MECHANICAL STRESS IN VITRO INDUCES INCREASED EXPRESSION OF MMPs 2 AND 9 IN EXCISED DUPUYTREN'S DISEASE TISSUE

J. F. TARLTON, P. MEAGHER, R. A. BROWN, D. A. McGROUTHER, A. J. BAILEY and A. AFOKE

From the Collagen Research Group, University of Bristol, the Department of Plastic Surgery, University College, London and the Department of Technology and Design, University of Westminster, London, UK

We have previously shown that the ability to mechanically extend Dupuytren's contractures in vivo by the Continuous Elongation Technique before surgery resulted in increased metalloproteinase activity. However, under these conditions it was not possible to show whether the response was proportional to the mechanical stimulus or was inflammatory cell mediated. Using an in vitro system of controlled extensions in which inflammatory involvement is absent, we have now shown that there is a clear correlation between the load applied to the tissue and the release of matrix metalloproteinase-2. The subsequent degradation of the collagen results in a loss of mechanical strength reported in the preceding paper.
Journal of Hand Surgery (British and European Volume, 1998) 23B: 3: 297–302

Dupuytren's disease is characterized by progressive and reversible deposition of excess fibrous collagen resulting in flexion of the fingers. The clinical management and pathology have been reviewed (Berger et al, 1994; MacFarlane et al, 1990) and the particular role of collagen described (Bailey, 1994).

Dupuytren's contracture has been shown to be slowly extensible in vivo when subjected to traction (Messina and Messina, 1991). This continuous elongation technique in which the fingers are slowly straightened on an external adjustable frame over a period of between 2 to 3 weeks was developed for patients with such severe contractures of the fingers that amputation was the only alternative. Since collagen fibres are virtually inextensible the ability to stretch these long term contractures, and the gradual reduction in resistance to the applied tension over time, indicated a change in the properties of the collagen. We reasoned that the response of the fibroblasts in the Dupuytren's tissue to the tension exerted was likely to be an increase in the degradative protease levels, and to produce some from of depolymerization thus weakening the collagen fibres. Indeed, we have demonstrated increased levels of interstitial collagenase (MMP-1), gelatinases A and B (MMPs 2 and 9) and the acid cathepsins B and L in tissue removed at fasciectomy following 2 to 3 weeks of the continuous elongation technique (Bailey et al, 1994). As might be expected there was considerable variation between subjects, and the unit force exerted on the tissue was not controllable and therefore highly variable. We were unable to relate directly the load exerted to the changes in enzyme levels, and, more importantly, to demonstrate any direct dependence on mechanical loading.

An alternative to the biomechanical thesis for the observed changes might be that the stretching resulted in damage to the tissue, inducing inflammatory infiltration by neutrophils, macrophages and monocytes, characterized by elevated levels of MMPs (Tarlton et al, 1997). Against this was the absence of any apparent tissue rupture and haemorrhage.

To resolve these questions we have investigated the effect of different levels of a known constant load on excised Dupuytren's tissue in vitro, eliminating the potential for cellular infiltrations. Any observed changes would therefore be due solely to biomechanical influence on the tissue and its cells.

MATERIALS AND METHODS

Tissue preparation

Tissue samples were removed from 21 patients with Dupuytren's disease at fasciectomy. The age range of the patients was 55 to 74 years with an average age of 62 years. The duration of the disease varied from 9 months to 30 years. Tissue was stored in saline moistened sterile swabs at 4°C for a maximum of 24 h until tested. Fatty tissue was removed and, using a specially constructed knife; at least two strips were cut from each sample with the long axis along the predominant fibre disposition and oriented as in the palma fascia. The thickness of the strips was 1.5 mm and the width was determined using a Nikon profile projector with 20 × magnification.

Mechanical loading

The tissue was subjected to defined creep loading in a custom built tensiometer which simulated the loading regime found in the continuous elongation technique of Messina and Messina (1991). A detailed description is given in the preceding paper (Afoke et al, 1998). Essentially strips of tissue, Dupuytren's cord or nodule, cut accurately on a template were fitted to the loading apparatus, oriented as in the palma fascia, by clamping the lower end to a fixed position, and the upper to the proximal end of a pivoting bar; all tissue crushed within the clamp was later discarded. To the distal end of the pivot were added predetermined weights corresponding to stresses of 0.25 MPa, 0.5 MPa and 0.75 MPa on the

specimen, and any creep was monitored using a linear displacement gauge. The entire tissue specimen was immersed in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) supplemented with penicillin (500 U/ml) and streptomycin (500 µg/ml) and maintained at 37°C in a humidified atmosphere for 18 hours. Control samples from each patient were maintained in culture as for the stretched tissue. Specimens were snap frozen in liquid nitrogen upon completion of the loading procedures, and stored at –20°C before biochemical analyses.

