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Aspects of the cell biology of the rheumatoid synovial lesion

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I am indeed honoured to deliver the Heberden Oration in 1980, particularly since it was 25 years ago that Walter Bauer was the Heberden Orator.¹ He was then Jackson professor of clinical medicine at Harvard Medical School and chief of the medical services at the Massachusetts General Hospital. Bauer had an enormous influence on the disciplines of rheumatology and internal medicine not only in the United States but throughout the world as well. He also had remarkable skills as a physician and was an extraordinary bedside teacher. Early in his career he became interested in the scientific basis of medicine and in 1927 came to London to work in the laboratory of Henry Dale. The following year he returned to Boston and accepted Cecil Drinker's invitation to head a new unit called the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. With his characteristic energy he proceeded to organise a broad programme of research in diseases of bones and joints.² Bauer believed that in addition to the importance of having scientists and clinicians working side by side there was a need in his department for some individuals who could *combine* skills in research and clinical practice. He took the position that 'scientific rigor and traditional humanitarianism are complementary aspects of medicine' and that some individuals must attempt 'to embrace both points of view.'³ He thought that the mysteries of joint diseases might be solved by study of connective tissue macromolecules, and with this aim he recruited young scientists interested in collagen, mucopolysaccharides and glycoproteins to work with him, while at the same time he helped and encouraged them to establish their own independence. Two of these individuals, Jerome Gross and Roger Jeanloz, professors at Harvard Medical School, are still engaged in exciting research in the Lovett Group at the Massachusetts General Hospital.

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I was thus extremely fortunate to come under the influence of the powerful personality of Walter Bauer when I joined the House Staff of the Massachusetts General Hospital in 1951. Gradually I developed my own interests in metabolic diseases and calcium and phosphorus metabolism. It was inevitable that I move from the mineral phase of bone to the matrix and, in 1958, I established a long term collaboration and friendship with Melvin Glimcher, then a young orthopaedic surgeon who subsequently became chief of the Orthopaedic Services first at the MGH and then at Childrens Hospital Medical Center. With this background, in 1961 Bauer thought that I could direct the Arthritis Unit and, with considerable trepidation, I accepted.

Collagenases and connective tissue degradation

Short, Bauer, and Reynolds⁴ had just completed a detailed long-term study of rheumatoid arthritis, and described the nature of this chronic inflammatory disease. I had become interested in how collagen is degraded in physiological as well as pathological bone resorption, and with encouragement from Jerome Gross I tried to identify collagenase activity in bone homogenates, but was unsuccessful. About that time Gross had begun to investigate the mechanisms of limb regeneration in amphibia (see review by Gross⁵). He too had been unable to find an enzyme in extracts of tadpole tissues capable of degrading native collagen at neutral pH and physiological temperature. He reasoned that, at any one time, the concentration of the putative enzyme might be below limits of detection, possibly because of binding to tissue collagen. With Charles Lapière he then devised an organ culture system using tissue explants placed on collagen gels and discovered that the collagen was lysed around these fragments of living tissue.⁶ Subsequent studies proved that the tissue released a specific collagenase capable of cleaving native collagen across the 3 chains of the

helical molecules at a point 3/4 the distance from the amino terminal end (Fig. 1).⁵⁻⁷

By 1966 it was possible to begin to explore some of the mechanisms of joint destruction in rheumatoid arthritis adapting approaches then being developed in Gross's laboratory. John Evanson joined the Arthritis Unit at that time and in collaboration with John Jeffrey we cultured fragments of rheumatoid synovium and found that all samples examined produced a collagenase with properties similar to those described for the amphibian enzyme.^{8,9} With David Woolley and others Evanson has continued his studies on collagenases, some of which are described in a symposium held recently at the University of Manchester.¹⁰

Further observations on the possible role of collagenase in the destruction of articular structures were made with Edward Harris and Donald DiBona. We demonstrated that active collagenase activity could be detected in synovial fluids from some patients with rheumatoid arthritis, establishing that collagenase is actually produced *in vivo*.^{11,12}

We were impressed by histological evidence which indicated that a diffuse, apparently proliferative synovitis is the dominant feature of the early lesions of rheumatoid arthritis, as originally described by Kulka, *et al.*¹³ In progressive disease this proliferation continues, and structures such as tendon and cartilage are eroded predominantly in regions contiguous with the surface of the cells of the advancing pannus (Fig. 2). This interface would thus be the site of the enzyme substrate interaction.¹⁴ It is therefore logical to assume that the cells of the pannus in this region interact and this interaction results directly or indirectly in degradation of the extracellular matrix. It is probable that the degradation is mediated (at least in part) by the action of collagenases and possibly by other neutral proteases on extracellular matrix macromolecules such as collagens and proteoglycans.¹⁵⁻¹⁷ It had also been shown by Robinson, McGuire, and Levine¹⁸ at the Massachusetts General Hospital and Brandeis University that prostaglandins are produced in large amounts by rheumatoid synovial fragments.

Fig. 1 Schematic representation of two collagen molecules in a collagen fibril to illustrate the site of cleavage by collagenase. An enzyme such as rheumatoid synovial collagenase cleaves across the 3 chains of the collagen molecule at the point indicated by the arrows. The collagen molecules include the 3 chains enclosed by the brackets. The type I collagen molecule shown here is comprised of 2 $\alpha 1$ chains (single line) and 1 $\alpha 2$ chain (double line). The nonhelical regions are shown by the wavy lines. Cross-links between 2 molecules are designated by the dots.

Fig. 2 Photomicrograph of a histological section of a metacarpophalangeal joint from a subject with classical rheumatoid arthritis. Cells of the pannus (Pan) have moved across the joint surfaces with articular cartilage (Car) on either edge. The granulations have also invaded subchondral bone (Bo) at the junctions designated by the arrows. Bar = 200 μ m.

