A BRIEF COMMUNICATION

Collagen Fragments Modulate Innate Immunity

AMELIA H. THOMAS,* ELAZER R. EDELMAN,†,‡,§ AND COLLIN M. STULTZ*,†,‡,‖,[1]

The Computer Science and Artificial Intelligence Laboratory, Cambridge, Massachusetts 02139; and †The Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and ‡The Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115; and §The Division of Cardiovascular Disease, Brigham & Women's Hospital, Boston, Massachusetts 02115; and ||The Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Activation of the innate immune response in diseases such as rheumatoid arthritis and atherosclerosis leads to the production of pro-inflammatory cytokines that can promote collagenolysis. While a number of studies suggest that inflammation plays a major role in initiating collagen degradation, the effect of collagen and collagen-degradation fragments on the inflammatory response is not well understood. We now demonstrate that different collagen fragments can either augment or suppress IL-1β production from human peripheral-blood monocytes. These data have wide-ranging implications for how amino acid variation in collagen affects disease and suggest that collagen degradation leads to the production of peptides that can modulate inflammation. Exp Biol Med 232:406–411, 2007

Key words: collagen; cytokines; inflammation; interleukin-1

Introduction

Inflammation plays an important role in many processes, protecting us against pathogens, and also potentially promoting disease progression (1). An overactive immune response has been implicated as the etiologic agent in a number of disorders including rheumatoid arthritis (RA) and atherosclerotic heart disease (AHD) (1). These diseases share a number of processes in common including activation of innate immunity and destruction of extracellular matrix. Degradation of type II collagen in cartilage, for example, is partly responsible for the clinical severity of RA, and excessive destruction of collagen types I and III in patients with AHD promotes atherosclerotic plaque rupture and myocardial infarctions (2–4).

A number of pro-inflammatory cytokines contribute to the pathogenesis of these disorders (5–7). The pro-inflammatory cytokine IL-1β can stimulate the expression of matrix metalloproteases (MMPs) in endothelial cells and smooth muscle cells—cells that play an important role in the pathogenesis of atherosclerosis (7). IL-1β has also been shown to promote the expression of collagenases from fibroblasts and chondrocytes, suggesting a role for this cytokine in the pathogenesis of RA as well (5). The importance of IL-1β in RA is underscored by the fact that inhibiting its effects can ameliorate signs and symptoms associated with RA (6).

There is an intimate connection between MMP expression and cytokine production. MMP-1, for example, not only cleaves native collagens, but it can also deactivate IL-1β through proteolysis (8). In addition, the gelatinases, MMP-2, MMP-3, and MMP-9, can activate IL-1β by cleaving the IL-1β precursor protein (9). While these data suggest that MMPs can modulate cytokine production, a definitive link between collagen degradation products and

C.M.S. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences.

[1] To whom correspondence should be addressed at MIT, The Stata Center, 32–310, 32 Vassar Street, Cambridge, MA 02139. E-mail: cmstultz@mit.edu

Received June 23, 2006.
Accepted September 13, 2006.

1535-3702/07/2323-0406$15.00

the production of IL-1β has not been established. Although it has been demonstrated that collagen fragments are chemotactic for human monocytes *in vitro*, it is not known whether collagen degradation products can activate these cells (10).

The initial step in the degradation of interstitial collagen is cleavage at a specific site by interstitial collagenases (11, 12). Subsequent unfolding of collagen monomers exposes additional scissile bonds that can then be cleaved (11). Consequently, collagenase-mediated collagen degradation produces a peptide mixture containing amino acid sequences of various sizes (13, 14). As the melting temperature of collagen-like peptides, which model subsequences from mature collagen, are typically several degrees below body temperature, collagen degradation fragments likely exist as unfolded peptide chains *in vivo* (15, 16).

