January 29, 2001

The Role of Nitric Oxide in the Formation of Keloid and Hypertrophic Lesions

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Abstract

Keloid and hypertrophic scars are a type of scarring pathology which is characterised by excess collagen deposition produced during the wound healing process. The mechanism by which this occurs is not understood and although hypertrophic scars can regress spontaneously, keloids do not, and currently no effective treatment exists. In this paper we hypothesise that nitric oxide, a free radical molecule synthesised by numerous mammalian cells, is involved in the formation of these scars. We suggest that the excess collagen production in keloid lesions can be attributed to higher than normal levels of nitric oxide, as the free radical is a known stimulus for fibroblast collagen synthesis. Furthermore, we propose that the basal epidermis is a source of this additional nitric oxide and we discuss this in relation to known histological characteristics of keloid and hypertrophic lesions.

Running title
Nitric oxide in keloid and hypertrophic lesions

Key words
Nitric oxide, Fibroblasts, Collagen, Keloid lesions, Hypertrophic scars.

HYPOTHESIS

Our hypothesis concerns the scarring pathologies of keloid and hypertrophic lesions. Both these scars are characterised by excess collagen deposition. In keloids this is parallelled by an acellular lesion extending beyond the confines of the original wound [1], and in hypertrophic scars there is a cellular state with the lesion remaining within the boundary of the insult, with eventual scar regression [1]. They are clinically difficult to distinguish and the exact mechanism by which they occur is not understood; currently no effective treatment exists, particularly in the case of keloids, which frequently reoccur following medical intervention. We suggest that the free radical nitric oxide plays a key role in the formation of these scars. Nitric oxide is synthesised by a wide spectrum of dermal cells, and during wound healing it stimulates fibroblasts to produce the extracellular matrix protein, collagen [2]. *In vitro* experiments have shown impaired healing following the application of a nitric oxide inhibitor [2]. Moreover, for both diabetes and protein-calorie malnutrition impaired healing there was a parallelled decrease in nitric oxide synthesis [3, 4]. This feature highlights a potentially important role for nitric oxide in excess collagen synthesis, corroborated by experimental work which demonstrates that transfection of the inducible nitric oxide synthase gene enhances collagen accumulation [5]. Consequently, we hypothesise that keloid scarring is associated with higher than normal nitric oxide secretion rates in the skin. To date there have been no studies which examine such a relation, but a number of features of the nitric oxide molecule and the keloid and hypertrophic lesions have lead us to propose this hypothesis.

Biological interpretation of lesional features in relation to nitric oxide

The basis of our hypothesis concerns the known effects of nitric oxide on collagen production, fibroblast phenotype, and vascular density. One widely reported histological characteristic of keloid scars is the absence of myofibroblasts, a contractile cell which is normally associated with scar formation [6, 7, 8]. Interestingly in the case of hypertrophic scar tissue, these cells are more abundant than normal. Studies have demonstrated that nitric oxide inhibits the transition of fibroblast to contractile fibroblast [2], whereas

Desmouliere *et al.* [9] have shown that the cytokine transforming growth factor-β (TGF-β), which also stimulates collagen production by fibroblasts, is a potent inducer of the myofibroblast phenotype. This suggests that the balance between TGF-β and nitric oxide could have much importance [8]. With regard to the two scarring pathologies, fibroblasts in keloid lesions have been shown to produce the same amount of TGF-β as normal fibroblasts [10], whereas hypertrophic fibroblasts exhibit an increased sensitivity towards the cytokine [10] and produce a reduced amount of nitric oxide in comparison to normal wound fibroblasts [2]. This leads us to hypothesise that a high nitric oxide level in keloidal tissue is blocking the phenotypic change of fibroblast to myofibroblast. To support this further, *in vitro* studies demonstrate that keloid fibroblasts do have the ability to express the α-smooth muscle actin characteristic of myofibroblasts [8], despite the fact that they do not express it *in vivo*. It has been suggested that this suppression is due to local microenvironmental factors found *in vivo* [8], we postulate that high levels of nitric oxide is a key component of this microenvironment. Moreover, it has been found that keloid tissue does not survive transplantation [11], supporting the notion that keloids are a local phenomenon.