The prostaglandins have been implicated in several aspects of synovial inflammation and could be important in mediation of bone resorption through stimulation of osteoclasts. Knowledge of the factors that modulate production of collagenase and prostaglandins by synovial cells might therefore provide therapeutic clues for controlling the destructive events which occur in rheumatoid arthritis.

Cell culture of the rheumatoid synovium

The rheumatoid synovium contains many different cells including fibroblasts, lymphocytes, macrophages, and cells of blood vessel walls in addition to the so-called synovial cells. It was thus essential to define the role of the cells in this heterogeneous population, and to identify which could be responsible for high levels of collagenase and prostaglandin production, 2 products likely to be involved in the degradative activities of the rheumatoid synovium. Harris and I in 1970 assayed for collagenolytic activity in medium from fibroblasts grown from rheumatoid synovial explants but could detect none, even when the cultures were incubated in the presence of colchicine, which we showed to stimulate collagenase production by synovial fragments.¹⁹ In 1974 Werb and Burleigh²⁰ found that rabbit skin or synovial fibroblasts in monolayer culture released collagenase into the medium, although no activity could be detected with human skin or synovial fibroblasts. Wahl *et al.*²¹ also found collagenase to be produced by cultured guinea-pig macrophages stimulated with endotoxin.

I had just returned from a sabbatical year in Oxford with Henry Harris when Jean-Michel Dayer arrived in the laboratory in 1974. We reasoned that culturing fibroblasts from explants, using conventional methodology, selects for cells which can grow well in artificial, serum-supplemented media. This method of obtaining fibroblasts from synovium might select against more slowly growing cells which could retain the capacity to produce high levels of collagenase. In order to avoid the negative selective effects of growing cells from explants we therefore dispersed the cells from synovectomy specimens using sequential digestion with clostridial collagenase and trypsin.^{22, 23} Although a variable percentage of these dispersed cells adhere to the culture vessel surface, all of the measureable collagenase and most of the prostaglandin are produced by these adherent cells. We were impressed that during the first 1–2 weeks of culture the predominant adherent cells are quite large (20–30 μm or greater in diameter) stellate in form, and with several branching processes (Fig. 3). In the same

Fig. 3 Photomicrograph using phase contrast optics of dispersed adherent cells of a primary culture of rheumatoid synovium. Stellate cells (Ste) and probable monocytes (Mon) are designated by the arrows. Bar = 18 μm .

cultures one can also recognise smaller cells which possess receptors for the Fc fragment of immunoglobulin (a macrophage marker). The large stellate cells do not display this or other conventional macrophage markers, i.e., they do not release lysozyme and do not have the characteristic ultrastructural features of macrophages.²⁴ We have assumed that these cells are fibroblast-like and could well be related to the type B synovial lining cells. In 1961 Hamerman, Stephens, and Barland²⁵ demonstrated in normal synovial lining the presence of delicately branching cells which stain histochemically for DPNH-diaphorase activity. There are many more of these cells in the inflammatory rheumatoid synovium and, in addition, the DPNH-diaphorase activity per cell is greater than normal. We do not yet have specific markers, however, which enable us to establish the identity of the cultured adherent cells with specific cells *in vivo*. It still remains a possibility that there are unique cells with special properties in the rheumatoid synovium which are related neither to macrophages nor fibroblasts. Evidence in support of this possibility has been presented by Krakauer and Zurier.²⁶ Woolley *et al.*^{27, 28} have shown that the large, stellate, adherent synovial cells demonstrate specific fluorescence, using fluorescein isothiocyanate-labelled antibodies to synovial collagenase, consistent with our suggestion that these cells are the source of the collagenase in rheumatoid synovial cultures. The findings of Woolley *et al.*^{27, 28} that macrophages in the primary cultures do not fluoresce are also consistent with the observations of Dayer *et al.*,²² Dayer, Russell, and Krane,²⁹ and Dayer, Robinson,

and Krane³⁰ that collagenase production persists in synovial cultures even after cells with Fc receptors can no longer be detected and lysozyme activity in the medium falls to very low levels.

The synovial cell cultures are usually prepared in the presence of fetal calf serum in the media. Under these circumstances, because of the presence of inhibitors, the collagenase released by the adherent synovial cells can be demonstrated only following activation with proteases such as trypsin; the collagenase is therefore considered to be in a latent form.²² In primary cultures of some specimens, in the absence of serum, sufficient enzyme may be produced so that activation is not required. We found that the latent enzyme has an apparent molecular weight of $\sim 60\,000$ – $70\,000$ daltons and the active enzyme (produced following incubation with trypsin) $\sim 35\,000$ daltons. Vater, Mainardi, and Harris³¹ demonstrated that the latent collagenase in serum-free culture media could also be activated with mercurial compounds such as mersalyl yielding an active enzyme with an apparent molecular weight of $\sim 33\,000$ daltons. These effects of trypsin and mercurials could be accounted for by dissociation of an enzyme-inhibitor complex or, alternatively, by activation of a protease which converts an inactive zymogen to an active enzyme. Until the amino acid sequence of the putative collagenase zymogen is elucidated, however, one cannot with absolute certainty distinguish between these 2 possibilities. If the collagenase has an important function in rheumatoid inflammation, then some mechanism must account for activation of the latent enzyme *in vivo*. Werb *et al.*³² showed that plasmin, produced by plasminogen activator released by cultured rheumatoid synovial cells, could activate the latent collagenase. Cell derived plasminogen activator could thus be important in controlling some of the destructive aspects of synovial inflammation.

The population of adherent cells cultured from rheumatoid synovium is heterogeneous. In early primary culture, levels of collagenase, PGE₂ and lysozyme are high in the culture media. After 1–2 weeks the smaller cells with macrophage markers diminish in number or disappear, associated with a decrease in lysozyme activity. (We consider lysozyme as a macrophage marker,³³ since polymorphonuclear leucocytes—which are also a source of this enzyme—are not present.) At this time levels of collagenase and PGE₂ can still be measured although they are lower than during the first week. It seemed reasonable to us that the decrease in collagenase and PGE₂ might be related to the loss from culture of the monocyte-macrophages or even lymphocytes that could stick loosely to the culture vessel or even

to the surface of the adherent cells. Interactions among these different cells could influence their functions, including release of degradative enzymes.

