Oxygen Free Radicals and Dupuytren's Disease

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Introduction

Dupuytren's contracture is associated with increasing age [1], sex (M > F) [1], diabetes mellitus [2,3], heavy alcohol consumptior [4], HIV infection [5], cigarette smoking [6], epilepsy [7] and Colles' fractures [8], but rarely with rheumatoid arthritis [9]. Two important features of the palmar fascia of Dupuytren's contracture are an increase in the number of fibroblasts [10], and an increase in the relative amounts of type III collagen [11]. It is likely that these phenomena are associated, as fibroblasts cultured at high density decrease type I collagen production and thus increase the relative amounts of type III collagen [12]. Changes in the glycosaminoglycan content can also be explained by high cell density [13].
The question remains: what are the stimuli for fibroblast proliferation? Evidence for localized ischemia [14] (van Lacken and Gropper, in preparation) and preliminary clinical results indicating that allopurinol may improve Dupuytren's contracture [15,16] suggest that xanthine oxidase-catalyzed free radical release may be important in the pathogenesis of Dupuytren's contracture.

What Is a Free Radical?

A free radical is any group of atoms capable of independent existence that contain one or more unpaired electrons (an unpaired electron is one that occupies an atomic or molecular orbital by itself). The presence of one or more unpaired electrons causes the species to be attracted slightly to a magnetic field and sometimes makes the species highly reactive.
Consideration of the above broad definition shows that there are many free radicals in chemistry and biology. Free radicals may be formed by radiolysis (decomposition of a chemical compound by the action of ionizing radiation), photolysis (decomposition of a chemical compound by the action of radiant energy from light), homolysis (splitting of a group of atoms into one or more groups of atoms), and during oxygen reduction reactions. They have half lives in the order of milliseconds. An important principle of free radical chemistry is

that the reaction of a free radical with a nonradical species produces a different free radical, which may be more or less reactive than the original radical. Reactivity depends on availability of reaction pathways as well as the "intrinsic" reactivity of the free radical species.

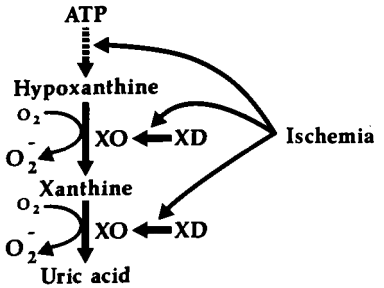
Oxygen Free Radicals

Oxygen as it occurs naturally has two unpaired electrons and hence qualifies as a radical. Oxygen is a good oxidizing agent, i.e., good at absorbing electrons from the molecule it oxidizes. If a single electron is added to the ground state O_2 molecule the product is the superoxide radical (O_2^-). Addition of a further electron will given O_2^{2-}, the peroxide anion (with no unpaired electrons), which readily becomes hydrogen peroxide (H_2O_2). Although H_2O_2 is not a free radical, it is an integral part of the free radical cascade and for this reason is often classified with free radical species. Traces of the transition metal ions Fe^{2+} and Cu^{2+} can catalyze the reaction of O_2^- with H_2O_2 to form the hydroxyl radical ($OH^{\cdot}$) (via the Haber-Weiss reaction).

Free Radicals in Medicine

Oxygen free radicals are becoming recognized as being important in more and more physiological and pathological processes. The roles for oxygen free radicals in the bactericidal activities of phagocytic cells and in mediating tissue damage after acute ischemia are now well established [17,18]. During ischemia the purine bases xanthine and hypoxanthine accumulate and endothelial xanthine dehydrogenase is converted to xanthine oxidase (Fig. 1). The purine base-xanthine oxidase reaction releases superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which in high concentrations are toxic to tissues and cultured cells.

To determine if free radicals could be important in the pathogenesis of Dupuytren's contracture we measured the concentration of substrates able to react with exogenous xanthine oxidase to produce O_2^- in Dupuytren's and control palmar fascia. These substrates are most likely to be hypoxanthine and xanthine; for clarity they are expressed as hypoxanthine concentrations. A sixfold increase in hypoxanthine was found in Dupuytren's palmar fascia compared with control palmar fascia (Fig. 2). In a single large piece of Dupuytren's tissue examined and sectioned, the hypoxanthine concentration increased with cell density and was two times greater in tissue classified as "nodule" 0.32 (SE 0.03) than in "cord" 0.14 (SE 0.03) μmol hypoxanthine/g wet weight; ($p < 0.005$). The mean xanthine oxidase activity in Dupuytren's contracture tissue from six patients was 13 (SE 6.1); range (1–41) mU/g wet weight. Samples of normal palmar fascia from control patients were too small for the xanthine oxidase activity assay [19].



