an example of their utility to discern fibrosis-related patterns, the temporal change in gene expression of cultured human fibroblasts to stimulation by serum demonstrated that, after examining 8600 genes at eight time points, distinct expression profiles can be detected for genes being regulated in a concerted manner.<sup>122</sup> Nearly 500 genes were found to be differentially regulated, with those related to the onset of cell proliferation such as immediate response genes (c-FOS, JUN B, MAP kinase) and cell cycle genes (PCNA, cyclin D1) being rapidly induced. Gene families involved in wound healing, and therefore pertinent to tissue fibrosis, were up-regulated. Other genes critical for tissue remodeling (elastin), intracellular signalling (TGF $\beta$ 3), vascular



**Figure 3** Peyronie’s plaque DNA microarray.

responses (endothelin I, vascular endothelial growth factor (VEGF)), inflammation (COX2, IL1- $\beta$ ), cytoskeletal reorganization (desmoplakin I), and many unidentified ESTs were induced in coordinated waves in the fibroblasts.

The universal utility of the microarray allows many diverse and relevant hypothesis-driven experimental paradigms to be examined in a coordinate manner. A variety of models, including analysis of colon cancer,<sup>123</sup> aging,<sup>124</sup> formation of multiple sclerosis lesions,<sup>125</sup> muscle wasting,<sup>126</sup> or effects of myostatin on muscle cell differentiation,<sup>127</sup> have been examined with this novel approach. In the case of the urogenital system, they have been employed for defining gene expression in renal cell carcinoma and the adult human renal cortex,<sup>128–130</sup> human prostate, prostatic cell lines and tumors,<sup>131–134</sup> genitourinary inflammation,<sup>135</sup> and other related studies. This will spur a massive effort on identifying profiles of gene expression for each pathological condition, particularly to define the coordinate expression of whole pathways, an impossible task for the pre-gene array procedures. Proteomics,<sup>136</sup> the extrapolation of the microarrays to the detection of protein expression, is the next stage.

Since no studies with this novel approach have been reported on the PD plaque or the Dupuytren's nodules, we applied DNA microarrays to define the differential profile of multiple gene expression in both conditions. For PD, we compared the mRNA levels in the plaque and the TA utilizing both the limited and selective Clontech assay and the ample spectrum Affymetrix procedure. In the first case, polyA + RNA was isolated,<sup>137</sup> and labeled with <sup>32</sup>P[dATP]. A DNA micro-array, consisting of 1176 cDNA probes was used to hybridize to both mRNA populations in separate reactions and the results determined by phosphorimaging and densitometric analysis. In the second case the DNA chip contains probe sets designed to detect individual transcripts which consists of 16–20 probe pairs with one perfect match probe (PM) that is complementary to a specific sequence and a mismatch (MM) probe that serves as a control for cross-hybridization. The biotin-tagged mRNA was hybridized with these microchips and the signal amplified with a fluorescent antibody to biotin. Then a software was applied that consists of an algorithm which analyzes and corrects the hybridization signals for each probe set in the form of absolute values for each control and experimental chips and determines the relative change in abundance for each transcript between both chips. Values were normalized to the expression of housekeeping gene controls (expected to remain constant over a wide variety of conditions), and by global normalization for the expression of all genes in the chip, to compensate for differences in the intensity of hybridization between the control and experimental chips. Only genes whose expres-

sion was changed by a factor of two or greater were considered. Finally, genes which are relevant to the biochemical pathways potentially involved in fibrosis and contracture were tabulated.

In the Clontech assay the expression of many genes was affected, but only eight genes were found up-regulated and four down-regulated with our stringent criteria (1% of the microarray). In the more extensive Affymetrix microchip assay, we found with these criteria that 14 genes were up-regulated and seven down-regulated, which represents 0.35% of this microarray. Our expectation was that the PD plaque would exhibit both fibrosis-related changes in gene expression as well as changes due to tissue remodeling, inflammation, and protein turnover. Our overall results combining both assays indicate that genes involved in collagen metabolism in the plaque, such as collagen I itself or TGF $\beta$ 1 were up-regulated, whereas genes involved in pathways opposing collagen accumulation, such as procollagenase IV, were down-regulated, thus contributing to fibrotic effects. As predicted from the considerable disorganization of elastic fibers in the PD plaque, elastase IIB was considerably up-regulated, and the inflammation chronically present in the lesion was represented by the marked elevation of MCP1 (monocyte chemotactic protein 1). Tissue remodeling and protein turnover were evident by the up-regulation of calpain and HSP28.