Prior studies have analyzed the effect of such mixtures of unfolded peptides on mammalian cells *in vitro* to decipher the effect of collagen fragments on inflammation and extracellular matrix degradation (10, 13). As a result, these approaches do not lead to precise conclusions about how the observed biologic response, if any, varies as a function of sequence differences in the degradative fragments. Therefore, to explore the relationship between peptide sequence and inflammation, we constructed collagen-like peptides that model regions of collagen with distinct amino acid compositions. Given the prior data relating collagen fragments to human monocytes, we studied the effect of collagen degradation products on IL-1β production from human peripheral blood monocytes (HPBMs) *in vitro* (10).

Materials and Methods

Peptide Preparation. Ten milligrams of peptide p1 (Lot #10054791, Genemed Synthesis Inc., San Francisco, CA), p2 (Lot #10059702, Genemed Synthesis Inc.), and p3 (Lot #10059701, Genemed Synthesis Inc.) were obtained with purity >98%. Each peptide was diluted with phosphate-buffered saline (PBS; 1 *M*, pH 7.2, 20012–043, Invitrogen Corp., Carlsbad, CA) yielding solutions of 10 mg/ml. The peptide solutions were incubated at 4°C for 24 hrs and then stored at −80°C until required. Glucagon (G2044, Sigma-Aldrich, St. Louis, MO) was stored, defrosted, and treated in an identical manner to the collagen-like peptides.

Monocyte Isolation and Culture. Our protocol for monocyte isolation was approved by the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects (COUHES approval number 0511001446). Informed consent was obtained from all subjects.

Human peripheral blood monocytes were isolated from whole blood supplied by a healthy male volunteer as follows. A 45-ml sample was drawn using 3.2% sodium citrate vacuum tubes. Whole blood was then diluted 1:2 with a 2.5-m*M* PBS sodium citrate solution (0.1 *M*, pH 5.0, sterile, S0231, Teknova Inc., Hollister, CA). Red blood cells were subsequently removed by density gradient separation using a 1:1 ratio of Histopaque-1077 (Sigma-Aldrich) and centrifuged for 30 mins at 400 *g* at 25°C. The plasma layer was removed by aspiration, and the buffy layer, composed of lymphocytes, monocytes, and platelets, was collected into two 50-ml conical tubes. To remove the excess histopaque, platelets, and plasma, the cells were centrifuged at 250 *g* for an additional 10 mins at 4°C. The supernatant was removed, and the cells were resuspended in 2.5-m*M* PBS sodium citrate and centrifuged again. The platelet-enriched supernatant was removed after the second wash, leaving a cell pellet in each of the two tubes.

The remainder of the monocyte isolation was carried out using an MS 130-041-201 column (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and Human Monocyte Isolation Kit II (MACS, Miltenyi Biotec) as outlined by the manufacturer. Subsequent fluorescence activated cell sorter (FACS) analysis of tissue culture samples confirmed a monocyte purity >82%. Monocytes were seeded at 5×10^5 per well and cultured in 10% fetal bovine serum (FBS) RPMI 1640 media and allowed to incubate at 37°C in 5% CO_2.

Incubating HPBMs with Different Peptides. As the collagen-like peptides under investigation can spontaneously form triple-helical structures in solution, we heated the peptide samples to 90°C for 2 mins prior to introducing them to HPBM cultures. This method helped to ensure that unfolded peptides, corresponding to the collagen-degradation products, were used for the experiments. Peptide solutions were then diluted with RPMI 1640 10% FBS at 37°C yielding a final concentration of 1 μg/ml. Serial dilutions of the peptide samples were performed to obtain the solutions at the desired concentrations. Lipopolysaccharide solution (LPS; 581-0101-L002, *Escherichia coli*–based, Alexis Corp., San Diego, CA) was diluted with 900 μl of 37°C 10% FBS RPMI 1640 media to make 1000 μl of a 0.1 mg/ml solution.