Possible cellular interactions in the rheumatoid synovium and identification of mononuclear cell factor

Intercellular communication among animal cells can be accomplished in several ways. Some cells communicate through direct contact and membrane-initiated signals or formation of cytoplasmic channels for transfer of some component from the cytoplasm of one cell to another.³⁴ Alternatively, communication could involve release of a soluble substance from one cell which would affect a second cell, directly or by potentiation of the effects of another ligand.

As an approach to this problem we initially utilised peripheral blood as a source of mononuclear cells that might have properties similar to those of the monocytes and lymphocytes in the rheumatoid synovium. These cells could then be cocultivated with the adherent synovial cells, or medium conditioned by the mononuclear cells could be added to the adherent cell cultures. The mononuclear cell population which layers in Ficoll diatrizoate gradients could be further subdivided by selective adherence to nylon wool, plastic, or glass surfaces or by utilising other properties such as the presence of immunoglobulins on the surface or the ability to form rosettes with sheep erythrocytes. Thus, the capacity of lymphocytes or monocytes to influence the function of the synovial cells could be examined.

Human peripheral blood monocytes alone, in the absence of high concentrations of concanavalin-A (con-A), do not produce measureable collagenase by the assay currently employed in our laboratory.²⁴ We have not yet detected collagenase produced by lymphocytes under any circumstances. When monocytes are cocultivated with adherent synovial cells, however, there is a striking increase in collagenase and PGE₂ production. An example of the collagenase stimulation is illustrated in Fig. 4. The stimulation is roughly proportional to the number of monocytes added; in the example shown in Fig. 5 a significant increase in collagenase production was elicited by addition of monocytes to synovial cells at a ratio as low as one monocyte to 20 synovial cells. Although we have not ruled out additional effects of cellular interactions requiring cell-cell contacts, the collagenase and prostaglandin stimulation can be accomplished by the addition of cell-free conditioned media from the mononuclear cell cultures to the synovial cells.^{23 24 29 30 35 36} We were aware of the

Fig. 4 Effects of cocultivation on collagenase production by adherent rheumatoid synovial cells and peripheral blood monocytes. Cells at the second passage were prepared as previously described²³ and incubated in serum-containing media 3 days prior to assays for latent collagenase (Dayer J.-M., Krane S. M., unpublished).

Fig. 5 Effects of increasing numbers of monocytes cocultivated with adherent rheumatoid synovial cells on collagenase production. This culture was obtained from a different specimen from that illustrated in Fig. 4 (Dayer, J.-M., Roelke, M. and Krane, S. M., unpublished).

studies of Wahl *et al.*²¹ which indicated that products released by guinea-pig lymphocytes stimulated with con-A or specific antigens increase release of collagenase by guinea-pig macrophages. I would like to stress, however, that in the synovial cell culture system described here, macrophages are *not* the

target cells. Furthermore, although we assumed in our initial report²⁹ that the stimulating substance was derived from lymphocytes, subsequent experiments utilising purified subpopulations of cells indicated that the monocyte macrophages are primarily responsible for these effects.²⁴ We have therefore termed the stimulating activity mononuclear cell factor, or MCF (Fig. 6).^{23 35-37} The active principle has been purified by gel filtration, ion exchange, and hydrophobic chromatography and by high performance liquid chromatography (HPLC).³⁸ The most highly purified (HPLC) material we have examined stimulates both collagenase and PGE₂ production. Since we are reasonably certain that even these preparations of MCF are still impure, we cannot rule out the possibility that there are different molecules, which copurify, which are responsible for the different biological properties. However, until the chemical structure of MCF is determined, we assume that the same molecule is responsible for these 2 biological effects.

We have found that MCF is resistant to heating to 65°C for 30 min or to exposure to 100 µg/ml trypsin. Nevertheless, on the basis of apparent size and charge properties we have considered MCF to be a protein. MCF is at least partially inactivated by

Fig. 6 Effects of 2 concentrations of mononuclear cell factor, partially purified by gel filtration, on collagenase and PGE₂ production by adherent rheumatoid synovial cells. Methods were those described by Dayer *et al.*²⁴; Dayer, J.-M., Krane, S. M., unpublished.

incubation with papain in 8 M urea, whereas it is not altered by incubation with the urea alone.³⁹ Furthermore, phenylglyoxal, which binds specifically to the side chain of arginine residues, also partially reduces biological activity.³⁹

Modulation of monocyte-macrophage effects

I have alluded to the results of our studies which have indicated that monocyte-macrophages are the cells primarily responsible for MCF production.³⁶ However, the monocytes interact not only with the nonlymphoid synovial cells but also with lymphocytes; these interactions affect release (and presumably synthesis) of MCF. There are many ways in which lymphocytes and macrophages influence respective cellular functions, the best known examples of which are production of mitogenic and migration-inhibitory factors.⁴⁰⁻⁴³

We observed that there is a difference, with respect to MCF production, between purified peripheral blood monocytes and unfractionated mononuclear cells.³⁶ Purified monocytes produce MCF in culture in the absence of added lectins such as pokeweed mitogen (PWM) or phytohaemagglutinin (PHA). Furthermore, MCF release is not increased in the presence of these lectins. However, these lectins do stimulate MCF release by unfractionated mononuclear cells. The unfractionated mononuclear cells contain T and B lymphocytes in addition to monocytes. Although purified populations of T lymphocytes show a mitogenic response to lectins they secrete very low or undetectable levels of MCF. In order to define the mechanism of MCF stimulation by lectins observed in unfractionated monocytes, purified monocytes were mixed with varying properties of T lymphocytes in the presence or absence of PWM. The data from one such experiment are summarised in Fig. 7. When monocytes are added back to the T cells, augmentation of MCF release is observed *if* PWM is present. Thus, there is a complex interaction of monocytes with T lymphocytes with respect to control of MCF production. Although we do not have direct evidence for a soluble factor from the T cells which stimulates the monocytes, this is a good possibility. Moreover, Huybrechts-Godin, Hauser, and Vaes⁴⁴ have shown that rabbit macrophages are stimulated by lymphocyte-conditioned media to release a factor which increases collagenase production by synovial fibroblasts. This collagenase-stimulating factor has several properties in common with MCF. We also have preliminary data suggesting that whereas the addition of small numbers of T lymphocytes to monocytes in the presence of PHA increases MCF release, higher proportions of T lymphocytes