The proliferation of fibroblasts and their differentiation into myofibroblasts observed earlier by individual expression assays was corroborated, on one side by the up-regulation of genes involved in cell proliferation (early growth response protein, c-myc; protomyosin  $\alpha$ ), or in the myofibroblast phenotype *per se* (22 kDa smooth muscle protein; ASMA; fibroblast tropomyosin, desmin). Very interestingly, the most highly up-regulated gene found in one calcified PD plaque, OSF-1 (osteoblast factor 1, also known as pleiotrophin or HBNF1), is a secreted heparin-binding protein thought to stimulate mitogenic growth of fibroblasts, neurons, osteoblasts, and other cells.<sup>138,139</sup> It may respond to tissue damage via PDGF and act in fibrosis to increase myofibroblast cell number and possibly contribute to calcification of the plaque since it acts as an osteoblast recruiter. The up-regulation of three of the genes, OSF-1, MCP-1, and ASMA, was confirmed by RT/PCR and Western blot. These profiles indicate myofibroblast abundance, fibrosis, ossification, and inflammation, thereby identifying potential therapeutic targets for the treatment of PD.

The DNA microarrays have also provided insight in the molecular pathophysiology of the related Dupuytren's disease, the fibromatosis of the palmar fascia present in 20% of patients with PD. As stated earlier, Dupuytren's nodules are thought to arise by mechanisms similar to the ones operating in PD, except that in the nodules, the last stage involves the substitution of myofibroblasts by a sparse popula-

tion of fibroblasts, rather than the persistence of myofibroblasts. This may be caused by anti-fibrotic repair processes of different intensity in both disorders. With the Clontech assay, Dupuytren's nodules showed, in comparison to control tissue, the expected up-regulation of collagen mRNA, as in PD.<sup>140</sup> Other genes that were up-regulated, as in PD, were the cell proliferation-related transforming protein rhoA, the 60S ribosomal protein, the protease involved in tissue remodeling, calpain, and the inhibitor of cell adhesion, alpha 1 catenin. However, the wound-healing promoters thymosin  $\beta$ -10 and -4, the anti-fibrotic genes decorin and collagenase IV, and the anti-apoptotic genes, defender against cell-death (DAD-1), and HSP27, were considerably up-regulated in Dupuytren's, whereas they were unchanged or reduced in PD. Therefore, it appears that collagen deposition remains active in both PD plaque and Dupuytren's nodules at the moment of surgical removal, with the Dupuytren's tissue having more intense anti-fibrotic, anti-apoptotic, and remodeling responses.

## Therapeutic implications

The DNA microarrays have made it possible to start defining in molecular terms the complex interplay of biochemical pathways operating in the pathophysiology of PD, in a manner that would have been impossible with the application of conventional assays of individual gene expression. In the near future, custom made DNA microchips or membranes will be produced specifically tailored to PD and fibrosis in general. Protein microchips are technically more difficult, and less amenable for the high through-put analysis applied to RNA, but they are already being applied in pilot studies, combined with highly sophisticated procedures for obtaining small groups of cells of interest in tissue sections, namely laser capture microdissection.<sup>141</sup> Individual gene expression assays (immunohistochemical and RT/PCR, mainly) will always be needed to corroborate and expand the microarray data on some genes, but they will be used to complement specific research goals rather than as wide screening tools. This knowledge will assist in designing new medical therapies to promote the involution of the PD plaque. Treatments will be developed utilizing the existing animal model of PD and its improvements to make it more similar to human PD, as well as derived tissue and cell cultures.

Based on the information discussed, we believe that a therapy for PD based on the biochemical pathways responsible for fibrosis and contracture will be focused on some of the targets listed below. The basic principle is that although the intensification of plaque degradation by manipulation of collagenolysis is the most obvious goal, results to

date suggest that collagen synthesis and tissue remodeling appear to be continue throughout the plaque life. This implies that the blockade of new collagen deposition through removal of pro-fibrotic factors can be an effective therapeutic strategy, since the endogenous collagenolytic and tissue repair mechanisms may then eliminate the existing excessive and/or abnormal fibers. The list of promising therapeutic targets to be investigated may include the following groups (see Figures 4 and 5, for potential targets):