In order to evaluate the potential for bias in selecting tissue for cellular nodule or less cellular cord before mounting to the apparatus, a specimen was selected for loading from each patient, along with control tissue, and each was maintained at 4°C but otherwise treated as previously. Under these conditions, minimal de novo synthesis of MMPs is to be expected, and therefore differences in measured expression are likely to represent the nature of the selected tissue rather than biochemical changes. Such differences would potentially represent disproportionate amounts of cord and nodule in the specimens, which may not be apparent by direct visual examination. Any such variation in characteristics may be used to correct inadvertent bias in specimen selection.

Extraction of enzymes

Frozen tissue specimens were pulverised to a fine powder using a micro-dismembrator, each having been precooled using liquid nitrogen. Powdered tissues were lyophilized and extract buffer, comprising 0.1% Brij-35 in 20 mM triethanolamine (pH 7.5), added at 1 ml per 50 mg dry tissue. Extraction was carried out at 4°C for 16 hours with constant agitation, and the suspension was centrifuged at 10 Krpm for 10 minutes in a microcentrifuge, in order to remove insoluble material.

Gelatin gel zymography

Gelatin substrate zymography was performed as previously described (Tarlton et al, 1997). In brief, aliquots of the enzyme extracts were mixed with equal volumes of 2× non-reducing SDS sample buffer, warmed at 37°C for 30 minutes and spun for 2 minutes at 10 Krpm in a microcentrifuge. Samples were loaded and run on 10% SDS-polyacrylamide gels supplemented with 0.75 mg/ml bovine skin gelatin. Gels were washed in 2.5% Triton X-100, and incubated for 16 hours, with agitation, at 37°C in MMP proteolysis buffer (50 mM tris/HCl pH 7.8, 50 mM $CaCl_2$, 0.5 M NaCl and 0.1 mM amino phenyl mercuric acetate (APMA)). Gels were stained with 0.1% Coomassie blue (R250, Bio-Rad) and destained until the zones of proteolysis or the stacking gel had cleared.

MMP-2 and MMP-9 standards (Biogenesis, Bournemouth, UK) at 1.25 ng per track were run alongside the samples in order to corroborate the identity of the enzymes. Also by including the MMP-2 on each of the gels, activities may be expressed in relation to this standard, and comparisons made between samples run on separate gels, if required. Nevertheless, in this study all samples collected from a particular patient were run and analysed on the same gel. Other corroborative procedures included inhibition studies using MMP inhibitors ethylenediaminetetraacetic acid and 1,10 phenanthroline, serine proteinase inhibitors phenylmethylsulphonyl fluoride and 4-(2-Aminoethyl)-benzenesulfonyl fluoride, hydrochloride, and the cysteine proteinase inhibitor trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane in the proteolysis buffers, as described previously (Tarlton et al, 1997). Further, to confirm the MMPs as gelatinases, differential affinities were evaluated for gelatin and casein, as described previously. In brief, extracts were incubated overnight with agarose linked to either gelatin or casein, the solid component separated by centrifugation. The pellet was extensively washed, and the bound gelatinases extracted using 2× non-reducing SDS sample buffer. The residues remaining from the initial separation were mixed with equal volumes of 2× non-reducing SDS sample buffer. Both bound and unbound fractions were analysed for gelatinase levels as above.

Demonstration of the activation status of the enzymes associated with the gelatinolytically clarified zones was achieved by APMA pretreatment of samples, with the switch from high to low molecular weight forms representing the conversion from pro- to active enzyme species.

