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Matrix metalloproteinases in the formation of human synovial joint cavities

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ABSTRACT

Matrix metalloproteinases (MMPs) have been implicated in tissue remodelling in growth and development. A histochemical study of human fetal limbs was undertaken to assess the presence, and consequently the possible role, of MMPs and their inhibitor TIMP-1 (tissue inhibitor of metalloproteinases-1) in synovial joint cavity formation. Cryostat sections of fetal limbs from 7 to 14 wk gestation were stained with specific antibodies to collagenase (MMP-1), gelatinases A (MMP-2) and B (MMP-9), stromelysin (MMP-3) and TIMP-1. Immunoreactive (IR) MMP-1, MMP-2 and MMP-3 were seen chiefly in chondrocytes, but in all cases in zones distant from the joint line before cavity formation. IR-MMP-1 and MMP-2 were also localised both in synovium and on the articular surfaces of joints after cavity formation. In addition IR-MMP-2 was seen in a 'collar' of perichondrium alongside the hypertrophic zone of chondrocytes and weakly in bone marrow spaces. IR-MMP-9 was seen in neutrophil leucocytes and in bone marrow spaces. IR-TIMP-1 was generally distributed in connective tissue cells. No IR-MMP (1, 2, 3 or 9) was seen along potential joint lines before or at the time of cavity formation, nor was there a specific decrease in IR-TIMP-1 at this site. These findings confirm a role for metalloproteinases in developmental processes such as cartilage remodelling and bone marrow space formation. MMP-1 and MMP-2 may be involved in the remodelling of developing synovial tissue and the articular surfaces subsequent to cavity formation. However, we have failed to find evidence to indicate that the loss of tissue strength at the joint line which allows synovial joint cavity formation relates to high local levels of MMPs.

Key words: Fetal development; synovium; collagenase.

INTRODUCTION

During synovial joint development in the fetus, the apparently continuous extracellular matrix of the developing skeleton separates to form spaces which will become synovial cavities (O'Rahilly & Gardner, 1978). This process suggests a change in tissue matrix at the joint line, from solid to fluid, but the changes in matrix metabolism involved remain to be established.

Several observations implicate changes in hyaluronan metabolism in joint cavity formation (Munaron, 1954; Craig et al. 1990; Archer et al. 1994; Edwards et al. 1994; Pitsillides et al. 1994). Munaron (1954) suggested that at the time of joint cavity formation the matrix at the joint line might be rich in soluble hyaluronan. Despite doubts expressed by others (Andersen & Bro-Rasmussen, 1961; Andersen, 1963), Craig et al. (1990) confirmed, in chicks, that a band of hyaluronan-rich matrix is present at the joint line concomitant with cavity formation. Pitsillides et al. (1994) demonstrated in the chick that cells bordering this band of hyaluronan-rich matrix have high activity of uridine diphosphoglucose dehydrogenase (UDPGD), and high content of hyaluronan synthase, both enzymes being involved in hyaluronan synthesis (Prehm, 1983). Human studies confirm these findings (Edwards et al. 1994). Moreover, cells of the interzone, within which the cavity forms, show

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Table 1. *Fetal tissue samples*

Sample no.	Region	Joints examined	Gestational age (to within 2 wk)
63	Hindquarter	Hip, knee, tarsus, digital	8
60	Arm	Elbow, wrist, carpus, cmc	8
64	Leg	Knee, ankle, subtalar, tarsal	10
58	Upper arm	Shoulder, elbow, wrist, carpus	10
61	Hand	Carpal#, cmc#, digital#	12
59	Leg	Knee#	12
62	Arm	Shoulder#, elbow#	12
65	Spine	Intervertebral discs	12
55	Foot	Ankle*, subtalar*, tarsal*	14
56	Hand	Carpal*, cmc*, mcp*	14
57	Leg	Knee*	14

Digital = metacarpophalangeal and interphalangeal; cmc, carpometacarpal; mcp, metacarpophalangeal.
# Showing partial separation of articular surfaces; * showing full separation of articular surfaces.

particularly high expression of the hyaluronan receptor CD44, and it has been suggested that an interaction between hyaluronan and CD44 may lead to reduced matrix cohesion at the joint line (Edwards et al. 1994).