Fig. 7 Effects of pokeweed mitogen (PWM) on MCF production by peripheral blood monocytes, T lymphocytes, and mixtures of these cells. MCF was measured as collagenase activity produced by adherent synovial cells which had been incubated with conditioned media from the peripheral blood cells (from Dayer et al.³⁶).

decrease MCF release (J.-M. Dayer, J. Bréard, L. Chess, and S. M. Krane, unpublished). These results suggest the possibility that there may be T cell 'help' and 'suppression,' analogous to effects of specific T lymphocytes observed in other systems.

Morley⁴⁵ has reviewed the large number of studies describing factors regulating production of prostaglandins by macrophages. Since mononuclear phagocytes have receptors for the Fc portion of IgG and con-A, the demonstration that aggregated IgG and purified Fc fragments and con-A all stimulate prostaglandin synthesis is of considerable interest (Fig. 8).⁴⁶ Monomeric IgG is less stimulatory than the aggregated immunoglobulin, and F(ab)₂ fragments do not alter prostaglandin synthesis by monocytes. Since endotoxin also enhances monocyte prostaglandin production it was critical to exclude the possibility that contaminating endotoxin is not responsible for the observed effects of aggregated IgG and Fc fragments. An approach we used was to take advantage of observations that polymyxin B has high affinity binding sites for bacterial lipopolysaccharides and that this binding by the antibiotic blocks biological effects of the lipopolysaccharides.^{47, 48} In cultured monocytes addition of polymyxin B blocks the PGE₂ stimulation of added lipopolysaccharide but does not inhibit the increase in PGE₂ synthesis produced by Fc fragments (Table 1). These effects attributable to the Fc portion of IgG have been confirmed by Rouzer *et al.*⁴⁹ using a different approach. They found that latex beads coated with immune complexes enhance PGE₂ synthesis by mouse macrophages whereas beads

Fig. 8 Effects of immunoglobulin G₁ (IgG₁) on PGE₂ synthesis by peripheral blood monocytes. IgG₁ either monomeric (IgG₁) or aggregated (aggr. IgG₁) at 50 µg/ml was added to adherent monocytes and PGE₂ measured in media after 3 days of incubation (from Dayer *et al.*²⁴).

Table 1 Effects of immunoglobulins and their fragments and endotoxin on MCF and PGE₂ production by peripheral blood monocytes

<i>Blood monocyte-macrophages</i>		<i>Synovial cells</i>
<i>Addition</i>	<i>PGE₂</i> <i>ng/ml</i>	<i>Collagenase</i> <i>units/10⁶ cells</i>
Experiment 1*		
None	0.2	0.1
IgG 50µg/ml	0.2	0.1
Fab 50µg/ml	0.2	0.1
Fc 50µg/ml	18.9	6.0
Con A 50µg/ml	16.3	7.1
Experiment 2**		
None	0.4	0.08
Endotoxin, 20µg/ml	22.3	6.03
Endotoxin, 20µg/ml plus polymyxin B, 5 µg/ml	0.4	0.01
Fc, 50µg/ml	12.7	3.96
Fc, 50µg/ml plus polymyxin B, 5 µg/ml	11.2	2.84

*Media from monocytes were diluted 1:40 before addition to synovial cells.
**Media from monocytes were diluted 1:10 before addition to synovial cells. From Dayer *et al.*²⁴

coated with F(ab)₂ fragments do not. Furthermore, Sephadex beads coated with immune complexes which cannot be phagocytosed also enhance prostaglandin synthesis. Thus, binding to the Fc receptor is sufficient to produce this enhancement. The observations of Passwell, Dayer, and Merler⁴⁶ and Dayer *et al.*²⁴ indicate further that immune complexes per se are not required, since the isolated Fc portion can replace the complexes. We presume that the Fc fragments exert this effect by binding to the Fc receptor, since they have this capacity, but it is conceivable that the fragments might enter the cell and function by some other mechanism. One must also conclude from the studies of Rouzer *et al.*⁴⁹ that the process of phagocytosis can be dissociated from effects on the Fc receptor even though prostaglandin synthesis by mouse peritoneal macrophages has been shown to be highly coupled to the process of phagocytosis.⁵⁰ It has also been shown that human polymorphonuclear leucocyte elastase can digest IgG.^{51 52} Therefore, it is possible that Fc fragments could be generated at sites of inflammation (such as involved joints in rheumatoid arthritis) and exert their effects directly on target cells. Other neutral proteases released by cells other than polymorphonuclear leucocytes might also liberate Fc fragments.

In view of these effects of aggregated IgG and Fc fragments on stimulating PGE₂ synthesis by monocytes, we were curious to know whether there would be a similar stimulation of MCF production by these ligands. Indeed, addition of aggregated IgG or Fc fragments markedly enhances MCF release by peripheral blood monocytes (Table 1; Fig. 9).²⁴ Synthesis of prostaglandin, however, is not essential for MCF release, since 95% reduction in medium PGE₂ levels by indomethacin does not alter MCF levels.