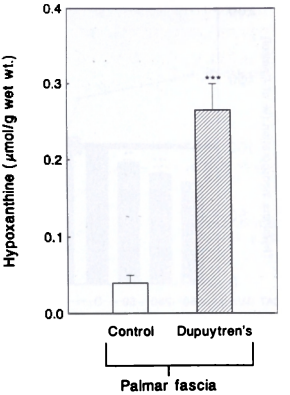


Fig. 1 *(left)*. The mechanism for ischemia-induced free radical damage. *ATP*, adenosine triphosphate; *XO*, xanthine oxidase, *XD*, xanthine dehydrogenase

Fig. 2 *(right)*. Hypoxanthine concentrations in control and Dupuytren's palmar fascia. n = 10 for both groups; mean (SEM), $***p < 0.001$

Oxygen Free Radicals Stimulate Fibroblast Proliferation

The effects of oxygen free radicals on cultured human fibroblasts were then examined. Passage 3–5 fibroblasts from skin biopsies or operative Dupuytren's contracture palmar fascia specimens were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum. Each well of a 1.6 cm 24 multiwell tissue culture plate was seeded with 4×10^4 fibroblasts and cultured for 48 h (near confluence). The medium was then replaced with 1.0 ml of media containing the agents to be tested. In thymidine incorporation experiments, this media also contained 1.0 μCi [^{3}H]thymidine and carrier thymidine to a final concentration of 5.0 μ*M* thymidine. After 4 h of incubation at 37°C the cell layer was harvested, processed, and the radioactive, acid insoluble fraction measured by liquid scintillation spectrometry to give an estimation of the rate of cell proliferation. Cell density was determined using a 1 mm^2 eyepiece graticule at 6 and 24 h. Cell morphology parameters were calculated at 4 h using a Zeiss modulator system for quantitative digital image analysis (MOP AM02) [20].

Oxygen free radicals were generated by three systems (xanthine oxidase and hypoxanthine, glyceraldehyde in phosphate buffered saline and H_2O_2). Oxygen free radicals in high concentrations ($>10^{-3}$ U/ml xanthine oxidase with 10^{-3} *M* hypoxanthine; $>10^{-4}$ *M* H_2O_2; $>10^{-3}$ *M* glyceraldehyde) visibly damaged cultured human fibroblasts, reduced cell density and inhibited thymidine incorporation. In contrast, lower concentrations of free radicals (10^{-4}–10^{-7} U/ml xanthine oxidase; 10^{-4}–10^{-6} *M* glyceraldehyde; 10^{-6} *M* H_2O_2) stimulated

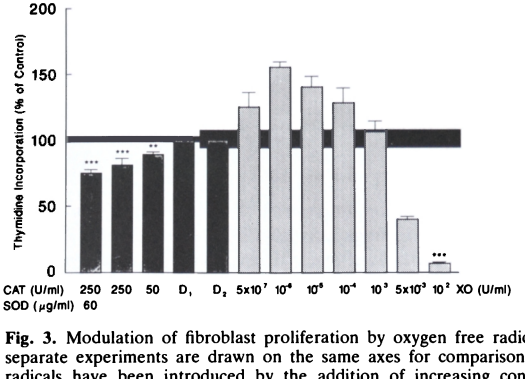


Fig. 3. Modulation of fibroblast proliferation by oxygen free radicals. The results of two separate experiments are drawn on the same axes for comparison. *Right*, exogenous free radicals have been introduced by the addition of increasing concentrations of xanthine oxidase (*XO*) to media containing 10^{-3} *M* hypoxanthine. Control (D_2), 10^{-2} U/ml xanthine oxidase heat denatured at 100°C for 15 min. Mean thymidine incorporated in control group was 13 pmol/10^6 cells. *Left*, no exogenous free radicals have been added and endogenous O_2^- or H_2O_2 has been scavenged by superoxide dismutase (*SOD*) and catalase (*CAT*), respectively. Control (D_1), 250 U/ml heat denatured CAT and 60 μg/ml heat denatured SOD. Mean thymidine incorporated in control group was 53 pmol/10^6 cells. Expressed as mean ± SEM % of control; $n = 6$. *Significantly different from control (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ by two-tailed Students's t-test). (From [20])

thymidine incorporation and increased mean cell area, maximum length and cell density (Fig. 3). Both the stimulatory and inhibitory effects of xanthine oxidase were inhibited if xanthine oxidase was heat inactivated for 10 min at 100°C or 10^{-4} *M* allopurinol (a competitive inhibitor of xanthine oxidase) was added, or O_2^- and H_2O_2 scavengers added (60 μg/ml superoxide dismutase and 250 U/ml catalase) [20]. The results were similar for each of the cell lines, and have been confirmed by other authors in other transformed and nontransformed fibroblast cell lines [21].

Superoxide Release by Cultured Fibroblasts

O_2^- release was estimated using the superoxide dismutase inhibitable reduction of cytochrome c [22,23]. For each parameter assessed half of the wells were incubated with 60 μg/ml superoxide dismutase and half without. The final volume of the reaction mixture was 1.0 ml. After incubation at 37°C for 80 min without agitation, reactions were terminated by addition of 1.0 ml of 2 m*M* N-ethylmaleimide. The amount of O_2^- release was determined by dividing the average difference in absorbance at 550 nm in samples cultured with and without superoxide dismutase by the extinction coefficient for reduction of cytochrome c.