While interactions between CD44 and hyaluronan could lead to reduced cohesion (Toole et al. 1984), it seems likely that for matrix to become fluid, fibrous components such as collagen must be removed. Enzymes such as acid phosphatases have been implicated in cavity formation (Milaire, 1947). However, their role in matrix degradation is unclear (Kulyk & Kosher, 1987) and recent observations cast doubt on a link between acid phosphatase activity and cavity site (Edwards et al. 1994). Current concepts of collagen removal focus on matrix metalloproteinases (MMPs) (Murphy & Reynolds, 1993).

MMPs are secreted both by mesenchymal and by haemopoietic cells (Murphy & Reynolds, 1993). Collagenases (MMP-1 and MMP-8) cleave native collagens types I, II and III at a single locus. Gelatinases (MMP-2 and MMP-9) cleave denatured collagens and type IV collagen. Stromelysins (including MMP-3) cleave several proteins, including proteoglycan core protein, type IV collagen and fibronectin. The action of MMPs is dependent on extracellular activation, and on the inhibitory effects of tissue inhibitors of metalloproteinases (TIMPs). A role for these enzymes in growth and development has been suggested by several studies. For instance, differential expression of MMPs is seen in growth plate cartilage (Brown et al. 1989) and during development of the mandibular condyle (Breckon et al. 1994).

The aim of the present study was to assess the distribution of MMPs and TIMP-1 in human synovial joints undergoing cavity formation.

MATERIALS AND METHODS

Fetal tissues were obtained from surgical termination of pregnancies for psychosocial indications, in accordance with the recent UK ethical guidelines. Joints examined and their gestational ages are given in Table 1. Limb segments were snap chilled in n-hexane cooled with solid carbon dioxide.

Sets of serial frozen sections were cut at −35 °C at a thickness of 7 μm, fixed in 5% paraformaldehyde for 30 min, permeabilised with Triton X-100 0.1% for 5 min and analysed immunohistochemically. One

Table 2. *Distribution of immunoreactive metalloproteinases and TIMP-1 in fetal limb tissues*

Site	MMP-1	MMP-2	MMP-3	MMP-9	TIMP-1
Potential joint line: before and during cavity formation	—	—	—	—	Equivalent to levels elsewhere
Developing synovium: after cavity formation	Weak	Weak	—	—	+
Articular surfaces: after cavity formation	—	Patchy, weak	—	—	+
Chondrocytes	Subarticular	Subarticular	Subarticular 12–14 wk	Weak, patchy, subarticular	+
Perichondrium	—	'Collar' alongside hypertrophic zone	—	—	+
Bone marrow spaces	—	Rim pattern, Weak	—	Rim pattern, Osteoclasts	+
Myeloid cells	—	—	—	Very strong	?*

* In the presence of general TIMP-1 staining the status of individual myeloid cells was uncertain.

sample section from each set was stained with 0.1% toluidine blue in 0.1 M acetate buffer (pH 6.2) and one with Harris's haematoxylin.

The following specific polyclonal antibodies were raised in sheep (Table 2): X670/10, to human gelatinase A (MMP-2) (Hipps et al. 1991); A560/8, to pig gelatinase B (MMP-9), which cross-reacts with human gelatinase B but not A (Murphy et al. 1989); Z494/7, to stromelysin, which reacts strongly with stromelysin 1 and weakly with stromelysin 2 (Allan et al. 1991); and S526/6, to TIMP-1 (Hembry et al. 1985). The characterisation of these antisera is detailed in the references given. Human interstitial collagenase was purified by the method of Whitham et al. (1986), injected into sheep and the resulting antiserum, X649/6, was characterised by Western blotting, inhibition and immunolocalisation as for the other antisera (Hembry et al. 1986). The antiserum does not cross-react with human neutrophil collagenase. Normal sheep serum was used as a control.

Sections were incubated with the following reagents with intervening washes in phosphate-buffered saline: primary antibody for 30 min, 0.3% H_2O_2 in methanol for 5 min, biotinylated donkey antisheep immunoglobulins (ICN Biochemicals, California) at 1/2500 in 20% normal human serum for 30 min, streptavidin peroxidase (DAKO) at 1/400 in 20% normal human serum for 30 min, and finally 0.08% ethylaminocarbazole (Sigma) in acetate buffer at pH 5.0, to which was added 25 ml of 30% w/v hydrogen peroxide, for 10 min. Rheumatoid arthritic synovia were stained using the same protocol as positive controls. Parallel studies were performed with streptavidin FITC conjugate (DAKO) at 1/50, with methyl green as counterstain.