Given the apparent regulation between TGF-β and nitric oxide, the absence of myofibroblasts in keloid lesions suggest the presence of a high nitric oxide concentration which can in turn account for the observed excess collagen deposition. The delayed appearance of these scars can also be linked to the TGF-β/nitric oxide system. TGF-β has been widely reported to down regulate nitric oxide. However, the transient nature of this cytokine, coupled with the fact that nitric oxide is present until healing is complete [2], allows for the possibility of a build up to a high nitric oxide concentration. In the case of hypertrophic scars the high number of contractile cells present suggests that the increased collagen is a consequence of the heightened sensitivity of hypertrophic fibroblasts towards TGF-β.

There has been little experimental work examining the relationship between nitric oxide and the degradation processes associated to collagenase activity. However, Murrell *et al.* [12] have demonstrated that nitric oxide activates human articular cartilage cells to upregulate collagenase. There has been much conflicting work concerning collagenase

levels in keloids, but the consensus leans towards studies which show collagenase activity in keloids is increased [13, 14] or normal [15, 16], but not diminished. Thus this coincides with our hypothesis of elevated nitric oxide in such lesions. Furthermore, with regard to hypertrophic lesions, several studies have provided evidence of reduced levels of collagenase [17, 18]. This could be linked to TGF-β activity as it has been found to down regulate collagenase expression.

In addition to the features of varied cell phenotype and excess collagen, both keloid and hypertrophic lesions exhibit a high vascularity, although the microvessels themselves are partially or fully occluded [8, 19]. This blood vessel occlusion has been associated with excess endothelial cell proliferation [20, 21, 22], and our hypothesis is also able to account for this feature. Recent studies have revealed that increased levels of nitric oxide can indirectly result in endothelial cell proliferation [23, 24, 25, 26, 27] . For example, *in vitro* experiments by Lee and co-works [24] have demonstrated that the nitric oxide synthesising enzyme, endothelial nitric oxide synthase (eNOS) is required for endothelial cell proliferation. Moreover Ziche *et al.* [25] have linked nitric oxide with the growth-promoting effects of vascular endothelial growth factor (VEGF). The combined results of these studies strongly suggests a link between nitric oxide and the excess endothelial cell proliferation observed in keloids, where higher levels of nitric oxide may result in this blood vessel occlusion via growth factor upregulation. The occlusion in hypertrophic scars may also be understood by relating it to the sensitivity of fibroblasts to TGF-β [28], a growth factor frequently associated with angiogenesis, albeit indirectly. Lowered nitric oxide production by hypertrophic fibroblasts leads to a reduced suppression of the effects of TGF-β in the early stages of wound healing [29]; one consequence of this will be increased endothelial cell proliferation.

Mathematical modelling to investigate the hypothesis

In addition to the biological findings which lend support to our argument, we have also devised a mathematical model to test the hypothesis [30]. The model provided a quantitative approach to examining our hypothesis, via the incorporation into our model of many of the key biological interactions involved in wound healing. Such an approach

allows us to overcome the problem of an absence of animal models which display keloid features. We can also avoid the problems associated with *in vitro* culturing of cells. The numerical simulations of our model, illustrated in Figure 1 compare normal, hypertrophic and keloid scarring. The profiles in column 1 demonstrate that including an increased background production of nitric oxide resulted in an acellular scar with a high collagen density together with a hypoxic wound environment, all characteristics of keloid lesions. The acellular nature of these lesions arose as a consequence of high collagen density limiting cell proliferation [31], where the production of this structural protein was stimulated by the nitric oxide.

The spontaneous regression characteristically observed in hypertrophic scars was also demonstrated by the model. By incorporating a high collagen level into the initial conditions which reflects the fibroblast sensitivity to TGF-β, we saw that once this stimulus had ceased the presence of lower nitric oxide levels resulted in the gradual regression of the scar to a collagen density similar to normal scar tissue, as observed clinically. We also investigated the effects of different keloid treatments, as illustrated in column 2 of figure **??**. We observe that by allowing the system to first evolve to a keloid equilibrium and then simulate surgical excision via decreasing collagen density, the keloid scar reestablishes. If however, in conjunction with surgery, a nitric oxide inhibitor is applied, achieved by reducing the background nitric oxide production, we see keloid recovery and a normal scar is established. The theoretical model which was used to generate these results is presented in the appendix.