Cells were washed with PBS and then exposed to 3 ml of peptide solution containing either 10% FBS RPMI 1640 media, lipopolysaccharide (3.3×10^{-7} *M*), collagen-like peptides (p1 10^{-7} *M*, p2 10^{-7} *M*, or p3 10^{-7} *M*), or LPS and collagen-like peptides. The combination of LPS and peptides were performed at three different concentrations for each peptide, 10^{-7} *M*, 10^{-8} *M*, and 10^{-9} *M*, all with the same concentration of LPS (3.3×10^{-7} *M*). Initial samples (150 μl) were taken in triplicate from each well, and the exposure media level returned to 3 ml using the appropriate peptide solution, which had been incubating along with the cell cultures. The samples were stored at −20°C until the sampling was complete. The sampling process was repeated every 4 hrs for 28 hrs. All samples were stored at −80°C until IL-1β assays could be performed (17).

Interleukin-1β Assay. Samples were assayed using enzyme-linked immunoabsorbent assay (ELISA) (Human

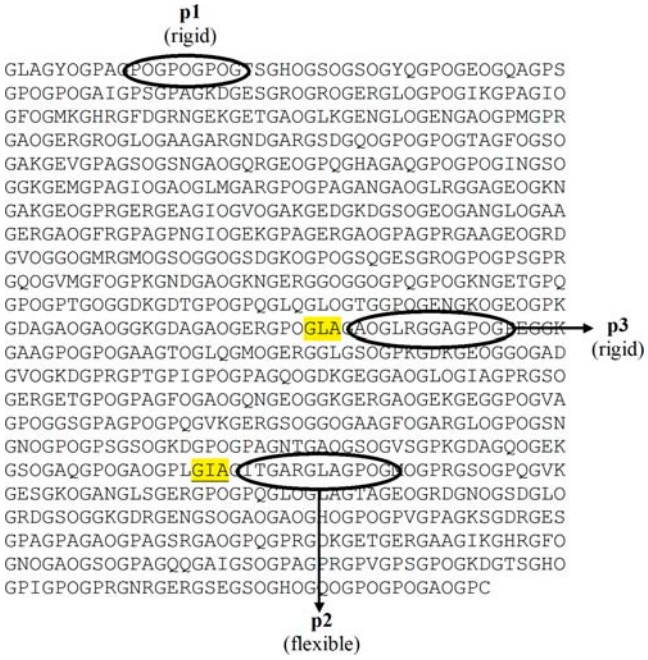

Figure 1. Amino acid sequence of human type III collagen, using 1-letter amino acid codes, and peptides used to mimic various subsequences in type III collagen. Peptide p1 models an imino-rich region of type III collagen, peptide p2 denotes a sequence adjacent to the unique MMP-1 collagenases cleavage site (underlined, highlighted region), and p3 models a region near a scissile bond recognized by gelatinases (yellow highlighted region). The initial cleavage of collagen occurs at the scissile bond adjacent to the region modeled by peptide p2, and it is believed that conformational flexibility of this region enables collagenases to gain access to this site. The region adjacent to peptide p3 is not cleaved until collagen has completely unfolded; therefore it is believed that such regions are relatively rigid and have concealed scissile bonds in the native triple-helical structure. O, hydroxyproline.

Interleukin-1β Colorimetric ELISA Kit; EH2IL1B, Endogen Searchlight, Pierce Biotechnology, Boston, MA), which has a sensitivity of <1 pg/ml and range of 10.2–400 pg/ml as described by the manufacturer. At least three plates were run per peptide concentration. All statistical analyses and plots were done with MatLab (The Mathworks, Inc., Natick, MA).

Results

The choice of collagen-like sequences to study was made based on a conformational analysis of collagen (18–20). Prior work suggests that regions of collagen that contain a significant number of proline and hydroxyproline residues (i.e., the *imino* acids) are relatively rigid, while regions containing a paucity of imino acids are relatively flexible in solution. Molecular modeling studies also imply that conformationally labile sequences within collagen may adopt a partially unfolded conformation with a solvent-exposed backbone (20). From a teleological standpoint, if fully exposed regions led to increased IL-1β production, then chronic inflammation would ensue, as these regions are, in principle, readily seen by inflammatory cells. Therefore, we postulated that conformationally labile sequences would have little effect on IL-1β production *in vitro*, whereas sequences corresponding to rigid, relatively hidden, regions may have more profound effects.