The distribution and orientation of collagen fibres were demonstrated using polarised light. Sections were stained with picrosirius red (Chayen & Bitensky, 1991) and viewed through crossed polarising filters.

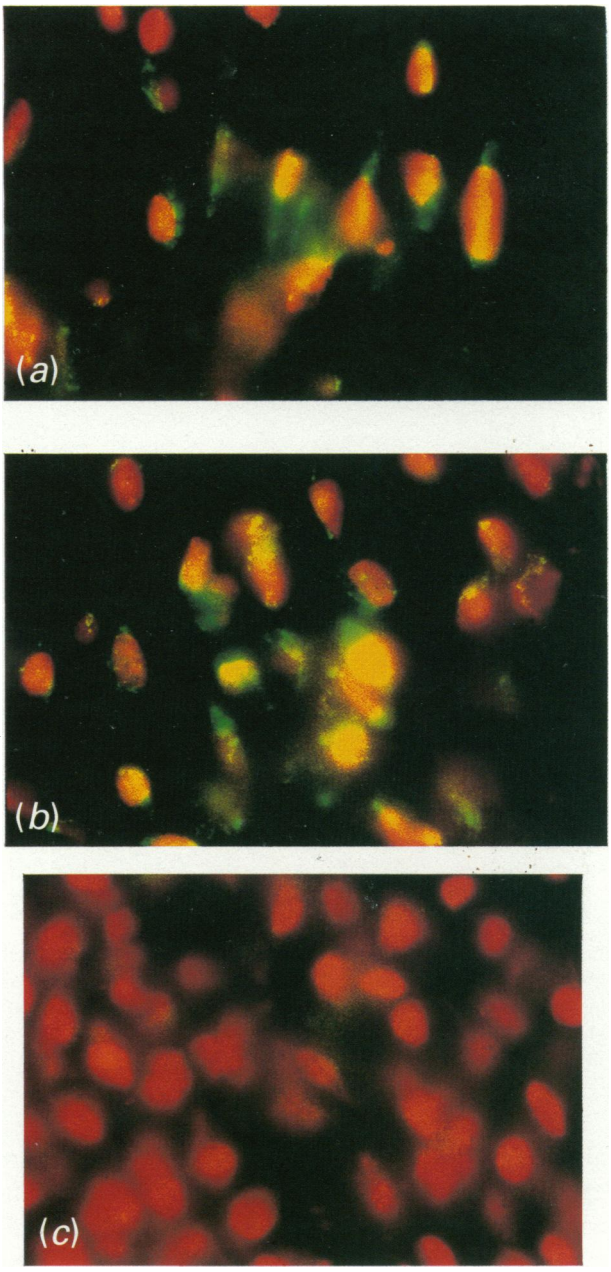

Fig. 1. Tibia at 12 wk gestation, showing IR-MMP3 (green). Nuclei stained red. (*a*) Proliferating chondrocytes. (*b*) Epiphyseal chondrocytes. (*c*) Potential knee joint line seen as a band of low cell density, top left to bottom right. ×400.

RESULTS

Comparison of staining patterns obtained with rheumatoid arthritic synovia with both enzyme and fluorochrome conjugates confirmed comparability with previous findings (Hembry et al. 1995).

A total of 65 joints were analysed, including examples prior to cavity formation, examples showing different degrees of partial cavity formation and examples after cavity formation for all major joints or small joint groups. Immunoreactive (IR) MMP-1 was seen within subarticular chondrocytes at all gestational ages, but particularly from 10 wk onwards. IR-MMP-1 was also seen locally in newly formed synovial tissue in some joints, but only at a stage following cavity formation, when synovium had separated from adjacent articular cartilage. IR-MMP-1 was not seen at the potential joint line, either in articular chondrocytes or interzone cells, prior to cavity formation.