Source of the proposed excess nitric oxide

To add completeness to our argument we also have to consider the source of the hypothesised additional nitric oxide that we suggest is present in keloids. A spectrum of information suggests that the basal epidermis is a likely candidate. We explain this by considering one of the major clinical characteristics of this scar, namely that the majority of keloids are found to occur in deeply pigmented skin [32]. There is also a higher incidence of keloids in people exhibiting hyperactivity of the pituitary gland [33], a physiological state associated with increased pigmentation. Furthermore, the lesions predominantly

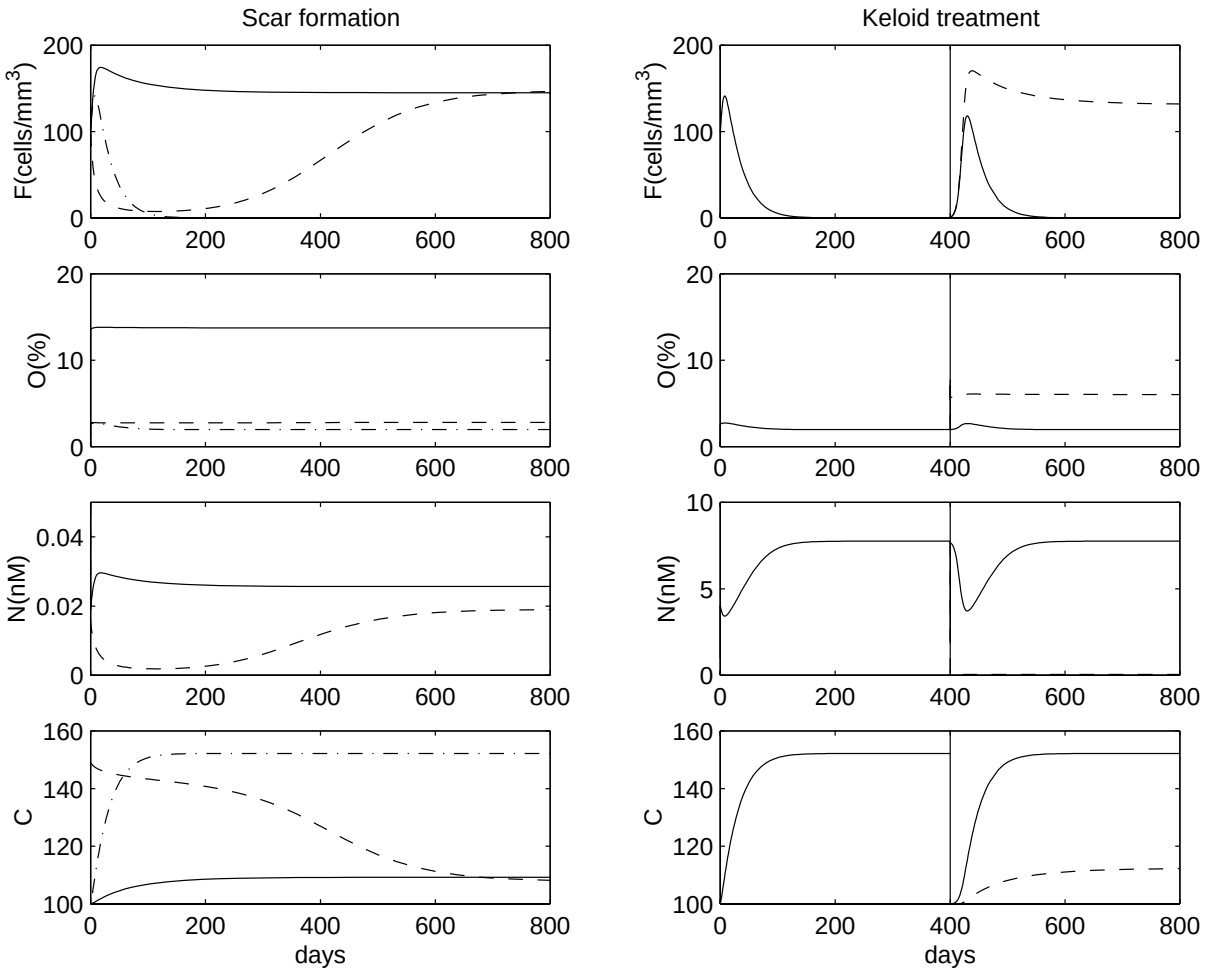

Figure 1: Numerical simulations of the model illustrating the temporal evolution of fibroblasts, $F(t)$, oxygen, $O(t)$, nitric oxide, $N(t)$ and collagen, $C(t)$ densities. The left column demonstrates the three types of scar formation, normal scar tissue, (—), hypertrophic (- -) and keloidal (-.-). As a reference the unwounded dermis is $C(t) \sim 100$ on the above scale. The second column presents the results of two types of keloid treatment, applied at 400 days post-wounding. The solid line (—) illustrates surgical excision. We note that the scar reoccurs and the keloid persists. The dashed line (- -) represents surgical excision combined with a nitric oxide inhibitor. The keloid does not reoccur and the skin evolves to normal scar tissue. (Parameters are discussed in the appendix).