- (a) NO/ROS (nitrosative/oxidative) ratio: the existing studies on antioxidant compounds (vitamin E, superoxide dismutase) to reduce ROS levels should be pursued and expanded with novel antioxidant agents. As an example, S-adenosyl-L-methionine (SAME) to increase glutathione levels, and polyenolphosphatidylcholine (PPC), to reduce lipid peroxydation, are in clinical trials for liver cirrhosis.<sup>142</sup> Our work on the antifibrotic effects of NO suggest that this strategy may be complemented with the stimulation of NO synthesis to destroy ROS (iNOS induction or gene transfer,<sup>148,149</sup> long-acting NO donors, L-arginine).
- (b) TGF $\beta$ 1 action: since this gene appears to be one of the primary effectors of fibrosis and contracture, the use of antisense gene transfer to reduce its synthesis,<sup>143</sup> antagonists to block interaction with its receptors (relaxin, synthetic peptides), neutralizing antibodies, or inhibitors of TGF $\beta$ 1 activation (latency-associated peptide (LAP), inhibitors of tissue transglutaminase), should be explored in PD as in other fibrotic conditions.<sup>54,143</sup> One interesting anti-TGF $\beta$ 1 strategy is based on the use of decorin, already reported as having beneficial effects in the animal model.<sup>144</sup>
- (c) Endothelin/AT2 system: endothelin receptor blockers, angiotensin-converting enzyme (ACE) inhibitors and angiotensin (AT) receptor antagonists,<sup>54</sup> may have effects similar to those reported for kidney and cardiac fibrosis, discussed above.
- (d) Myofibroblast apoptosis and differentiation: the NO-based therapy may induce myofibroblast apoptosis and the disappearance of the cell widely believed to intensify fibrosis and lead to the contracture. For this reason, inhibitors of fibroblast cell growth, such as colchicine,<sup>145</sup> should be tested in conjunction with potential inhibitors of their differentiation, such as INF $\alpha$ , effective for the treatment of keloid scars and inhibiting myofibroblast function, which has been recently applied for PD treatment.<sup>146</sup>
- (e) Collagen catabolism: an obvious target, though collagenase has not been successful.<sup>147,148</sup> New avenues should include promotion of endogenous collagenase expression and blockers of

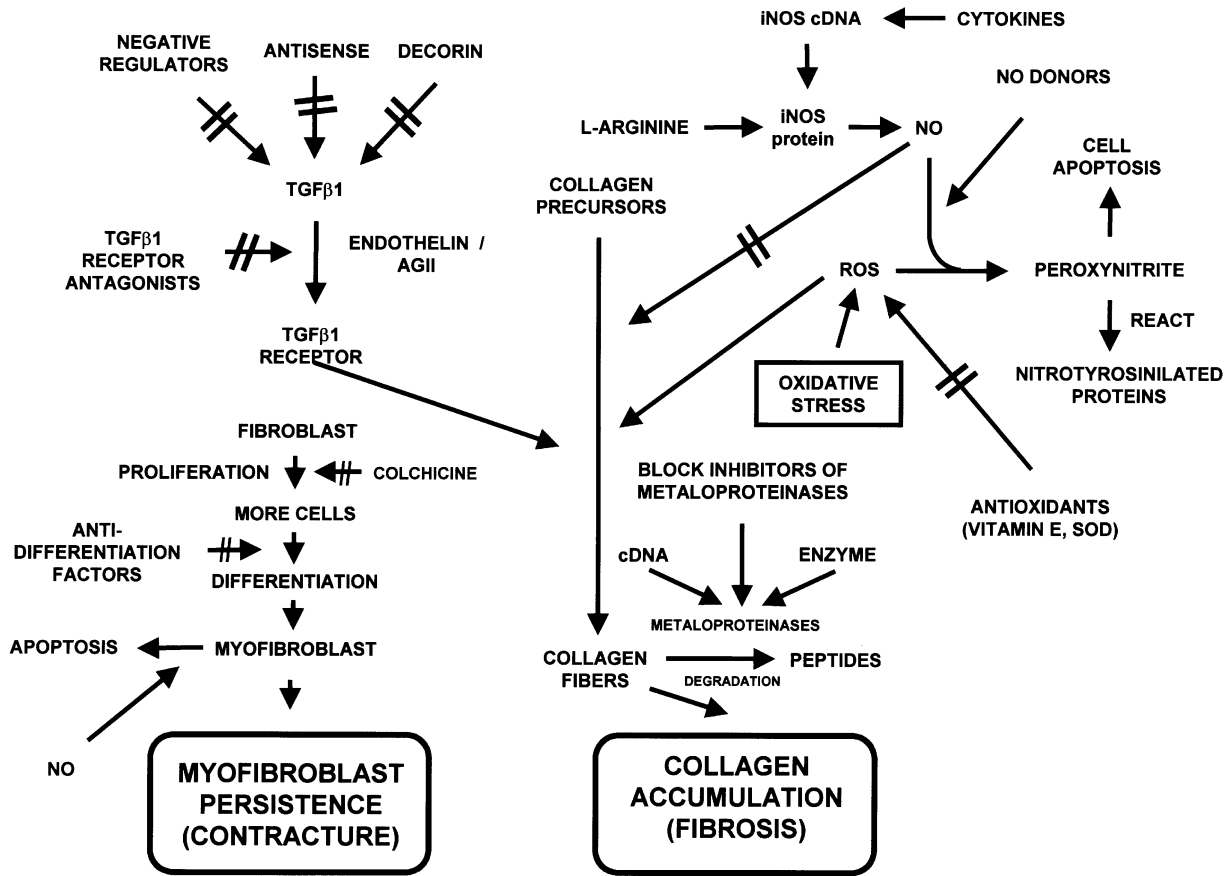


Figure 4 Putative molecular targets for the medical therapy of Peyronie's disease A. Myofibroblast and collagen accumulation.