IR-MMP-2 was seen in subarticular chondrocytes, at gestational ages from 10 wk onwards. IR-MMP-2 was also seen as a linear streak within perichondrium

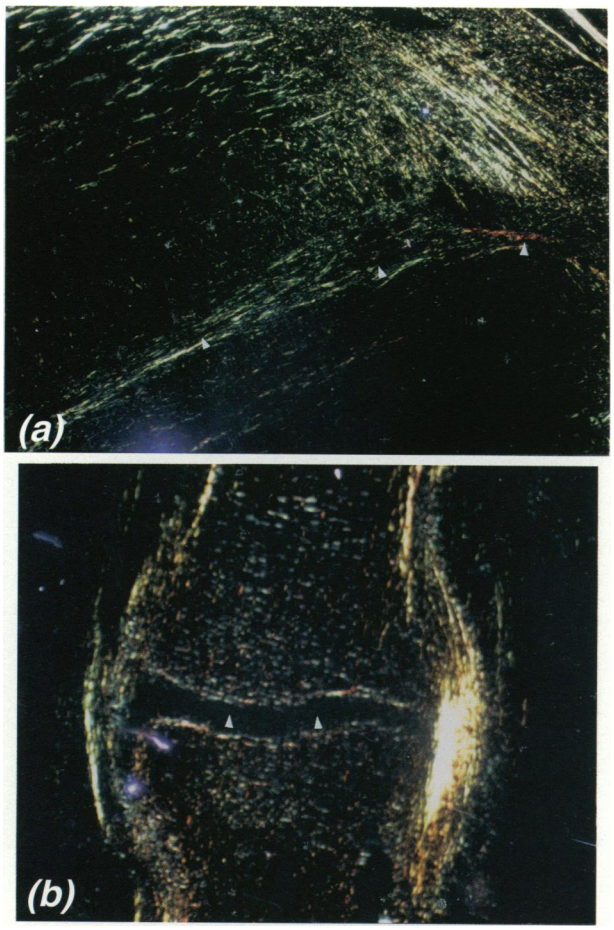

Fig. 2. (*a*) Talocalcaneal joint at 10 wk gestation showing collagen fibres running parallel to the potential joint line (white arrowheads), but no fibres crossing the joint line. × 100. (*b*) Proximal interphalangeal joint at 10 wk gestation. No collagen fibres are seen at the joint line (white arrowheads). × 100.

alongside the hypertrophic zone of chondrocytes, apparently representing a 'collar' around the lengthening cartilage. Some of this staining was intracytoplasmic, but some was also extracellular. IR-MMP-2 was present at low levels at the margin of bone marrow spaces within cartilage. Within joints IR-MMP-2 was seen locally both in newly formed synovium, in a similar distribution to IR-MMP-1, and on the articular surfaces of some joints after cavity formation. A proportion of the staining was intracellular, but staining was also seen in short streaks, in association with collagen bundles in synovium. On the articular surfaces staining was seen in the most superficial layer of extracellular matrix, and in particular at sites where the surface showed irregularities. As with IR-MMP-1, no IR-MMP-2 was seen at the potential joint line in joints before cavity formation.

IR-MMP-3 was present at very low levels (using rheumatoid tissue as a comparison), close to the limit of detection. Fluorescence confirmed that the staining was in the form of intracellular granules (Fig. 1*a*, *b*), which were present chiefly in chondrocytes in the subarticular zones in samples at 12–14 wk gestation. Cells at the potential joint line in the same sections showed no IR-MMP-3 (Fig. 1*c*).

IR-MMP-9 was seen in neutrophil leucocytes where these were present (e.g. in bone marrow and within vessels). IR-MMP-9 was seen in osteoclasts and at the margin of marrow spaces as a band of extracellular material.

Intracellular IR-TIMP-1 was generally distributed throughout connective tissue. This distribution included cells of the potential joint line in joints prior to cavity formation, and there was no indication of a specific reduction in IR-TIMP-1 at this site.

Using picrosirius red and polarising filters, joints prior to cavity formation showed no collagen fibres crossing the potential joint line. In some joints collagen was present in the interzone, but its orientation was parallel to the joint line (Fig. 2*a*). In other joints collagen was absent from the vicinity of the joint line (Fig. 2*b*).

DISCUSSION

Our findings suggest that MMPs are involved in a number of developmental processes including cartilage remodelling, marrow space formation and possibly a 'controlled sliding' between perichondrium and surrounding mesenchyme. However, within synovial joints, if MMPs play a role in matrix remodelling it appears to be largely after cavity formation.