arise in areas of the body where melanocyte concentration is highest [19]; for example, keloids are rarely found on the palms of the hands where melanocyte level is minimal [33]. Variations in pigmentation are due not to differences in the number of melanocytes, but rather to differences in the levels of the pigmentation chemical, melanin, and the way it is dispersed [33, 34]. This is related to nitric oxide, as the free radical activates tyrosinase, one of the main enzymes responsible for the biosynthesis of melanin [35]. It suggests that we would expect to observe higher levels of nitric oxide associated with darker skin pigmentation, to account for the extra melanin observed.

When a wound occurs the melanocytes in that region are destroyed and are not regenerable [36], however the keratinocytes which produce the nitric oxide used in melanin synthesis do regenerate [37]. Hence, postwounding we have additional free nitric oxide which we hypothesise is in excess in keloids. Another factor which can account for enhanced levels of nitric oxide arises from the fact that melanin absorbs UV radiation [38]. The absence of melanin in the wound space implies that there is additional UVB stimulus for keratinocyte synthesis of nitric oxide [37]. Furthermore, a number of studies have associated a thickened epidermis with keloid scars which is stretched as the scar grows [8, 19]. This implies the possible existence of an increased keratinocyte population. Not only would this offer additional cellular sources of nitric oxide, but also the increased cell numbers could be attributed to the nitric oxide as it stimulates the proliferation of keratinocytes [39].

In conclusion we predict that in individuals predisposed to keloid lesions, the natural nitric oxide levels are relatively high, and the release of these quite significant additional quantities of the free radical can be sufficient to produce a high enough concentration of nitric oxide to provide a stimulus for collagen synthesis in the neighbouring dermis below. This could then lead to an elevated excess scar characteristic of keloids.

Implications for treatment

To date there is no entirely effective treatment for keloid lesions, however, one of the more successful existing therapies involves excision combined with intralesional administration of corticosteroids. This has a high response rate, although recurrence is still common [40].

We can interpret the effectiveness of this treatment in terms of our hypothesis because corticosteroids are known to suppress inducible nitric oxide synthase (iNOS) [41], one of the enzymes which synthesise nitric oxide. Thus the therapy partially inhibits nitric oxide production and in some mild cases of keloids this could be sufficient to prevent recurrence. However, we can account for the absence of complete success of corticosteroids by noting that they do not inhibit constitutive nitric oxide synthase (cNOS) [41] and it is this enzyme which is involved in keratinocyte production of nitric oxide. Since we postulate that keratinocytes are the principal source of the excess nitric oxide which we predict is present in keloids, it follows that corticosteroids would only have a limited inhibitory effect on nitric oxide production. Instead, our hypothesis suggests that the administration of a more general nitric oxide inhibitor such as L-NMMA would be more effective. Studies by Schaffer *et al.* [42] reveal that the administration of such nitric oxide inhibitors would be best applied via a constant intraperitoneal infusion, as the short biological half life of NOS inhibitors prevent oral administration and daily intraperitoneal injection being as effective. In their animal studies 100 mg/kg body weight/day of MITU was well tolerated and reduced hydroxyproline content by 33%.

Our hypothesis does not put forward a treatment strategy for hypertrophic lesions; however, it may be possible to obtain an effective method for differentiating between keloid and hypertrophic scars, which to date has proven to be clinically very difficult. By preventing misdiagnosis, we can avoid such events as the development of a new growth of scar tissue which can occur if tension is applied following hypertrophic regression.

Testing the hypothesis

In order to test the hypothesis we propose a comparative clinical study in which nitric oxide levels are measured directly from the skin surface of keloid lesions, normal skin and from intermediate scar types exhibiting characteristics of both keloid and hypertrophic scar tissue. Our hypothesis implies the results of such a study would demonstrate elevated levels of nitric oxide in keloid lesions in comparison to normal skin. This can then be used as a guide to dosage levels of a nitric oxide inhibitor which could, in the long term, be a possible treatment strategy to be applied along with excision therapy. There is

already in existence a viable non-intrusive method for the measurement of skin nitric oxide secretion, which has already been applied to measure the nitric oxide production from psoriatic plaques [43]. Thus, this offers a viable means of testing the hypothesis of increased nitric oxide levels associated to keloid scarring.

