Collagenase production by rheumatoid synovial cells: morphological and immunohistochemical studies of the dendritic cell

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SUMMARY The dendritic cells of dissociated, adherent rheumatoid synovial cell cultures are recognised by their distinctive morphological features—compact cytoplasm around the nucleus and long branched cytoplasmic extensions. Such cells usually composed approximately 10% of the total adherent cell population but could vary from as few as 2% to as many as 40% with different synovial specimens. Histological studies have shown the cells to contain many mitochondria and large spherical cytoplasmic inclusions which often distort the dendritic extensions. Although lysosomes were observed, no evidence for phagocytic activity was obtained. Immunolocalisation studies by means of a monospecific antibody to human collagenase have shown that the dendritic cell attached to a collagenous substratum produces and releases this enzyme in vitro. In contrast collagenase was detected in only a few of the fibroblast- and macrophage-like cells, and it was always intracellular. It is proposed that the dendritic cell may have an important role in the pathophysiology of the rheumatoid joint, particularly with regard to collagenase-mediated cartilage destruction.

The ability of rheumatoid synovial collagenase to degrade cartilage collagen and insoluble tissue collagens (Harris and McCroskery, 1974; Woolley *et al.*, 1975a, 1975b), and the recent immunolocalisation of this enzyme at the cartilage-pannus junction of rheumatoid joints (Woolley *et al.*, 1977) support the view that collagenase plays an important role in the pathophysiology of the rheumatoid joint. The collagenase used in most studies to date has been obtained from the serum-free culture medium (Evanston *et al.*, 1968) of rheumatoid synovial tissue explants. However, Dayer *et al.* (1976) recently reported that monolayer cultures of dissociated, adherent rheumatoid synovial cells released relatively large quantities of enzyme and prostaglandins. Such cultures also revealed an unusual cell type, described as 'stellate' (Dayer *et al.*, 1976) or 'dendritic' (Werb *et al.*, 1977) which was characterised

morphologically by its long, branched cytoplasmic extensions.

The 'stellate' cells were originally recognised in relatively long-term cultures (3-25 weeks) which contained fetal calf serum, and the observation was made that collagenase production might be related to the presence of these cells (Dayer *et al.*, 1976). As other adherent cell types, for example, macrophages, either disappeared or lost certain properties in such long-term cultures we have examined short-term cultures (up to 5 days) of adherent rheumatoid synovial cells in which serum was replaced by lactalbumin hydrolysate after the first 24 h (Werb *et al.*, 1977). This culture system, with its full complement of adherent rheumatoid synovial cells of varying morphology, allowed us to examine which cells were responsible for collagenase production. We report here on the various adherent cell types observed in serum-free cultures and describe our collagenase immunolocalisation studies which implicate the dendritic cell with its unusual morphology as that largely responsible for collagenase production in vitro.

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Materials and methods

RHEUMATOID SYNOVIAL CELL CULTURES

Fresh rheumatoid synovium obtained at time of synovectomy was minced and dissociated by enzymatic digestion as described by Dayer *et al.* (1976). Cells were plated on to glass coverslips (22 × 22 mm) in plastic culture wells 32 mm in diameter (Costar 6-well cluster dishes, Microbiological Associates) at a concentration of 2.5×10^5 cells per well in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, penicillin/streptomycin and L-glutamine added (Werb *et al.*, 1977). After 24 h at 37°C in an atmosphere of 5% CO₂ in air the culture medium was changed. The cultures were washed 5 times with Hank's buffered salt solution (HBSS), thereby removing dead and non-adherent cells, and finally replenished with 2 ml DMEM supplemented with 0.2% lactalbumin hydrolysate in place of serum. This culture medium was replaced every 24 or 48 h and cultures were usually maintained for up to 8 days.

For immunofluorescent and scanning electron microscope studies the cells were plated on to collagen-coated coverslips prepared by adding 1.5 ml of a collagen solution (equivalent to 1 mg collagen in 0.12 M NaCl and 0.02% sodium azide) to coverslips in each well and incubating at 37°C. The thermally reconstituted collagen gels were then allowed to dry and washed 5 times with HBSS prior to use. For histological and enzymatic studies the cells were plated out and treated in the same way on glass coverslips without collagen.