Interactions with matrix components

Cells not only interact with other cells but are also influenced by contacts with the extracellular matrix. Cell adherence, spreading, replication, and synthetic functions may all be altered by specific matrix components. Collagens or proteoglycans may serve in this role either directly or through the mediation of specific glycoproteins such as fibronectin⁵³ or chondronectin.⁵⁴ Gospodarowicz and Ili^{55 56} have shown that an extracellular matrix can serve as a proliferative stimulus for endothelial cells. The mechanism whereby cell-matrix contacts influence cell function could theoretically be mediated through binding to specific receptors on the cell surface. Data supporting the existence of such receptors on

Fig. 9 Effect of immunoglobulin on MCF production by peripheral blood monocytes. Conditions were similar to those described in Fig. 8. The monocyte conditioned media were then added to adherent rheumatoid synovial cells for 3 days and MCF activity determined by collagenase release (from Dayer *et al.*⁵⁴).

the surface of fibroblasts or the interstitial collagens have been presented.^{57, 58}

It has been possible to demonstrate that collagens alter the production of collagenase in our in-vitro cell culture model in several ways. Biswas and Dayer⁵⁹ incubated adherent synovial cells, or human or rabbit skin fibroblasts with types I, II, or III collagens, added in solution to the culture media. It is probable that the collagen molecules added do not remain in solution at neutral pH and 37°C but polymerise into fibrils. Nevertheless, the presence of the collagens stimulates basal production of latent collagenase by these cells (Table 2). Furthermore, the collagens potentiate the collagenase response to MCF. It should not be assumed however, that the collagens function in a *specific* feedback capacity to regulate their own degradation,

Table 2 Effect of collagen on synovial cell response to MCF

Addition	Collagenase units/10 ⁶ cells
None	0.3
Type I collagen, 0.6 mg/ml	1.5
MCF	18
MCF plus type I collagen, 0.6 mg/ml	120

Synovial cells were incubated for 8 days under conditions described. From Biswas and Dayer.⁵⁹

since there are parallel increases in PGE₂ levels in the culture media as well. These observations are thus consistent with other data presented indicating that prostaglandin and latent collagenase production by synovial cells increase in parallel under the influence of a variety of different stimuli.

We have also looked for collagen effects on mononuclear cells. The rationale for the design of these experiments is based in part on the reasonable likelihood that the monocyte macrophages could interact with the extracellular matrix in a fashion similar to that of the synovial cells. Trentham *et al.*⁶⁰ have reported that patients with rheumatoid arthritis exhibit significant cellular immune responses to types II and III collagens, as measured by the levels of leucocyte migration inhibitory factor (LIF) in the medium produced by cultured peripheral blood mononuclear cells. Stuart *et al.*⁶¹ have also observed that increased medium levels of a lymphocyte-derived chemotactic factor for monocytes result from exposure of cultured peripheral blood mononuclear cells to collagens. This response is not restricted to types II and III collagens, however, and is seen in cells from normal subjects as well. The experimental approach in the studies of Dayer *et al.*⁶² on mononuclear cells was different from that of Biswas and Dayer⁵⁹ in that the collagens were first dried on to the surface of the culture vessels prior to addition of the mononuclear cells from the Ficoll/diatrizoate gradients. It was found that incubation with human, pepsin solubilised types II and III collagens increases production of MCF and PGE₂ by the mononuclear cells (Table 3). Type I collagen is distinctly less stimulatory. However, whereas Trentham *et al.*⁶⁰ had found that

Table 3 Effect of collagen on MCF and PGE₂ production by mononuclear cells

	Blood mononuclear cells		Synovial cells	
	Addition	PGE ₂ ng/ml	Collagenase units/10 ⁶ cells	PGE ₂ ng/ml
Experiment 1	None	10.5	5.8	91.
	Type II collagen	60.7	14.5	271.

From Dayer *et al.*⁶².

stimulation of LIF release by types II and III collagens is restricted to cells from rheumatoid subjects (confirmed in these studies), the collagen-induced increase in mononuclear cell MCF and PGE_2 production is seen in normal as well as rheumatoid cells.⁶² Furthermore, LIF is lymphocyte derived whereas MCF has its source in the monocyte; indeed, the collagen effects are observed in the most purified monocyte populations we have prepared. Thus, in the synovial cells, several different stimuli yield parallel increases in collagenase and prostaglandin. In the monocyte these same stimuli yield increases in MCF and prostaglandin.

Effects of cellular interactions on hormonal responses

I have discussed the results of studies which indicate that functions of component cells of the pannus are modulated by interactions with other cells, mediated at least in part, by soluble factors such as MCF or, perhaps, certain proteases. Modulation also involves interactions of cells with their extracellular matrix. The cells that comprise the pannus would also be exposed to local hormones (e.g., prostaglandins) or hormones carried to the site of the lesion via the circulation (e.g., parathyroid hormone PTH).

The concept that PTH might have a permissive role in focal bone resorption and osteoporosis such as that resulting from local immobilisation had been proposed by Burkhart and Jowsey.⁶³ Although it is generally assumed that PTH has its primary actions on target cells in bone (osteoclasts, osteoblasts, and possibly osteocytes) and kidney, it is apparent that PTH receptors are present in cultured dermal fibroblasts⁶⁴ and adipocytes as well.⁶⁵

We therefore examined responses of the adherent synovial cells to PGE_2 and PTH and found that all cultures tested increase cAMP content when exposed to PGE_2 and some to PTH as well.^{35 64} Newcombe *et al.*⁶⁶ and Castor *et al.*⁶⁷ had previously demonstrated that cells cultured from explants of rheumatoid synovium increase cAMP levels when incubated with prostaglandins of the E series. Since cyclic nucleotides influence many cellular functions, hormone induced changes in cAMP levels could be responsible for modulating cellular interactions in rheumatoid synovial tissues. Any modification of these hormone-induced changes in cAMP content would therefore provide additional mechanisms for regulating proliferative and destructive features of the rheumatoid lesion.