Standard curve analyses of gelatinase dilutions were made in order to evaluate the proportionality of the zymographic procedure, and to ensure that tissue extract analyses were performed within the linear region of the standard plot. For this purpose, a range of loadings of MMP-2 standard from 0.1 ng to 5.6 ng/lane were run on gels, analysed as described and gave coefficients of regression > 0.95 (data not shown). Gels were scanned according to methods adapted from those previously described (Tarlton and Knight, 1996) using an Agfa Studioscan colour flatbed scanner in transmittance mode. Fotolook scanning software was utilized for the scan, set on high resolution (> 200PPI) with a correction curve applied to maintain linearity of the scanner response across the entire intensity range. The image was transferred to NIH Image 1.5 analysis software, in which the proteolytically clarified zones were measured for both area and intensity and their values plotted and quantified according to displacement area.

Data for the stretched specimens were analysed as a percentage increase from the unstretched tissue, and statistical analysis was performed using the paired two-tailed Student's t-test. Values of $P < 0.05$ were regarded as significant.

RESULTS

Identification of matrix metalloproteinases

The major enzyme species identified were found to co-migrate with purified human MMP-2 at Mr 66 kDa,

58 kDa and 45 kDa, and MMP-9 at Mr 90 kDa, with similar molecular weights to those previously described for these gelatinases (Meikle et al, 1995). ProMMP-2 was present in all specimens, and all but one also expressed activated MMP-2. ProMMP-9 was expressed in 58 out of a total of 112 specimens examined taken from the tissue of the 21 patients, and five specimens demonstrated levels of activated MMP-9.

APMA pretreatment resulted in molecular weight shifts from the Mr 90 kDa to Mr 80 kDa, 75 kDa and 68 kDa bands, consistent with conversion of proMMP-9 to activated forms of the enzyme (Bu and Pourmotabbed, 1995; Ogata et al, 1995; Trancart et al 1992). Similarly, the band at Mr 66 kDa decreased in favour of a species which migrated with a Mr of 58 kDa; this again is consistent with a conversion from pro- to active MMP-2. Furthermore, APMA pretreatment resulted in elevation of the 45 kDa activated MMP-2 fragment, as did prolonged storage at 4°C.

Expression of MMPs

Figure 1 summarizes the data obtained for Mr 66 kDa proMMP-2 extracted from specimens stretched at 0.75 MPa, expressed as a proportion of the measured levels in both the 0.75 and 0.25 MPa groups. Figure 2 shows a similar comparison between specimens loaded at 0.75 and 0.5 MPa. In each case the predicted result that would indicate no difference between the two stretched groups is 0.5, and any increase in MMP-2 levels due to differences in stretching would give a result > 0.5. The difference was clearly shown by comparing the measured levels viz., the means for 0.75 MPa (Mean, 1092 [Standard Error of Mean, 100]) and 0.5 MPa (930 [102]) stretched groups, although this was not statistically significant ($P = 0.135$). There was also a difference between 0.75 MPa (1212 [121]) and 0.25 MPa (848 [114]) and this was shown to be highly significant ($P = 0.009$). The

progressive nature of the effect was also demonstrated by the differences seen between the 0.5 MPa (940 [104]) and 0.25 MPa (858 [117]) groups, although this did not attain statistical significance ($P = 0.448$). MMP-9 expression demonstrated a similar trend, with the greatest difference in levels seen for those specimens stretched at 0.75 MPa (209 [69]) compared with 0.25 MPa (125 [59]), this being the only result with statistical significance ($P = 0.013$).

Comparisons between the other stretched groups demonstrated a trend towards greater expression with increased loading, with 0.75 MPa (173 [65]) against 0.5 MPa (152 [63]), and 0.5 MPa (160 [66]) against 0.25 (112 [61]), but not demonstrating statistical significance ($P = 0.737$ and $P = 0.369$ respectively). The variable expression of MMP-9 seen throughout the experiment was reflected in the spread of the data for the stretched groups.

The experiment to evaluate selection bias demonstrated clear differences between the tissue taken for stretching and that selected for control procedures. Figure 3 illustrates how the actual expression of MMP-2 falls below the expected result, and paired analysis clearly demonstrates this, with specimens for stretching (1399 [171]) having lower levels than controls (1729 [167]); $P = 0.056$). Data for MMP-9 confirmed this bias with selected tissue (389 [172]) less than controls (1113 [492]), but not attaining statistical significance ($P = 0.052$).