endogenous metalloproteinase inhibitors,<sup>147</sup> as well as more effective ways to deliver the enzyme (tissue uptake facilitators, electroporation).<sup>148</sup>

(f) Inhibitors of post-translational processing of collagen: this strategy is being actively pursued for other fibrotic conditions, Therapies would include blocking proline hydroxylation, the

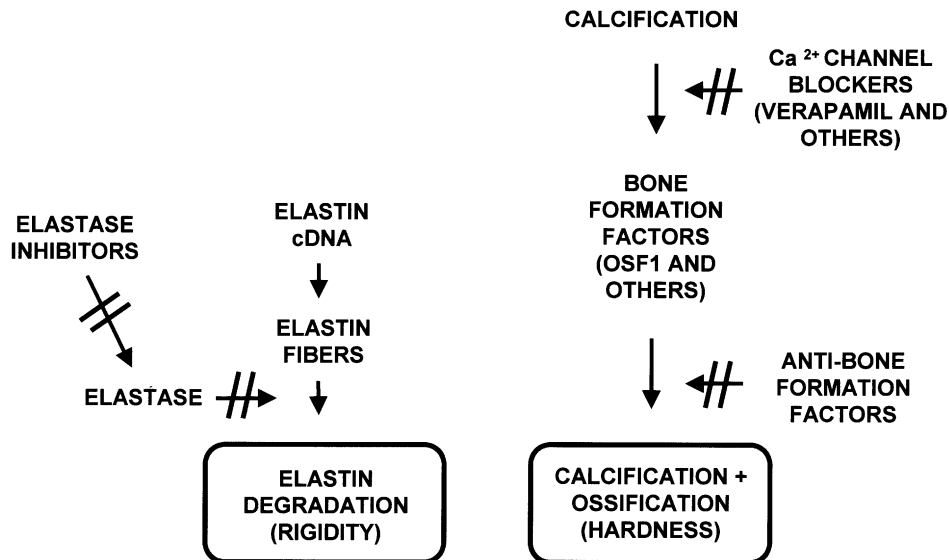


Figure 5 Putative molecular targets for the medical therapy of Peyronie's disease B. Elastin degradation and ossification.

cleavage of C- and N-terminal extension peptides by specific proteinases, and the inhibition of lysyl oxidase catalyzed inter- and intra-chain crosslinking, all steps operating in the deposition of collagenase-resistant fibers in fibrosis.<sup>54</sup> A number of agents are currently in animal and clinical trials.

- (g) Inhibitors of elastin degradation: not yet explored, but possibly elastase inhibitors may be useful, particularly considering that elastin peptides resulting from its degradation may be strong ROS inducers.<sup>150</sup> The integrity of elastic fibers is very important for the function of the TA.
- (h) Calcification and ossification: Calcium channel blockers other than verapamil<sup>151</sup> may be effective, albeit by a mechanism more complex than simply preventing calcification. The considerable elevation of OSF-1 in the ossified PD plaque suggests that the blockade of this or related genes, or of the differentiation of stem cells in the plaque<sup>34</sup> may avoid its irreversible hardening at late stages.
- (i) Ancillary pathways: the promotion of fibrin degradation by conventional fibrinolytic intervention may remove a persistent pro-fibrotic factor; the considerable MCP-1 expression in PD<sup>137,152</sup> suggests that current anti-inflammatory approaches (corticosteroids, antihistamine) should be expanded to include novel non-corticosteroid anti-inflammatory agents. However, this may be counteractive if it blocks iNOS expression and NO synthesis. Thymosin  $\beta$ , currently used for promoting wound healing,<sup>153,154</sup> may have prospects in PD, based on the natural up-regulation of this gene observed in the Dupuytren's nodules.<sup>140</sup>

In summary, the investigation of the expression of multiple genes in the tunica albuginea during the development of the PD plaque, far from being a technically complicated, clinically irrelevant subject, is an important research front in the study of PD. With the help of DNA and protein microarrays, these results will soon be translationally applicable to the care of individual patients and will allow the design and follow-up of novel pharmacological treatments. The knowledge gained in PD may have implications for understanding and treating many other fibrotic and abnormal wound healing disorders.

## Acknowledgements

The experimental studies conducted by our group were funded primarily by a grant from the Eli and Edythe L. Broad Foundation, and supported for some approaches by NIH grants R01DK-53069 and G12RR-03026.

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