PHASE-CONTRAST MICROSCOPY

Examination of the adherent rheumatoid synovial cells by phase contrast microscopy was performed on coverslips fixed for 5 min at room temperature in 1.5% glutaraldehyde (Fisher Chemicals) buffered with 0.1 M sodium cacodylate, pH 7.5. A Zeiss Photomicroscope III was used, and photographs were made using either Kodak Plus-X or High Speed Ektachrome films.

STAINING PROCEDURES

Janus green and acridine orange at concentrations of 1 µg/ml DMEM were added to the cell cultures for 2 min at room temperature for the vital staining of mitochondria and lysosomes, respectively. For nucleic acid staining, glutaraldehyde-fixed cells were stained with 0.1% toluidine blue in 0.1 M phosphate buffer (pH 6.2), or cells fixed with 4% formalin were stained with haematoxylin and eosin.

ANTIBODY TO RHEUMATOID SYNOVIAL COLLAGENASE

Sheep antibody to purified rheumatoid synovial collagenase was shown to be pure and specific as

judged by double diffusion, immunoelectrophoretic, and enzyme adsorption techniques (Woolley *et al.*, 1976, 1977). For immunohistochemical studies specific IgG antibody was separated from other immunoglobulins by affinity chromatography on a collagenase-Sepharose 4B column using 50 mM sodium citrate buffer, pH 3.8, to dissociate the antigen: antibody complex. The purified immune IgG was dialysed against phosphate-buffered saline (PBS) containing 0.05% sodium azide as preservative.

Double diffusion in agarose gels (1%, w/v) containing 50 mM Tris-HCl buffer, pH 8.0, 0.17 M NaCl and 10 mM CaCl₂, using antibody dialysed against the same buffer, was carried out as described previously (Woolley *et al.*, 1977).

COLLAGENASE IMMUNOHISTOCHEMISTRY

For immunohistochemical studies on adherent synovial cells collagen-coated coverslips were fixed at various times of culture by immersion in 80% ethanol for 30 s and air-dried. After washing in phosphate buffered saline (PBS) the cells were treated with the collagenase antibody (sheep anti-human collagenase immunoglobulins, 100 µg/ml PBS) for 45 min at room temperature and washed for 1 h with 3 changes of PBS. FITC-labelled rabbit anti-sheep IgG (Wellcome Reagents) was then added for 30 min at 60:1 PBS dilution. The coverslips were then washed for 1 h in the dark with 3 changes of PBS, mounted with glycerine/PBS, and examined immediately with a Zeiss Photomicroscope III fitted with epifluorescent illumination. Fluorescence micrographs were made with Kodak High Speed Ektachrome ASA125 film with developing uprated to ASA250.

All studies on collagenase immunolocalisation were accompanied by controls containing non-immune sheep IgG (200 µg/ml) in place of the antibody and the FITC-conjugated antibody reagent alone at a 60:1 dilution. No nonspecific FITC staining was observed with these controls. Similarly, controls with blocking antisera (unlabelled rabbit anti-sheep IgG antibody) applied after exposing the cells to the collagenase antibody were also negative. Moreover, when the antibody IgG was absorbed by incubation with excess purified collagenase for 1 h at 22°C no positive FITC-fluorescence was subsequently observed with cells which demonstrated positive fluorescence with untreated collagenase antibody.

Counterstaining of the cells with eriochrome black was often used to stain the whole cell cytoplasm orange-red and ethidium bromide was used to specifically counterstain the cell nuclei (Poole *et al.*, 1973).

Unfixed cryostat sections (8 μm) of rheumatoid synovial tissue were treated with equivalent amounts (100 μl of 1050 units/ml; 1 unit degrades 1 μg collagen fibrils/min at 37°C) of latent and trypsin-activated enzyme for 5 h and 10 min respectively at 37°C in DMEM. The sections were then washed for 30 min with DMEM at room temperature (22°C) and fixed with 80% ethanol and examined with the FITC-antibody technique.