The 0.75 MPa stretched group demonstrated greater levels of MMP-2 (1185 [109]) than the unstretched controls (988 [135]), although these differences were not significant ($P = 0.2$). The bias in MMP-2 expression identified above was presented as a model of the expected values should no differences result from stretching the tissues. However, if the actual results are compared with this "null hypothesis" (Fig. 4), the differences in the stretched versus unstretched specimens are amplified ($P = 0.016$).
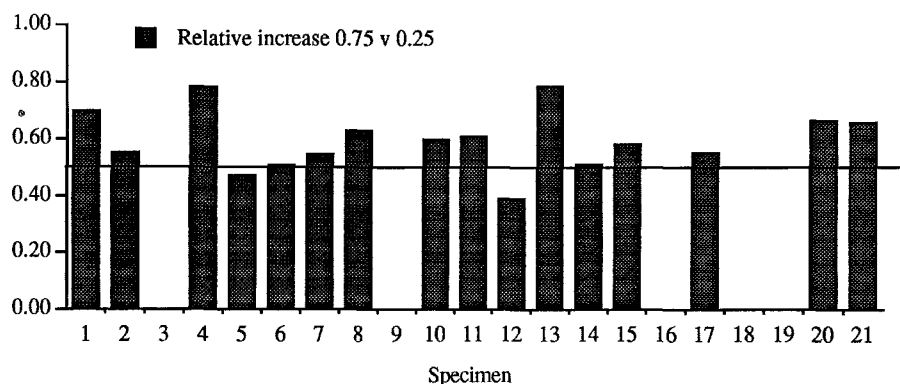

Fig 1   Relative differences in proMMP-2 levels between specimens stretched at 0.75 MPa and 0.25 MPa. ProMMP-2 (0.75 MPa) expressed as a proportion of total levels in both treatment groups. Equality is indicated at a value of 0.5.
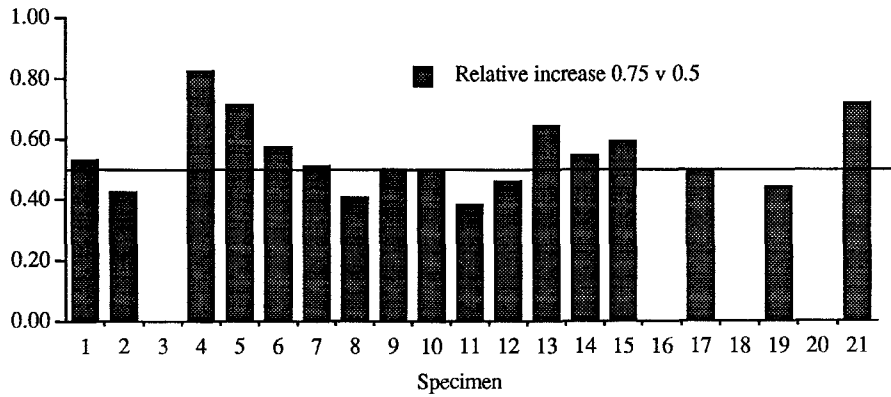
Fig 2   Relative differences in proMMP-2 levels between specimens stretched at 0.75 MPa and 0.5
         MPa. ProMMP-2 (0.75 MPa) expressed as a proportion of total levels in both treatment
         groups. Equality is indicated at a value of 0.5.
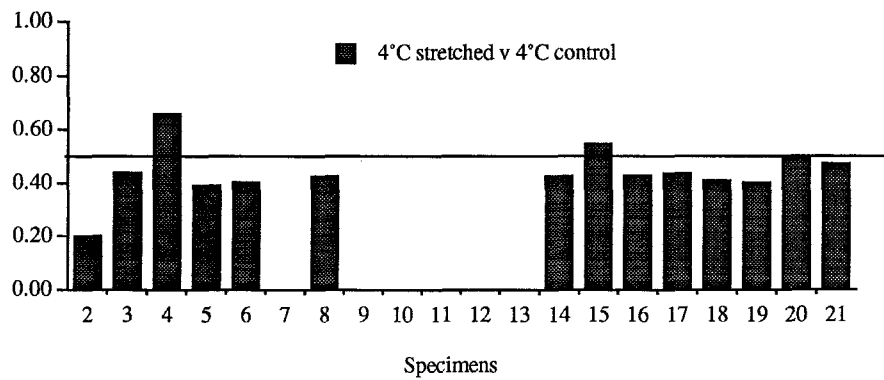