There are limitations to the analysis of events leading to joint cavity formation on the basis of histochemistry alone. However, previous studies have identified changes at the potential joint line at a stage immediately before cavity formation (Edwards et al. 1994; Pitsillides et al. 1994). In such joints the potential joint line is populated by cells with prominent UDPGD activity and CD44 expression. No such line of cells is identified by localisation of metalloproteinases. Thus it appears that the formation of the joint line at a precise site within the interzone is not due to a high local expression of MMPs, or a local reduction in TIMP-1.

On the other hand, in joints showing separation of articular and synovial surfaces IR-MMP-1 and IR-MMP-2 were seen in consistent patterns. Both were found in newly formed synovial tissue, suggesting a role in remodelling. IR-MMP-2 was also seen along the articular surfaces, and particularly at sites of

irregularity, suggesting that the enzyme may be important in the formation of a smooth articulating surface.

These observations may seem surprising, in that collagen present at the joint line prior to cavity formation might be expected to inhibit tissue separation. Collagen is present in the matrix throughout the length of the blastema in chick limbs prior to cavity formation (Craig et al. 1987). However, avian joints differ from human joints in that much of the interzone, which contains type I collagen, becomes fibrocartilage rather than synovium. Moreover, the mode of interzone growth (Lewis, 1977) suggests reasons why collagen may not contribute to interzone strength in a proximodistal direction. Apparently as a result, the interzone becomes increasingly attenuated centrally, with both cells and matrix components taking up an orientation parallel to the potential joint line. The early interzone comprises 3 layers, 2 outer chondrogenic layers and a middle layer. Just before cavity formation the middle layer may be lost centrally (Andersen, 1963), leaving 2 apposed chondrogenic layers, independent in terms of the fibrous components of their matrix. The present findings with picrosirius red support this. It appears that collagen in the interzone before cavity formation is exclusively oriented parallel to the joint line. Thus cohesion at the joint line may depend only on hyaluronan/cell surface interactions. We conclude that the separation of tissues at the time of cavity formation remains constant with the concept of an increase in local hyaluronan levels in the presence of CD44 expressing cells (Edwards et al. 1994; Pitsillides et al. 1994), as originally proposed by Underhill & Dorfman (1978) and Toole et al. (1984).

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REFERENCES

ALLAN JA, HEMBRY RM, ANGAL S, REYNOLDS JJ, MURPHY G (1991) Binding of latent and high Mr active forms of stromelysin to collagen is mediated by the C-terminal domain. *Journal of Cell Science* **99**, 789–795.

ANDERSEN H (1963) Development, morphology and histochemistry of the early synovial tissue in human fetuses. *Acta Anatomica* **58**, 90–115.

ANDERSEN H, BRO-RASMUSSEN F (1961) Histo-chemical studies on the histogenesis of the joints in human fetuses. *American Journal of Anatomy* **1087**, 90–115.

ARCHER CW, MORRISON H, PITSILLIDES AA (1994) Cellular aspects of the development of diarthrodial joints and articular cartilage. *Journal of Anatomy* **184**, 447–456.

BRECKON JJW, HEMBRY RM, REYNOLDS JJ, MEIKLE MC (1994) Regional and temporal changes in the synthesis of matrix metalloproteinases and TIMP-1 during development of the rabbit mandibular condyle. *Journal of Anatomy* **184**, 99–110.

BROWN CC, HEMBRY RM, REYNOLDS JJ (1989) Immunolocalisation of metalloproteinases and their inhibitor in the rabbit growth plate. *Journal of Bone and Joint Surgery* **71A**, 580–593.

CHAYEN J, BITENSKY L (1991) *Practical Histochemistry*, pp. 61–2. Chichester: Wiley and Sons.

CRAIG FM, BENTLEY G, ARCHER CW (1987) The temporal and spatial patterns of collagen I and III and keratan sulphate in developing chick metatarsophalangeal joint. *Development* **99**, 383–391.

CRAIG FM, BAYLISS MT, BENTLEY G, ARCHER CW (1990) A role for hyaluronan in joint development. *Journal of Anatomy* **171**, 17–23.