SCANNING ELECTRON MICROSCOPY

Collagen-coated coverslips with adherent synovial cells were fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) buffered in 0.1M HEPES (Sigma Chemical Corp.) at pH 6.7 for 45 min at room temperature. After 3 washes in the fixative the coverslips were immersed in 1% osmium tetroxide (Ladd Research Industries, Inc., Burlington) for 2 h on ice in a fume hood. After 3 rinses in HEPES the coverslips were dehydrated in successive baths of 30%, 75%, 95%, and 100% ethyl alcohol at room temperature. Samples were then dried in a critical point drying apparatus with liquid CO_2 and mounted on aluminium stubs (Ladd Research Industries, Inc.) with silver conducting paint (Ted Pella Co., Tuscin, California). A 10 Å (1 nm) thickness of gold was sputter-coated on to each specimen. Samples were viewed in a Coates and Welter Instrument Corporation Model 102 Field Emission Scanning Electron Microscope.

COLLAGENASE ASSAYS

^{14}C -labelled guinea-pig skin collagen (6000 dpm per mg) was purified from neutral salt or 0.5 M acetic acid extracts as previously described (Werb *et al.*, 1977). Culture medium harvested from the cell cultures was assayed for both spontaneously active and latent collagenase. The latter was activated *in vitro* by exposure to trypsin (TPCK Sigma, 10 μg per ml for 30 min at 25°C) with subsequent addition of a fourfold excess of soybean trypsin inhibitor (Sigma). The assay conditions for the ^{14}C -labelled reconstituted collagen gel were those described previously (Werb *et al.*, 1977).

Results

MORPHOLOGY AND HISTOCHEMISTRY

Approximately 50% of dissociated rheumatoid synovial cells adhere to glass or plastic surfaces when cultured for 24 h at 37°C in DMEM plus 10% fetal calf serum. Nonadherent cells such as lymphocytes, red cells, and polymorphonuclear leucocytes were removed from the culture by gentle washing after 24 h with HBSS followed by DMEM plus 0.2% lactalbumin hydrolysate. The adherent cells com-

prised 3 principal morphological types described as fibroblast-like, macrophage-like, and dendritic. The fibroblast-like cells make up the bulk of the cell population and appear usually as elongated cells, occasionally with several spiky cytoplasmic processes, and often containing phase-dense cytoplasmic granules and vesicles in a perinuclear region. The macrophage-like cells are usually rounded and appear flattened with ruffled edges and have a large cytoplasm/nucleus ratio. These cells constitute only a small percentage of most dissociated adherent rheumatoid synovial cell populations. The third type of cell is clearly recognised by its multi-branched cytoplasmic processes and has been described as a 'stellate' or 'dendritic' cell (Dayer *et al.*, 1976; Werb *et al.*, 1977).

Fig 1a is a phase-contrast micrograph which illustrates these 3 types of synovial cell. The proportion of dendritic cells in adherent cell cultures varied with each tissue specimen, from as few as 2% to as many as 40%, but usually represented approximately 10% of the total cell population. No significant change in the proportions of the cell types was observed during the first 5 days of culture, and as there was no change in cell density (owing to the lack of cell division in serum-free culture medium) it would appear that cells were not undergoing morphological transformations once they had adhered to a glass, plastic, or collagen substratum.

Various forms of the dendritic cell were observed as illustrated in Fig. 1b. Most were characterised by long, branched cytoplasmic processes, varying in number from 2 or 3 to many (for example, I and II of Fig. 1b). Usually all had flattened, ruffled, terminal extensions to the dendritic processes. Most of the dendritic cells observed were mono- or binucleate, with each oval-shaped nucleus usually containing at least 2 prominent nucleoli. Occasionally multinucleated dendritic cells were observed. In addition to the dendritic cells having compact cytoplasm around the nuclei, the cytoplasmic processes often showed bulbous protrusions where the plasma membrane was displaced by large, rounded inclusions. On vital staining with Janus green some inclusions were positively stained, suggesting that they are mitochondria (Fig. 1c). In contrast, the fibroblast- and macrophage-like cells revealed only a few Janus green-positive inclusions. When the cell cultures were incubated with latex particles for 24 or 48 h there was no evidence of phagocytosis by the dendritic cells. Vital staining with acridine orange to detect lysosomal structures showed their presence in all cell types. Lysosomes of the dendritic cells were of much the same size as those of the macrophage- and fibroblast-like cells,