Steven Goldring, Dayer, and I have looked into this question by measuring PGE_2 - and PTH-induced changes in cAMP content in adherent synovial cells (1) under conditions of cocultivation

Table 4. Effects of cocultivation of mononuclear cells and synovial cells on hormone responses

Preincubation	Test incubation		
	Buffer	PGE_2	PTH
		1 μ g/ml cAMP pmoles/well	1 μ g/ml
Monocytes, alone	0.46	3.93	0.96
Synovial cells, alone	1.15	117	4.65
Monocytes plus synovial cells	1.40	7.15	10.5

Cultures were maintained for 48 hours prior to the test incubation. Cultures were then washed and exposed under test incubation conditions for 15 min in the presence of isobutylmethylxanthine, a phosphodiesterase inhibitor. From Goldring *et al.*⁶⁴

with mononuclear cells and (2) after incubation with MCF. When mononuclear cells alone or synovial cells alone are incubated with PGE_2 or PTH, cAMP content usually increases as shown by the example in Table 4. After cocultivation, whereas there is an increase in the magnitude of the PTH-induced cAMP response, there is blunting of the magnitude of the PGE_2 -induced cAMP response.⁶⁴

It is apparent that the pattern of change in response to PGE_2 and PTH is very different in synovial cells after cocultivation with mononuclear cells compared to synovial cells alone. These differences are not accounted for by changes in phosphodiesterase activity, since inhibitors of this enzyme are included in the test incubations. Rather, the differences probably result from alterations in hormone binding affinity, numbers of receptor sites, or possibly changes in coupling of hormone-receptor complexes with adenylate cyclase. Since PTH stimulates bone resorption, increased sensitivity to effects of PTH could be an additional modulator of connective tissue destruction or repair in rheumatoid arthritis.

Although we do not yet have experimental verification for any proposed explanation for the increase in cAMP content in response to PTH in cocultures, a possible mechanism for the decrease in cAMP content in response to PGE_2 can be offered. We have previously reported that there is a relationship between endogenous PGE_2 levels in synovial cell culture media and sensitivity to exogenous PGE_2 , as measured by changes in the magnitude of the cAMP response.³⁵ In those cultures where the PGE_2 is highest, the response to added PGE_2 is lowest. These results are not accounted for simply by receptor occupancy, since the changes are similar even after extensive washing of the cells prior to testing. It has been demonstrated in several systems that the concentration of a given ligand

influences the number or affinity of receptors on cell surfaces for that same ligand (homologous desensitisation or down-regulation) (see review by Catt *et al.*⁶⁸). When the mononuclear cells are cocultivated with the synovial cells there is a striking increase in medium PGE₂ levels. This increase probably accounts for the decrease in the response of the cells to exogenous PGE₂. Confirmation of this interpretation is provided by the examination of cultures to which indomethacin is added in a concentration that nearly completely inhibits endogenous PGE₂ synthesis. This results in a marked increase in the magnitude of the PGE₂-induced cAMP response, suggesting that suppression of endogenous PGE₂ synthesis prevents desensitisation of PGE₂ receptors.

I previously indicated that the effects of coculture on PGE₂ synthesis can be reproduced by incubating the synovial cells with MCF, suggesting that cell-cell contact is not essential for this stimulation. We have also concluded that cell-cell contact is not essential for the changes in PGE₂ responses that result from cocultivation. Preincubation of synovial cells with partially purified MCF results, in all instances, in a decrease in the magnitude of the cAMP responses when cells are test-incubated with exogenous PGE₂. As in the cocultivation studies, addition of indomethacin during the preincubation prevents the subsequent loss of PGE₂-induced cAMP responses. In addition, when cells are preincubated with indomethacin in the presence of MCF there is further potentiation of the cAMP response to PGE₂ (Fig. 10). These stimulating effects of indomethacin are then reversed by additions of exogenous PGE₂ during the preincubation (Fig. 11). Furthermore, the addition of both indomethacin and MCF shifts the dose-response curve to the left and lowers the threshold concentration of exogenous PGE₂ necessary for eliciting an increase in cAMP content.³⁵ It thus appears that MCF acts directly to alter the sensitivity of the synovial cells to PGE₂. As yet, we have no direct measurements of PGE₂ binding to support our hypothesis that this increase in sensitivity most likely involves an increase in either the number of PGE₂ receptors or an alteration in binding affinity. How this could be accomplished is not known, but it is tempting to speculate that some change in the structure of the plasma membrane is responsible. Hirata and Axelrod⁶⁹ have suggested that several different substances which affect cellular functions are capable of initiating chemical and physical changes in membrane lipid structure which lead to redistribution of receptors on cell surface membranes. Hirata and Axelrod⁶⁹ have attributed these responses to ligand-induced alterations in phospholipid

Fig. 10 Effect of incubation conditions on cAMP response of synovial cells to challenge with PGE₂. Cells were incubated for 3 days in medium alone (Control), MCF partially purified by gel filtration, indomethacin (Indo) 14 μ M, or MCF plus indomethacin. Cells were then washed and incubated with test buffer alone (Basal) or 1 μ g/ml PGE₂ for 10 min and cAMP determined in cells plus medium (unpublished; design was similar to experiments described by Dayer *et al.*³⁵).

methylation mediated by two membrane-associated methyl transferases which catalyze the methylation of phosphatidylethanolamine to phosphatidyl choline. The methylation is then thought to result in translocation of the phospholipids from the inner to the outer aspect of the cell membrane. This process may be coupled to Ca⁺⁺ influx, activation of phospholipase with release of arachidonic acid, lysophosphatidyl choline, and prostaglandins. Methylation could result in decrease in membrane microviscosity (increase in fluidity) which could in turn result in enhanced hormone-receptor-adenylate cyclase coupling and increases in generation of cAMP.