EDWARDS JCW, WILKINSON LS, JONES HM, SOOTHILL P, HENDERSON KJ, WORRALL JG et al. (1994) The formation of human synovial joint cavities: a possible role for hyaluronan and CD44 in altered interzone cohesion. *Journal of Anatomy* **185**, 355–367.

HEMBRY RM, MURPHY G, REYNOLDS JJ (1985) Immunolocalisation of tissue inhibitor of metalloproteinases (TIMP) in human cells. Characterisation and use of a specific antiserum. *Journal of Cell Science* **73**, 105–119.

HEMBRY RM, MURPHY G, CAWSTON TE, DINGLE JT, REYNOLDS JJ (1986) Characterisation of a specific antiserum for mammalian collagenase from several species: immunolocalisation of collagenase in rabbit chondrocytes and uterus. *Journal of Cell Science* **81**, 105–123.

HEMBRY RM, BAGGA MR, REYNOLDS JJ, HAMBLEN DL (1995) Immunolocalisation studies on six matrix metalloproteinases and their inhibitors TIMP-1 and TIMP-2 in synovia from patients with osteo- and rheumatoid arthritis. *Annals of the Rheumatic Diseases* **54**, 25–32.

HIPPS DS, HEMBRY RM, DOCHERTY AJP, REYNOLDS JJ, MURPHY G (1991) Purification and characteristics of human 72 kDa gelatinase (type IV collagenase). Use of immunolocalisation to demonstrate the non-coordinated regulation of the 72 kDa and 95 kDa gelatinases by human fibroblasts. *Biological Chemistry Hoppe-Seyler* **372**, 287–96.

LEWIS J (1977) Growth and determination in the developing limb. In *Vertebrate Limb and Somite Morphogenesis* (ed. D. A. Ede, J. R. Hinchcliffe & M. Balls), pp. 215–228. Cambridge: Cambridge University Press.

KULYK WM, KOSHER RA (1987) Temporal and spatial analysis of hyaluronidase activity during development of the embryonic chick limb bud. *Developmental Biology* **120**, 535–541.

MILAIRE J (1947) In *Normal and Abnormal Embryological Development* (ed. C. H. Frantz), p. 61. Washington, DC: National Research Council.

MUNARON G (1954) Osservasione istofisiche ed istochimiche sul mesenchimima intermedio delle articolazioni embryonali e suio derivati. *Bolletino del Societa di Biologia Sperimentale* **30**, 919–922.

MURPHY G, REYNOLDS JJ (1993) Extracellular matrix degradation. In *Connective Tissue and its Heritable Disorders* (ed. P. M. Royce & B. Steinmann), pp. 287–316. New York: Wiley-Liss.

MURPHY G, WARD R, HEMBRY RM, REYNOLDS JJ, KUHN K, TRYGGVASON K (1989) Characterisation of gelatinase from pig polymorphonuclear leucocytes. A metalloproteinase resembling tumour type IV collagenase. *Biochemical Journal* **258**, 463–472.

O'RAHILLY R, GARDNER E (1978) The embryology of movable joints. In *The Joints and Synoval Fluid* (ed. L. Sokoloff), vol. 1. New York: Academic Press.

PITSILLIDES AA, ARCHER CB, PREHM P, BAYLISS MT, EDWARDS JCW (1994) Hyaluronan synthesis in joint cavitation. *Journal of Histochemistry and Cytochemistry* **43**, 263–273.

PREHM P (1983) Synthesis of hyaluronan in differentiated teratocarcinoma cells. II. Mechanisms of chain growth. *Biochemical Journal* **211**, 191–198.

TOOLE BP, GOLDBERG RL, CHI-ROSSO G, UNDERHILL CB, ORKIN RW (1984) Hyaluronate-cell interactions. In *The Role of Extracellular Matrix in Development* (ed. R. L. Trelstad), pp. 43–66. New York: Alan R. Liss.

UNDERHILL CB, DORFMAN A (1978) The role of hyaluronic acid in intercellular adhesion of cultured mouse cells. *Experimental Cell Research* **117**, 155–164.

WHITHAM SE, MURPHY G, ANGEL P, RAHMSDORF H-J, SMITH BJ, LYONS A et al. (1986) Comparison of human stromelysin and collagenase by cloning and sequence analysis. *Biochemical Journal* **240**, 913–916.