Fig. 1 *Morphology of dissociated, adherent rheumatoid synovial cells.* 1a. Phase-contrast micrograph of the mixed cell population comprising fibroblast-like (f), macrophage-like (m), and dendritic cells (d). $\times 500$. 1b. Two examples of dendritic cells stained with toluidine blue showing the long branched cytoplasmic processes and compact cytoplasm around the nuclei. $\times 1000$. 1c. Phase-contrast micrograph of cells exposed to Janus green. The dendritic cell contains many positively-stained inclusions. $\times 1000$

but were much smaller than the rounded inclusions which distorted the plasma membrane of the extensions.

COLLAGENASE IMMUNOLOCALISATION STUDIES

When cultured in DMEM plus 0.2% lactalbumin hydrolysate the adherent rheumatoid synovial cells produced collagenase in both active and latent forms (Werb *et al.*, 1977). In an attempt to determine which of the 3 cell types was responsible for enzyme production the cells were cultured on collagen-coated coverslips in DMEM. After 2, 3, or 4

days cultures were fixed in 80% ethanol and the immunolocalisation of collagenase was examined by the indirect or sandwich technique. Fig. 2a shows a phase-contrast micrograph of a mixed population of cells after 3 days in culture. When this cell culture was subjected to antibody treatment, positive FITC fluorescence was observed only with the dendritic cells (Figs. 2b and 2c), suggesting that these cells are responsible for collagenase production at this stage of culture. Fluorescence was clearly associated with the dendritic extensions and the perinuclear region.

As shown in Fig. 3a, in any 1 culture not all dendritic cells showed immunofluorescence at the same time. Here 1 dendritic cell is intensely fluorescent

Fig. 2 *Collagenase immunolocalisation studies of the synovial dendritic cells adherent to collagen-coated coverslips.* 2a. Phase-contrast micrograph illustrating the mixed-cell population used in the immunofluorescent studies shown in 2b and 2c. Note that the FITC-fluorescence is restricted to cytoplasmic extensions which clearly indicate the morphology of a mono- and binucleate dendritic cell respectively. No positive fluorescence is seen with surrounding cells. $\times 500$

whereas an adjacent similar cell is not. Two more examples of dendritic cells yielding positive FITC-fluorescence are shown in Figs. 3b and 3c. These are both binucleate cells, fixed on day 4 of culture, showing extracellular as well as intracellular fluorescence. When fluorescence was occasionally seen with fibroblast- and macrophage-like cells it was always localised to the intracellular, perinuclear region (Figs. 3d, 3e, 3f).

When the same cell culture was plated on to glass coverslips not coated with collagen, the FITC-fluorescence of the cells was considerably reduced and limited to the perinuclear region. It thus seems likely that the more extensive fluorescence seen when these cells were cultivated on collagen gels represented extracellular enzyme bound by its substrate after release. Support for this interpretation was obtained by the observation of fluorescence which took the

Fig. 3 Collagenase immunofluorescence of adherent rheumatoid synovial cells. 3a, 3b, and 3c. FITC-fluorescence associated with dendritic cells after 4 days in culture. $\times 960$. 3d, 3e, and 3f. FITC-fluorescence confined to a perinuclear, intracellular location in fibroblast-like cells (3d, 3f) and macrophage-like cells (3e). $\times 1440$.

form of 'footprints' of dendritic cells which had apparently been detached prior to antibody treatment (Fig. 4a). More conclusive evidence for extracellular enzyme is presented in Fig. 4b, which shows a dendritic cell with punctate FITC deposits of collagen-bound collagenase radiating from the cell, but from which the dendritic processes have either been withdrawn or displaced laterally. This photograph clearly illustrated the association of the extensions (arrowed and counterstained with eriochrome black) with the tracks of extracellular, collagen-bound enzyme.