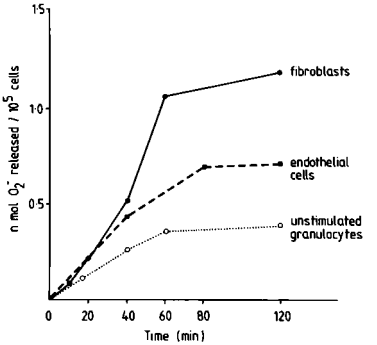


Fig. 4. Time course of O_2^- release by cultured fibroblasts compared with endothelial cells and unstimulated granulocytes. Each point is the mean of a triplicate incubation. (Adapted from [20])

In this system there was a superoxide dismutase inhibitable reduction in cytochrome c that increased with time and cell density. The shape of the O_2^- release vs fibroblast seeding density curve was similar to that previously observed for stimulated granulocytes[23], while the time course of O_2^- release by cultured fibroblasts was similar in form and magnitude to both unstimulated granulocytes and endothelial cells [20,23] (Fig. 4). Superoxide release doubled when cultured fibroblasts were agitated at 2 cycles/s. It is important to note that the stimulatory effect of phorbol myristate acetate (PMA) on O_2^- release by fibroblasts was 10- to 15-fold less than that observed in hagocytic cells. The lower magnitude of O_2^- release compared with phagocytes is in keeping with its role in connective tissues. The source of O_2^- release by cultured fibroblasts is likely to be a plasma membrane bound NADPH oxidase [24]. Xanthine oxidase catalyzed reactions were not a significant source of O_2^-, as addition of $10^{-4}\,M$ allopurinol in the presence of $10^{-4}\,M$ hypoxanthine did not alter O_2^- release.

Modulation of Fibroblast Proliferation by Oxygen Free Radicals

The rate of O_2^- release by cultured fibroblasts was equivalent to that which stimulated fibroblast proliferation, while the rate of O_2^- release by phagocytic cells was equivalent to that which damaged and inhibited fibroblast proliferation. Furthermore, fibroblast proliferation could be reduced by inhibiting endogenous free radical release (Fig. 3). These and other observations of inhibition of fibroblast chemotaxis with free radical scavengers [25] suggest that oxygen free radicals may have the ability to modulate fibroblast proliferation and chemotaxis.

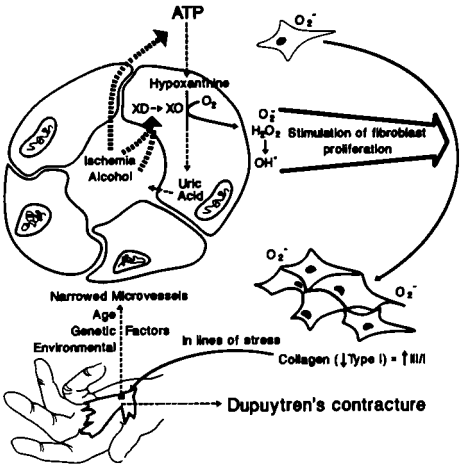


Fig. 5. Hypothesis for Dupuytren's contracture. (Adapted from [10])

Hypothesis

These biochemical and morphological observations have thrown new light on Dupuytren's contracture. They suggest that a number of factors (genetic, age, sex, race) may lead to the characteristic thickening of endothelial cells, lamination of the basal lamina and narrowing of the lumen of microvessels in the palmar fascia. Localized ischemia and alcohol cause adenosine triphosphate (ATP) breakdown to the purine bases, hypoxanthine and xanthine, and the conversion of xanthine dehydrogenase to xanthine oxidase with resultant oxygen free radical (O_2^-, $OH^\cdot$) and H_2O_2 release.

HIV infection and cigarette smoking are also associated with oxidant stress. Oxygen free radicals may damage pericytes and lead to pericyte regeneration, with consequent further layering of basal laminae, and fibroblast proliferation. Oxygen free radicals released by the fibroblasts themselves may also further stimulate fibroblast proliferation. The collage produced by fibroblasts in areas of high cell density (nodules) is initially highly disorganized and relatively depleted of type I collagen but gradually becomes aligned in lines of stress resulting in the characteristic fibrous "cords" that are palpated beneath the palmar skin and extend into the fingers of patients with Dupuytren's contracture. Progressive fibroblast proliferation and collagen deposition may further compromise microvessels with a positive feedback effect consistent with the progressive nature of the condition (Fig. 5).

Clinical Application

The above scenario suggests that agents which decrease oxygen free radical release may inhibit or prevent Dupuytren's contracture. Allopurinol (a competitive xanthine oxidase inhibitor) has shown early promise in mild contractures, while a group of patients who are likely to consume large amounts of steroidal and nonsteroidal anti-inflammatory agents (patients with rheumatoid arthritis) have a very low prevalence of Dupuytren's contracture [9].

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