Acknowledgements. CAC was supported by a studentship from the Engineering and Physical Sciences Research Council. We thank Prof. Jonathan Sherratt (Centre for Theoretical Modelling in Medicine, Heriot-Watt University, Edinburgh) for his helpful suggestions.

A Theoretical model

Our theoretical model of the wound healing process focuses on the remodelling stage of repair, with a time scale starting approximately 20 days post-wounding. We include only the key components of the system: Fibroblast cell density, $F(t)$ (cells mm^{-1}); collagen density, $C(t)$ ($\times 5.4\mu$g mg^{-1} dry weight); nitric oxide concentration, $N(t)$ (nM) and oxygen, $O(t)$ (%), and we use a time scale, t of days. The equations below describe the effect different biochemical and cellular interactions have on the rate of change of each of our variables. The terms are loosely based on the ideas of mass action.

$$\boxed{\text{Fibroblasts:}} \quad \frac{dF}{dt} = \overbrace{F(1-Fk_1-Ck_2)r_1}^{\text{cell mitosis with growth restricted by collagen density}} - \overbrace{d_1F}^{\text{natural decay}} \times \overbrace{q(O)}^{\text{effect of oxygen levels on the cell cycle}} \tag{1}$$

$$\boxed{\text{Collagen:}} \quad \frac{dC}{dt} = \overbrace{h(C)g(N)F}^{\text{production by NO stimulated fibroblasts}} - \overbrace{d_2CF}^{\text{degradation}} \tag{2}$$

$$\boxed{\text{Nitric Oxide:}} \quad \frac{dN}{dt} = \overbrace{Ff_0}^{\text{prod}^n \text{ by fibroblasts}} - \overbrace{d_7NO}^{\text{reaction with blood vessels}} - \overbrace{d_5N}^{\text{natural decay}} - \overbrace{d_6FN}^{\text{react}^n \text{ with fibroblasts}} - \overbrace{k_7Nv(1+s(N))}^{\text{NO react}^n \text{ leading to blood vessel dilat}^n} + \overbrace{K}^{\text{background production}} \tag{3}$$

$$\boxed{\text{Oxygen:}} \quad \frac{dO}{dt} = \overbrace{k_4v}^{\text{oxygen produced by vasculature}} \times \overbrace{(1+s(N))}^{\text{NO stimulated dilation}} - \overbrace{d_3NO}^{\text{react}^n \text{ with Nitric Oxide}} - \overbrace{d_4O}^{\text{natural decay}} \tag{4}$$

The details of the model derivation and explicit functional forms are discussed in Cobbold *et al.* [30].

The initial conditions which were used to generate normal scarring in Figure 1 are as follows: $F(0) = F_0 = 100$ cells/mm^3, $C(0) = C_0 = 100$ $\times 5.4\mu g$/mg dry weight, $N(0) = N_0 = 1$ nM and $O(0) = O_0 = 0$ %. The parameter values used are given as: $r_1 = 0.92, d_1 = 0.12, d_2 = 0.000075, d_3 = 800, d_4 = 3800, d_5 = 500, d_6 = 20, d_7 = 800, k_1 = 0.0004, k_2 = 0.006, k_3 = 0.5, k_4 = 5280, k_5 = 4, k_6 = 1.2, k_7 = 185, v = 7, a_1 = 1/300, m = 20, f_0 = 2.79, K = 0$. These values are discussed further in Cobbold *et al.* [30]. Parameters for hypertrophic lesions are the same as those used for normal scarring with the exception of: $v = 1, f_0 = 0.79$, reflecting blood vessel occlusion and decreased fibroblast production of nitric oxide, respectively. Initial conditions remain the same except for: $C(0) = C_0 = 149$ ($\times 5.4\mu$g/mg dry weight), representing the early increased sensitivy to TGF-β. Similarly for keloids, parameters and initial conditions are as in normal scarring

except for: $v = 1, K = 20000$, again reflecting blood vessel occlusion and also the increased background nitric oxide production.

A further observation which arose from this model was a possible explanation for Chu *et al.* [29] not detecting significantly increased collagen production in hypertrophic scars. They studied developed scars of 9 mths to 2 yrs old, which as we can see from the profiles in Figure 1, by this stage often collagen has begun to regress accounting for the absence of increased collagen production. This time scale of 1-2 years has frequently been reported as a time at which regression begins to occur.

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