Since the synovial cell cultures produced both active and latent forms of collagenase and predominantly the latter at the early stages of culture (Werb *et al.*, 1977) it was important to determine what form of enzyme was being detected by the antibody. Double diffusion studies were used to assess immunoreactivity of the antibody with concentrated latent collagenase, activated latent enzyme, and an equivalent amount of active enzyme derived from primary cultures of synovial explants (Woolley *et al.*, 1975b). Six latent enzyme preparations were examined, and 4 produced a precipitin response with the antibody. However, when activated by trypsinisation, latent enzyme preparations produced an increased precipitin response showing a reaction of identity with that produced by an equivalent amount of active enzyme from culture medium of synovial explants (Fig. 5). This finding suggests that the latent collagenase is itself weakly immunoreactive but that the activation process clearly unmasks more antigenic sites. However, as latent enzyme preparations may contain some denatured collagenase, or may undergo

some degree of autoactivation (for example, Stricklin *et al.*, 1977) during immunodiffusion, the immunoreactivity of latent synovial collagenase remains uncertain. It is known that enzyme rendered inactive by complexing with the serum inhibitors α_2 -macroglobulin or β_1 -antitrypsin does not react with antibody in double diffusion experiments (Woolley *et al.*, 1976, and 1977).

Further studies using the immunolocalisation technique to detect latent and active enzyme are illustrated in Fig. 6. Here frozen sections of unfixed rheumatoid synovial tissue, previously shown to have no immunofluorescent reaction, were exposed to equivalent amounts of latent and trypsin-activated collagenase. After fixation in 80% ethanol the sections were subjected to the fluorescent antibody technique. Intense FITC-fluorescence was observed in those sections exposed to active enzyme (Fig. 6a), whereas those exposed to latent enzyme showed relatively little FITC-fluorescence.

SCANNING ELECTRON MICROSCOPY

The unusual morphology of the dendritic cell with its cytoplasmic inclusions and the collagenase immunolocalisation studies prompted further morphological examination of this cell type. Accordingly scanning electron microscopy was carried out, and in Fig. 7a a dendritic cell extended on a collagen film is shown with many spherical inclusions projecting prominently from the dendritic extensions of the cell. The flattened, ruffled terminal extension of each dendritic process is also illustrated. The spherical inclusions often give a 'knobbly' appearance varying in size, and sometimes packing the process along the whole length (Fig. 7b). The long thin cell process

Fig. 4 Detection of extracellular collagenase in rheumatoid synovial cell cultures. 4a. A 'footprint' of positive FITC-fluorescence on a collagen-coated coverslip indicates the outline of a dendritic cell, which was presumably secreting collagenase prior to detachment. 4b FITC-tracks of extracellular collagenase radiating from a cell counterstained with eriochrome black. Only 2 cytoplasmic extensions are observed and these are arrowed. $\times 1440$.

Fig. 5 Immunodiffusion of the specific antibody to rheumatoid synovial collagenase against the latent and activated forms of enzyme obtained from cell cultures. Equivalent amounts of latent enzyme, trypsin-activated enzyme and active enzyme obtained from serum-free tissue culture of explanted rheumatoid synovium, were run against the sheep antibody (Ab, 20 mg IgG/ml). Two latent enzyme preparations (E_{L1} and E_{L2}) are compared with equivalent amounts of active enzyme in A (20 units) and B (30 units). The immunoreactivity of the latent form of collagenase was markedly weaker than that of the active forms. $E'1$ and $E'2$, trypsin-activated latent enzyme; E_{EXP} active enzyme from cultured explants

Fig. 6 FITC-immunofluorescence of rheumatoid synovial tissue sections after exposure to either latent or trypsin-activated collagenase. Frozen tissue sections were exposed to equivalent amounts of active and latent enzyme and examined as described in 'Materials and methods'. Treatment with active enzyme was followed by intense FITC-fluorescence (6a), whereas prior exposure to latent enzyme gave very weak fluorescence (6c) when compared with the control tissue section without enzyme (6b). $\times 480$.

with their broadened terminal extensions and tiny microprocesses which appear to adhere to the collagen fibrils clearly indicate a large surface area.