Table 1. Peptide Sequences Used in this Study

Peptide	Sequence
p1	POGPOGPOGPOGPOGPOGPOGPOGPOGPOG
p2	POGPOGPOGITGARGLAGPOGPOGPOGPOG
p3	POGPOGPOGAOGLRGGAGPOGPOGPOGPOG
p0	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT

Three collagen-like peptides formed the basis of this work, two of which have been the focus of prior investigations on the structure of collagen (18). The first peptide, p1, consists of repeating POG (where O is hydroxyproline) triplets and therefore models an imino-rich segment of collagen—a region thought to adopt a rigid triple helical structure (Fig. 1) (19). The second peptide, p2, models an imino-poor region that likely has considerable conformational flexibility (Fig. 1) (19). Last, we constructed another collagen-like peptide, with an amino acid sequence different from peptides p1 and p2, which models a distinct region of collagen that likely represents a relatively rigid segment (peptide p3, Fig. 1). A series of POG triplets were added to the N and C termini of each peptide sequence, as this improves each peptide's solubility, while helping to maintain the expected overall amino acid composition of collagen (Table 1).

These peptides were designed to model conformationally labile and conformationally rigid regions that are thought to exist in all interstitial collagens; i.e., types I, II, and III (11).

In addition to peptides p1 to p3, we also tested the effect of a noncollagenous peptide on HPBMs. These data enable us to determine whether any observed biologic response is specific to the collagen-like peptides in question, or a general feature of amino acid sequences of similar length. A literature search for naturally occurring peptides suggested that the sequence of human glucagon would be suitable (peptide p0, Table 1) (21). To determine how peptides p0 to p3 affect IL-1β production *in vitro*, we incubated HPBMs with each peptide and measured IL-1β production *via* an ELISA. We note that as collagen fragments derived from MMP-mediated degradation are likely unfolded at body temperature (15, 16), peptide samples were heated prior to incubating them with HPBMs. This method ensures that HPBMs also see these peptides in their unfolded state. Last, in order to choose an appropriate concentration for these studies we rely on prior measurements of the concentration of collagen type II fragments in the synovial fluid of patients with RA; i.e., approximately 200 ng/ml (22, 23). Therefore, we incubated HPBMs with the peptides described above at concentrations of 10^{-7} *M* (~270 ng/ml), 10^{-8} *M* (~27 ng/ml), and 10^{-9} *M* (~2.7 ng/ml).

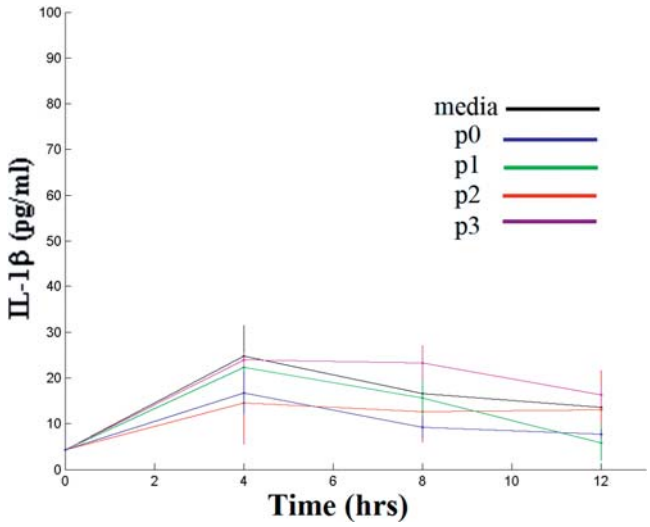

Figure 2. IL-1β production in the presence of peptides p0, p1, p2, and p3.