There is also a reasonable possibility that the arrangement of the cytoskeleton influences the cellular responses, possibly by indirect effects on the

Fig. 11 Effect of exogenous PGE_2 on subsequent response of adherent synovial cells to PGE_2 challenge. Conditions were those described in Fig. 10 except that half of the wells were preincubated with 100 ng/ml PGE_2 3 days prior to challenge. The increase in cAMP response that resulted from preincubation with indomethacin or MCF plus indomethacin was abolished by preincubation with PGE_2 as well (unpublished; design was similar to experiments described by Dayer et al.³⁵).

plasma membrane. Several compounds which perturb the cytoskeleton increase collagenase production in cultured cells and tissues. For example, colchicine stimulates collagenase synthesis in organ cultures of rheumatoid synovium¹⁹ and in rodent macrophages.⁷⁰ Harris, Reynolds, and Werb⁷¹ and Johnson-Muller and Gross⁷² have found that cytochalasin B also increases collagenase production in cultured cells. In addition, synovial fibroblasts are stimulated by the tumour promoter, phorbol myristate acetate.⁷³ Not only is basal collagenase production increased by these agents, but we have also found that cytochalasin B, or colchicine potentiate the MCF stimulation of both the collagenase and PGE_2 production by synovial cells.⁷⁴ The drug trifluoperazine, which binds to the Ca^{++} calmodulin complex in the cell^{75, 76} also potentiates the response of synovial cells to MCF. Interpretation of the specificity of the effects of drugs such as these must be made with caution since the binding of the phenothiazines may be more related to their hydrophobicity rather than to their tranquilliser properties.⁷⁷ However, trifluoperazine seems only to enhance the collagenase-stimulating effects while it blunts the PGE_2 -stimulating effects of MCF.

Although one might question the biological significance of in-vitro actions of these diverse compounds, it should be recalled that specific receptors for many drugs have been demonstrated in several different tissues. The existence of such receptors has initiated attempts to identify endogenous ligands which could compete with the labelled drug for specific binding sites. The best known example of a drug interacting with a receptor for an endogenous ligand is offered by the opiate peptides. Shoyab and Todaro⁷⁸ have found that a variety of mammalian cells and tissues contain high affinity receptors for biologically active phorbol esters such as phorbol myristate acetate (one of the compounds which enhances collagenase production). Sherline, Schiavone, and Brocato⁷⁹ have also presented evidence for the existence of an endogenous inhibitor of labelled colchicine binding to tubulin. It seems likely that further understanding of the biological consequences of the binding of drugs such as these might reveal features of control of collagenase production by cells.

The observations on effects of cyclo-oxygenase inhibition on potentiating basal and MCF-induced cAMP responses to PGE_2 suggest another paradox associated with the use of drugs such as indomethacin. When PGE_2 synthesis is decreased, ambient concentrations fall. However, inhibition is probably never 100%, with doses commonly employed in therapy. Since the decreases in concentration of PGE_2 are associated with homologous sensitisation (up regulation) to the effects of PGE_2 then the resultant lower levels of PGE_2 could have effects equal to or greater than those in the uninhibited state. This possibility is difficult to prove or disprove in the patient with rheumatoid arthritis, but might account for the somewhat disappointing results of therapy observed with chronic use of cyclooxygenase inhibitors.

Additional consequences of monocyte-synovial cell interactions

There are other effects of mononuclear-synovial cell interactions besides modulation of collagenase production. For example, MCF derived from the monocytes affects DNA synthesis and proliferation in synovial cells.³⁵ A function of mononuclear cell products in modulation of synovial cell proliferation provides a potentially important regulatory mechanism whereby cell-cell interactions in the rheumatoid pannus could determine the cell number and therefore the mass of the pannus. Addition of increasing amounts of mononuclear cell medium alone to synovial cells actually results in decreased uptake of [³H] thymidine and cell proliferation (Table 5). These inhibitory effects of MCF, however,

Table 5 Effects of preincubation conditions on proliferation of synovial cells

Preincubation conditions	Cell number	[³ H]thymidine uptake
	$\times 10^{-4}$	cpm
Medium alone	5.73	501
Indomethacin, 10 μ M	6.54	677
PGE ₂ , 1 μ g/ml	5.56	350
Indomethacin, 10 μ M, plus PGE ₂ , 1 μ g/ml	5.46	274
MCF	5.94	497
MCF, plus indomethacin, 14 μ M	7.27	862
MCF, plus indomethacin, 14 μ M plus PGE ₂ , 1 μ g/ml	5.69	334

Cells were plated at 4×10^4 cells/well. After 3 days cells were washed and incubated under conditions shown for 4 more days. [³H]thymidine was added to replicate wells 1 day before termination of the experiment. From Dayer *et al.*³⁵

are not direct ones but appear to be related to the MCF-induced stimulation of endogenous PGE₂ synthesis, since indomethacin reverses the MCF suppression of proliferation. Our further observations that these stimulatory effects of MCF plus indomethacin are reversed by the addition of small amounts of exogenous PGE₂ add further support for the concept of a critical role for PGE₂ in mediating the inhibition of cell proliferation by MCF.³⁵ Korn, Halushka, and LeRoy⁸⁰ have also observed that mononuclear cell conditioned media suppress fibroblast proliferation. Evidence that this suppression is related to concomitant stimulation of endogenous PGE₂ synthesis by the fibroblasts is provided by observations that growth inhibition is reversed by the addition of cyclo-oxygenase inhibitors. There are several other reports documenting suppression of cell proliferation in different cells which can be ascribed to prostaglandins.⁸¹⁻⁸⁵ It is likely that the prostaglandin-induced increases in intracellular cAMP content account for these effects on growth.^{86, 87} MCF itself does not produce acute increases in cAMP content of synovial cells but levels of the cyclic nucleotide do rise after several hours of exposure, which can be accounted for by increases in medium PGE₂ levels resulting from cellular synthesis.³⁵ These observations are similar to those reported earlier by Castor *et al.*⁶⁷ using preparations of connective tissue activating peptides (CTAPs) which also stimulate prostaglandin synthesis. The CTAPs are extracted from different cells such as platelets, lymphocytes, tumour cells and neutrophilic leucocytes.⁸⁸ The chemical and biological relationship of the CTAPs to MCF has not yet been determined, however.