Discussion

This paper has described our examination in detail of some features of the dendritic cells observed in monolayer cultures of isolated rheumatoid synovial

cells adhering to and spreading on glass, plastic, or a collagen substratum. The cells are characterised as having relatively large single or double refractile nuclei surrounded by compact cytoplasm and long branched cytoplasmic processes. The extensions often contain large spherical or ovoid inclusions as well as mitochondria and lysosomes. The large inclusions often modify the smooth outline of the plasma membrane into bulbous excrescences,

Fig. 7 Scanning electron micrographs of cultured dendritic rheumatoid synovial cells extended on a collagen substratum. Note the flattened terminal extensions with fine microprocesses of the cell illustrated in 7a. Bar, 20 μ m. Both cells have the characteristic spherical inclusions which distort the plasma membrane giving a 'knobbly' appearance seen particularly well in 7b. Bar, 10 μ m.

appearing as 'knobbly' processes when observed with scanning electron microscopy.

It is important to emphasise that within our classification of 'dendritic cells' there appeared to be heterogeneity in both a morphological and functional sense. Cell shapes varied, and the different distributions of cellular inclusions suggest that we may be looking either at several species of the same cell or at a cell type which is capable of morphological changes which might be correlated with different biological activities. In any single culture, as judged by immunofluorescence, not all the dendritic cells were producing collagenase at the same time, even though all were presumably subject to the same environmental conditions. So far we have no information on the time course of collagenase production by individual cells throughout the culture

period, and we cannot exclude the possibility that there is a subpopulation of collagenase-producing dendritic cells.

The immunohistochemical study showed that the dendritic cells were those largely responsible for collagenase production in monolayer cultures, and a preliminary account of these observations has been reported (Woolley *et al.*, 1978). Our findings from immunodiffusion and immunofluorescent studies of both active and latent enzyme preparations suggest that most of the positive FITC- fluorescence represents active enzyme which is collagen-bound. This is also supported by our observations of little or no fluorescence during the first 2 days of culture—when only latent enzyme is found in the culture medium. As active enzyme begins to appear in the culture, this is paralleled by an increase in the amount of positive immunofluorescence.

Our interest in the dendritic cells in rheumatoid synovial cell cultures grew from our observations, stimulated by the studies of Dayer *et al.* (1976), that the dendritic cells found in adherent cell populations after enzymatic dissociation could not be seen growing out from primary explants of rheumatoid synovial tissue. Either they represent a unique cell type or they have assumed this appearance in response to some step in the preparative technique, for example, the collagenase/trypsin treatment during the process of dissociation. Alternatively, the dendritic morphology may result from the influence of inflammatory mediators on certain cells, possibly in a manner analogous to the morphological transformation of cultured bone cells exposed to humoral influences such as hormones and cyclic nucleotides (Miller *et al.*, 1976). The observation that the proportion of dendritic cells in each culture did not change significantly during the first 5 days of culture suggests that this cell is probably not undergoing a sequential morphological transformation similar to that described for cultured macrophages of rheumatoid synovial fluids (Mackay *et al.*, 1974).

The dendritic cells are morphologically similar to those reported in mouse spleen cell cultures by Steinman and Cohn (1973), who also described many mitochondrial structures within the cytoplasmic extensions of the cells. However, a functional characterisation of these cells is not available. Transformation of cultured bone cells to a stellate form by hormones, purine nucleosides/nucleotides, and cytochalasin B has been achieved in vitro (Miller *et al.*, 1976), but again nothing is known about the biochemical function of this altered cell. Similarly, the possible relationship of the synovial dendritic cell to reticuloendothelial cells of similar appearance is uncertain for reasons detailed by

Steinman and Cohn (1973) for their mouse spleen dendritic cells.

As collagenase has been shown to have an important role in cartilage destruction in the rheumatoid joint (Harris and McCroskery, 1974; Krane, 1975; Woolley and Evanson, 1977) further studies on the distribution and physiology of the dendritic cell, especially with regard to the effects of intermediates of chronic inflammation such as lymphokines (Dayer *et al.*, 1977a) and prostaglandins (Dayer *et al.*, 1977b) on morphology and enzyme production would hopefully improve our understanding of the role of this cell in the rheumatoid lesion.

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