Fig 3   Bias in selection of specimens for stretching or control treatment. Lower activity in specimens
         selected for stretching is demonstrated by proportional levels falling below 0.5, the value at
         which stretched and control specimens are equal.
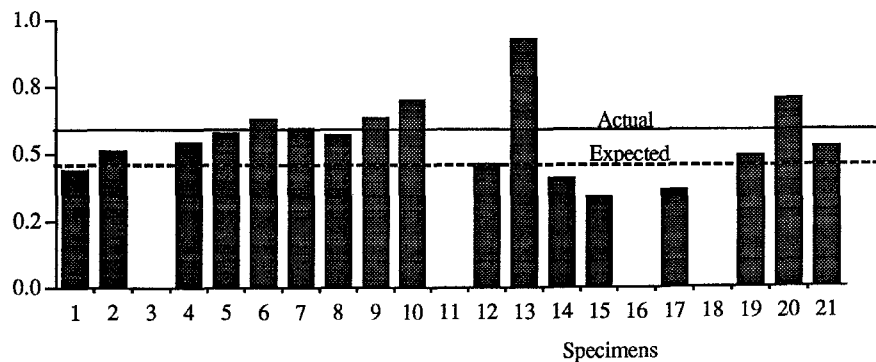

Fig 4   ProMMP-2 levels in 0.75 MPa stretched and unstretched specimens. Comparison of actual
         measured values with those expected if no stimulation is apparent, and taking selectivity bias
         into account. Actual expressions (mean, 0.555) are significantly higher ($P = 0.017$) than those
         expected in the absence of induction (mean, 0.439).

DISCUSSION

The obvious loss of strength of the tissue, or continuous creep, clearly illustrated in the preceding paper (Afoke et al, 1998), demonstrates a weakening through the depolymerization of the collagen fibres, the major mechanical support of the tissue, during the tension exerted at constant load in vitro.

Significant differences in the MMP levels were obtained between 0.75 MPa and 0.25 MPa, but not between 0.75 MPa and 0.5 MPa, and between 0.5 MPa and 0.25 MPa, indicating a proportionality in the stimulation of MMP production, and possibly a threshold loading at approximately 0.5 MPa. Comparisons with controls at 37°C were not strongly significant, but when corrected to eliminate bias in selection of cord or nodule tissue the differences were significant.

Depolymerization of the collagen fibres and ultimate complete degradation, is achieved by a group of enzymes, primarily the matrix metalloproteinases (MMPs) and the acid cathepsins. As well as raising expression of proteases, mechanical stress may cause proliferation of the fibroblast population. Indeed, previous reports on the stretching of dental sutures demonstrated proliferation of cells and a consequent increased expression of collagenase (Meikle et al, 1980).

MMP 2 and 9 are markers of this degradation of collagen, and can be accurately quantified, and consequently were monitored in this study. We demonstrated an increase in the levels of both MMP-2 and MMP-9 with in vitro stretching. This result is consistent with the increases we previously demonstrated during in vivo stretching employing the Messina continuous elongation technique (Bailey et al, 1994).

The precise molecular mechanisms and the levels at which collagen disruption occurs are unknown. However, these may be related to the collagen fibril "slip" process which was predicted as a necessary function by previous authors (Brown and Byers, 1989; Harkness and Harkness, 1973; Nemetschek et al, 1980; Nimni and Harkness, 1988; Woessner, 1982). If so, identification of the mechanical and chemical signals which initiate this process would be surgically important, eg in tissue expanders, scar stretching and in adolescent tendon growth. The apparent threshold response to mechanical loading•seen in this study potentially could explain the paradox that collagen fibre growth occurs whilst the tissue remains inextensible for functional purposes. In the case of the Messina stretching procedure, the Dupuytren's tissue was clearly subject to tensile loading from the patient, albeit intermittent and cyclical rather than high static loads. This study suggests that the stimulus for expansion is the application of a supraphysiological level of creep load.

MMP-9 is associated with cell types which have an infiltrative or invasive phenotype, such as neutrophils and monocytes/macrophages and is significantly elevated during inflammatory events. The increase in the levels of MMP-9 in this in vitro study is clearly not a consequence of an inflammatory response, demonstrating that such a change identified in vivo may also occur in the absence of inflammation. This is consistent with the lack of haemorrhage that is found with the technique of Messina and Messina (1991).

MMP-2 is the most widespread of the metalloproteinase and is expressed by connective tissue cells such as fibroblasts, osteoblasts, osteoclasts and chondrocytes. The large increase in MMP-2 levels in the Dupuytren's tissue clearly indicates a mechanical effect on the fibroblasts and myofibroblasts in the cords and nodules of this tissue. The mechanism by which mechanical stress can trigger expression of enzymes is currently a major topic of research.