Peptides p0 to p3 had no significant effect on IL-1β production when incubated with HPBMs alone, suggesting that these sequences do not activate the innate immune response by themselves (Fig. 2). However, as patients with inflammatory diseases like RA may have inappropriate activation of innate immunity leading to tissue destruction, we used LPS, a potent activator of the innate immune response, to determine the effect that each peptide would have on activated monocytes (5, 7, 24). As expected, HPBMs incubated with LPS alone produce elevated levels of IL-1β (Fig. 3A, black line). The addition of the noncollagenous peptide, p0, had no significant effect on IL-1β production (Fig. 3A, blue lines). Although some augmentation of IL-1β levels is observed when a relatively high concentration of peptide p0 is used, this increase is not statistically significant. By contrast, there was an additive effect when either peptide p1 or p3 was incubated with HPBMs (Fig. 3B). While augmentation is observed with both peptides, the greatest effect is associated with peptide p3, that is, elevated IL-1β levels in the presence of peptide

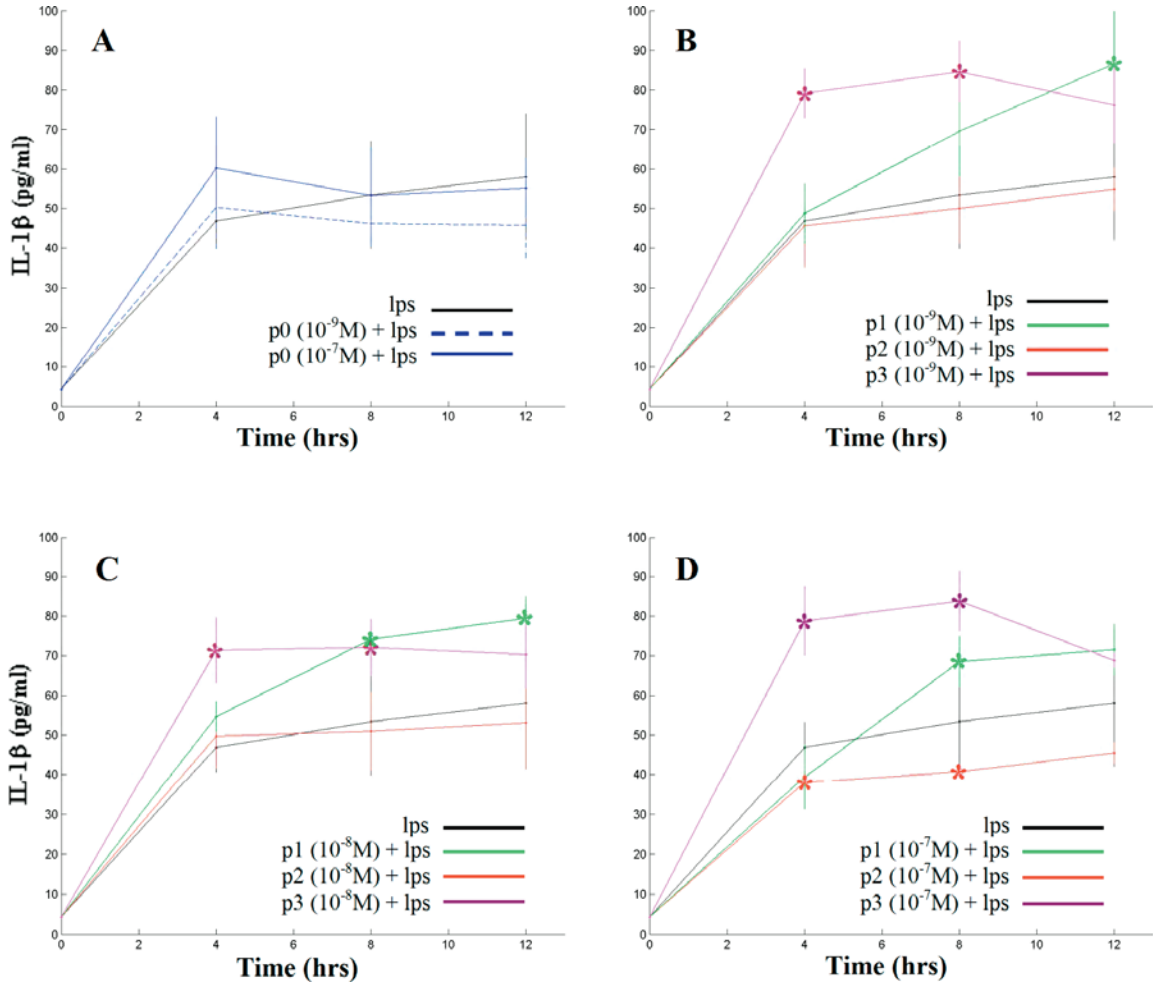

Figure 3. IL-1β production from HPBMs in cell culture. All points represent the mean ± standard deviation of at least three samples. (A) IL-1β production in the presence of glucagon (peptide p0). (B–D) IL-1β production in the presence of peptides p1, p2, and p3. Peptide concentrations are explicitly shown. *Denotes points where IL-1β concentration is significantly different than obtained with LPS alone (Student's *t* test for comparing means with unequal variances, $P < 0.05$).

p1 begin at a later time relative to that seen with peptide p3. Peptide p2, on the other hand, does not augment IL-1β levels (Fig. 3B and C). Moreover, at a relatively high concentration, peptide p2 suppresses LPS-induced IL-1β production (Fig. 3D). Of interest, although peptides p2 and p3 differ by only 4 amino acids (Table 1), they have very different effects on LPS-mediated IL-1β production.

Discussion

Inflammatory mediators play an important role in many disorders of collagen metabolism, and the literature is replete with data suggesting that cytokines affect the progression of such diseases. The converse of this statement, however, is less well established. That is, the effect of structural proteins such as collagen, and their degradation products, on inflammation in general has not been thoroughly investigated.