The mitogenic effects of MCF revealed by blocking target cell prostaglandin synthesis with indomethacin are analogous to the potentiation of PGE₂-induced cAMP responses observed in cells similarly pre-

incubated. One might also speculate that these alterations in mitogenesis are attributable to changes in the structure of plasma membranes. Factors which increase membrane 'fluidity' (decrease viscosity) not only enhance hormone-stimulated adenylate cyclase activity but also increase mitogenesis.

Prostaglandins synthesised by synovial cells in this cell culture system act in turn to affect additional cellular functions. Collagenase production is itself responsive to changes in endogenous PGE₂ levels, although in many cultures marked inhibition of PGE₂ synthesis does not alter release of the protease.^{74, 89} In other cultures, indomethacin blunts, but does not abolish, the collagenase response to MCF. Under the latter circumstances, addition of small amounts of exogenous PGE₂ restores the collagenase response. We are not certain what characteristic of the synovial cell cultures determines the indomethacin effects on collagenase. The behaviour of the human rheumatoid synovial cell cultures, however, is very different from that of rodent macrophages. Wahl *et al.*⁹⁰ observed that when the latter are incubated with endotoxin, collagenase levels markedly increase but the increase is totally blocked by indomethacin. Addition of PGE₁, PGE₂, or dibutyryl cAMP then restores collagenase production.^{89, 91} I propose that the failure of indomethacin to regularly suppress collagenase production in the rheumatoid synovial cell cultures could be explained by an increased sensitivity to PGE₂ (heterologous and homologous sensitisation of PGE₂ receptors) as previously discussed.

Other synthetic functions of the cultured synovial cells modified by MCF and prostaglandins include synthesis of connective tissue macromolecules such as collagens and fibronectin. Levels of labelled medium proteins in cultures of cells incubated with labelled amino acids are increased by MCF.^{92, 93} The major high molecular weight bands seen on SDS-PAGE are collagens (type I and III) and fibronectin. Addition of indomethacin in the absence of MCF enhances collagen and fibronectin synthesis. The presence of both MCF and indomethacin further enhances synthesis. Addition of small amounts of exogenous PGE₂ then suppresses the stimulation, suggesting a regulatory role for PGE₂ in macromolecule synthesis (Fig. 12).

Relationship of MCF to other mononuclear cell products

It is likely that MCF is related to other monocyte products which have been characterised on the basis of various biological effects on target cells different

Fig. 12 Interrelationship of MCF and PGE_2 production by synovial cells. The increased levels of PGE_2 produced by synovial cells in response to MCF act back on the synovial cells. Indomethacin by blocking cyclooxygenase and decreasing PGE_2 synthesis, can unmask certain effects of MCF on synovial cells such as increasing cAMP response to PGE_2 and stimulating cell division.

from those studied in our synovial cell model. One of these products is lymphocyte activating factor (LAF), also known as interleukin 1.^{42 43 94} We have just reported that highly purified preparations of murine LAF have biological activity similar to that of MCF in the rheumatoid synovial culture system and, conversely, partially purified preparations of human MCF have LAF activity.³⁹ Furthermore, both preparations are inactivated by papain in 8M urea and by the arginine reagent, phenyl glyoxal. That MCF and LAF are identical has not been established, however, and it remains possible that the 2 activities could be attributable to similar polypeptides with sequence homologies or to distinct molecules with overlapping biological properties. The situation could be analogous to that recently described for the interferons.^{95 96} Interferons are members of a family of genes. These genes code for proteins which have amino acid sequence homologies, yet are distinct from each other. These differences in structure, in turn, result in different biological properties. Thus, until we obtain amino acid sequences, and even the sequences of the genomic DNA that code for the respective polypeptides, we cannot resolve the question of identity of molecules which have been characterised mainly on the basis of specific but restricted biological properties. The relationship of MCF to factors produced by rheumatoid synovium such as those described by Dingle⁹⁷ and Meats, McGuire, and Russell⁹⁸ therefore, is also yet to be determined.

Summary and conclusions

1. I have described a system in which cells from active rheumatoid synovium are dispersed with proteolytic enzymes to yield a population of

adherent cells. The predominant cells in early stages of culture have a distinctive morphological appearance characterised by large size and numerous branching processes. We have used the term dendritic and stellate to describe them. Cultures containing these cells, which are probably fibroblast related, produce high levels of prostaglandin (PGE_2) and latent collagenase. The collagenase and PGE_2 are thought to play a role in the degradation of extracellular matrix macromolecules that is a major feature of chronic active inflammatory synovitis.

2. The function of these adherent stellate cells in culture can be modulated by interactions with lymphocytes and monocytes in a manner which may be a model for the cellular interactions in vivo. These interactions are at least in part mediated by soluble cell factors, among which a monocyte-derived principle of molecular weight 14 000–25 000 daltons appears to be most important. This factor which we have termed mononuclear cell factor (MCF) is similar in its properties to interleukin 1 (lymphocyte activating factor).

3. Responses to hormones capable of regulating connective tissue remodelling are also influenced by cell-cell interactions in this model system. Depending upon the conditions, one can observe increases or decreases of the cAMP response to hormones such as PGE_2 and parathyroid hormone in the synovial cells. These responses are modified further as a consequence of exposure of the cells to cyclooxygenase inhibitors such as indomethacin.

4. Interactions of monocyte-macrophages with other cells such as T lymphocytes, or with immune complexes (or Fc fragments of immunoglobulin G), or with macromolecules of the extracellular matrix (types II and II collagens) further modulate cell function. The biological effects include stimulation of MCF release, increases in PGE_2 and collagenase production as well as changes in cell proliferation and effects on synthesis of collagens and fibronectin.

5. Although our understanding of the detailed mechanisms of the complex interactions in this model system is incomplete, we hope eventually to use the information derived to design more novel therapeutic approaches than are currently available to deal with the destructive lesion which characterises rheumatoid arthritis.

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