This in vitro study has demonstrated that the success of the continuous elongation technique is indeed due to mechanical stress on the fibroblastic cells of the Dupuytren's disease tissue resulting in the release of degradative enzymes which weaken the collagen and allow stretching.

References

Afoke A, Meagher PJ, McGrouther DA, Bailey AJ, Brown RA (1998). Biomechanical characterization of tissues in Dupuytren's disease tissue. Journal of Hand Surgery, 23B: 291–296.

Bailey AJ. Collagen changes in Dupuytren's disease. In: Berger A, Delbruck A, Brenner P, Hinzmann R (Eds): *Dupuytren's disease*. Berlin, Springer-Verlag, 1994: 37–48.

Bailey AJ, Tarlton JF, Van Der Steppen J, Sims TJ, Messina A (1994). The continuous elongation technique for severe Dupuytren's disease: A biochemical mechanism. Journal of Hand Surgery, 19B: 522–527.

Berger A, Delbrück A, Brenner P, Hinzmann R (Eds): *Dupuytren's disease – pathobiochemistry and clinical management*. Berlin, Springer-Verlag, 1994.

Brown RA, Byers PD (1989). Swelling of cartilage and expansion of the collagen network. Calcified Tissue International, 45: 260–261.

Bu CH, Pourmotabbed T (1995). Mechanism of activation of human neutrophil granules and secretory vesicles. Journal of Biological Chemistry, 270: 18563–18569.

Harkness MLR, Harkness RD. Changes with age in some mechanical properties of skin in the rat. In: Vogel HG (Ed.): *Connective tissue and ageing*. Amsterdam, Excerpta Medica, 1973: 219–221.

MacFarlane RM, McGrouther DA, Flint MH (Eds): *Dupuytren's disease*. Edinburgh, Churchill Livingstone, 1990.

Messina A, Messina J (1991). The TEC (Continuous elongation technique) for severe Dupuytren's contracture of the fingers. Annals of Hand Surgery, 10: 247–250.

Meikle MC, Sellars A, Reynolds JJ (1980). Effect of tensile mechanical stress on the synthesis of metalloproteinases by rabbit coronal sutures in vitro. Calcified Tissue International, 30: 77–82.

Meikle MC, Bord S, Hembry RM, Reynolds JJ (1995). The synthesis of collagenase, gelatinase-A (72 kDa) and -B (95 kDa) and TIMP-1 and -2 by human osteoblasts from normal and arthritic bone. Bone, 17: 255–260.

Nemetschek TH, Riedel H, Jonak R, Nemetschek-Gansler H, Bordas J, Koch MHJ, Schilling V (1980). Functional properties of parallel fibre connective tissue with special regard to viscoelasticity. Virchows Archiv A. Pathological Anatomy and Histology, 386: 125–151.

Nimni ME, Harkness RD. Molecular structure and functions of collagen. In: Nimni ME (Ed.): *Collagen*. Boca Raton, CRC Press, 1988, Vol. 1: 1–77.

Ogata Y, Itoh Y, Nagase H (1995). Steps involved in activation of the pro-matrix metalloproteinase-9 (progelatinase B) tissue inhibitor of metalloproteinase-1 complex by 4-aminophenylmercuric acetate and proteinases. Journal of Biological Chemistry, 270: 18506–18511.

Tarlton JF, Knight PJ (1996). Comparison of reflectance and transmission densitometry, using document and laser scanners, for quantitation of stained western blots. Analytical Biochemistry, 237: 123–128.

Tarlton JF, Vickery CJ, Leaper DJ, Bailey AJ (1997). Post-surgical wound progression monitored by temporal changes in the expression of matrix metalloproteinase-9. British Journal of Dermatology, 137: 506–516.

Trancart MM, Chalmeign N, Giradot C, Zarpanelian C, Prigent D (1992). Gelatinase is the main matrix metalloproteinase involved in granuloma-induced cartilage degradation. International Journal of Tissue Reactions, 14: 287–294.

Woessner JF. Uterus, cervix and ovary. In: Weiss JB, Jayson MIV (Eds): *Collagen in health and disease*. Edinburgh, Churchill Livingstone, 1982: 506–527.