Structural proteins such as collagen impart tensile strength and durability to tissues, yet data are mounting that these proteins can affect a number of physiologic processes. Our results demonstrate that collagen fragments can be either pro-inflammatory or anti-inflammatory depending on their amino acid sequence. While LPS-induced IL-1β production is augmented in the presence of peptides p1 and p3, IL-1β is reduced in the presence of peptide p2. The relatively small difference in amino acid sequence between peptides p2 and p3 argues that subtle amino acid variation in collagen fragments can lead to very different effects on the inflammatory response.

Peptides p1 and p3 augment IL-1β production at all concentrations studied (Fig. 3B–D), suggesting that this response saturates at low peptide concentrations (Fig. 3B). By contrast, peptide p2 suppresses IL-1β only at concentrations close to that observed in the synovial fluid patients with active RA (Fig. 3D) (22, 23). This finding implies that the suppressive effect of peptides such as p2 is only exerted when there is excessive cartilage destruction. As such, the effect of immunosuppressive peptides, such as peptide p2, may only have a significant effect when there is excessive cartilage destruction, thereby providing a mechanism for limiting the extent of joint inflammation.

To our knowledge, this study is the first to establish a direct link between collagen fragments and cytokine production from human monocytes. At concentrations similar to that seen in the synovial fluid of patients with RA, we find that some peptides can augment IL-1β production, while others suppress IL-1β levels. Given the relatively small difference between the amino acid sequences of peptides p2 and p3, this effect is quite sensitive to amino acid sequence. Furthermore, as we chose peptides for this study based on a conformational analysis of collagen, these findings also demonstrate that interesting biologic insights can be derived from fundamental structural analyses of collagen. More importantly, these results argue that collagen fragments can be either pro-inflammatory or anti-inflammatory depending on their amino acid sequence. Consequently, the extent of joint inflammation in patients with RA, for example, may depend on whether collagenolysis leads to a preponderance of inflammatory or anti-inflammatory peptides. Therefore, in addition to being an important structural protein, collagen is likely to be an active player in inflammatory processes that impact disease.

1. Henson PM. Dampening inflammation. Nat Immunol 6:1179–1181, 2005.
2. Elliott S, Cawston T. The clinical potential of matrix metalloproteinase inhibitors in the rheumatic disorders. Drugs Aging 18(2):87–99, 2001.
3. McDonnell S, Morgan M, Lynch C. Role of matrix metalloproteinases in normal and disease processes. Biochem Soc Trans 27:734–740, 1999.
4. Celentano DC, Frishman WH. Matrix metalloproteinases and coronary artery disease: a novel therapeutic target. J Clin Pharmacol. 37:991–1000, 1997.
5. Smolen JS, Redlich K, Zwerina J, Aletaha D, Steiner G, Schett G. Pro-inflammatory cytokines in rheumatoid arthritis: pathogenic and therapeutic aspects. Clin Rev Allergy Immunol 28:239–248, 2005.
6. Abramson SB, Amin A. Blocking the effects of IL-1β in rheumatoid arthritis protects bone and cartilage. Rheumatology 41:972–980, 2002.
7. Young JL, Libby P, Schonbeck U. Cytokines in the pathogenesis of artherosclerosis. Thromb Haemost 88:554–567, 2002.
8. Ito A, Mukaiyama A, Itoh Y, Nagase H, Thøgersen IB, Enghild JJ, Sasaguri Y, Mori Y. Degradation of interleukin 1β by matrix metalloproteinases. J Biol Chem 271:14657–14660, 1996.
9. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4: 617–629, 2004.
10. Postelthwaite AE, Kang AH. Collagen- and collagen peptide-induced chemotaxis of human blood monocytes. J Exp Med 143:1299–1307, 1976.
11. Fields GB. A model for interstitial collagen catabolism by mammalian collagenases. J Theor Biol 153:585–602, 1991.
12. Overall CM. Molecular determinants of metalloproteinase substrate specificity. Mol Biotechnol 22:51–86, 2002.
13. Vankemmelbeke M, Dekeyser PM, Hollander AP, Buttle DJ, Demester J. Characterization of helical cleavages in type II collagen generated by matrixins. Biochem J 330:633–640, 1998.
14. Eckhardt A, Mikšík I, Charvátová J, Deyl Z, Forgács E, Cserháti T. Proteomics of collagen peptides: a method to reveal minor changes in post-translationally modified collagen by HPLC and capillary electrophoresis. J Liq Chromatogr Relat Technol 28:1437–1451, 2005.
15. Persikov AV, Yujia X, Brodsky B. Equilibrium thermal transitions of collagen model peptides. Protein Sci 13:893–902, 2004.
16. Danielsen CC. Thermal stability of human-fibroblast-collagenase-cleavage products of type-I and type-III collagens. Biochem J 247(3): 725–729, 1987.
17. Peluso G, Petillo O, Melone MAB, Mazzarella G, Ranieri M, Tajana GF. Modulation of cytokine production in activated human monocytes by somatostatin. Neuropeptides 30:443–451, 1996.
18. Brodsky B, Persikov A. Molecular structure of the collagen triple helix. Adv Prot Chem 70:301–339, 2005.
19. Fan P, Li M, Brodsky B, Baum J. Backbone dynamics of (ProHyp-Gly)10 and a designed collagen-like triple-helical peptide by 15N relaxation and hydrogen-exchange measurements. Biochemistry 32: 13299–13309, 1993.
20. Stultz CM. Localized unfolding of collagen explains collagenase cleavage near imino-poor sites. J Mol Biol 319:997–1003, 2002.
21. Bermudez LE, Wu M, Young LS. Effect of stress-related hormones on

macrophage receptors and response to tumor necrosis factor. Lymphokine Res 9:137–145, 1990.

22. Elsaid KA, Jay GD, Chichester CO. Detection of collagen type II and proteoglycans in the synovial fluids of patients diagnosed with non-infectious knee joint synovitis indicates early damage to the articular cartilage matrix. Osteoarthr Cartil 11:673–680, 2003.
23. Elsaid KA, Jay GD, Warman ML, Rhee DK, Chichester CO. Association of articular cartilage degradation and loss of boundary-lubricating ability of synovial fluid following injury and inflammatory arthritis. Arthritis Rheum 52:1746–1755, 2005.
24. Heumann D, Roger T. Initial responses to endotoxins and Gram-negative bacteria. Clinica Chimica Acta 323:59–72, 2002.