Effects of carrageenin on human synovial cells in vitro: morphology, hyaluronic acid production, growth, and the lysosomal system

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SUMMARY Some in-vitro effects of the arthritogenic polysaccharide carrageenin were studied on cells from human synovium. Synovial cells were isolated from intact human knee joints, and cell lines were developed by passaging with trypsin. Carrageenin was ingested by the cells but did not significantly affect cell growth, numbers of lysosomes, intracellular lysosomal enzyme activity (N-acetyl-β-D-glucosaminidase), or release of lysosomal enzyme from cells. Carrageenin produced a reduction in net hyaluronic acid synthesis. It also induced a striking morphological change in a high proportion of synovial cells, characterised by increased spreading over the culture surface and apparent condensation of the cytoplasm into a pattern of ridges. Nonrheumatoid and rheumatoid synovial cells behaved similarly to one another.

The phytoextract carrageenin, when injected into foot pads or joints of certain animals, induces arthritis with many features of rheumatoid arthritis in man (Gardner, 1960; Muirden and Peace, 1969). Carrageenin has recently been used in rabbits to study processes occurring in synovial and cartilage tissue during induction of arthritis (Lowther and Gillard, 1976; Gillard and Lowther, 1976; Lowther et al., 1976). Cartilage was found to lose proteoglycans while lysosomal enzymes increased in the synovium. The contribution of the synovial intima to the total enzyme activity was uncertain and could not be distinguished from that of the infiltrating inflammatory cells, although the intimal cells show villous hyperplasia (Gardner, 1960) and considerable phagocytic activity in carrageenin arthritis (Muirden and Peace, 1969).

After discussion with Lowther and his colleagues it was decided to study the effects of carrageenin on synovial lining cells by utilising cells in culture, thus avoiding the secondary effects of inflammation.

Materials and methods

Cell types. Human synovial cells were isolated from cadaver joints by the procedure of Fraser and Catt (1961). Rheumatoid synovial cells were obtained from patients with persistent effusions (Clarris et al., 1977). Methods of management of synovial cell lines have been described previously (Fraser and McCall, 1965). Cell lines were used while in the euploid phase of their life history (Clarris and Fraser, 1967).

Human embryonic lung fibroblasts (MRC 5), isolated originally in the MRC Laboratories, Hampstead, London, were kindly supplied by Mrs J. Stanley, Commonwealth Serum Laboratories, Australia.

CULTURE

Human serum was obtained aseptically from fasting donors. Fetal calf serum (FCS), Eagle's basal medium (EBM), trypsin and salt solution (Hanks's balanced salt solution-HBBS; Dulbecco phosphate-buffered saline PBS) were obtained from Commonwealth Serum Laboratories.

Carrageenin. Native (mixed λ and χ) carrageenin was kindly donated by Professor D. Lowther. Preparation, purification, and demonstration of biological activity have been described elsewhere (Lowther and Gillard, 1976). Stock solutions were prepared in saline or water and sterilised by boiling for 5 minutes.

Cell growth. Replica cultures were prepared in 50 ml polystyrene culture flasks (Falcon Plastics, 25 cm² culture area). After 24 hours in 10 to 20% FCS, which allowed attachment and spreading, cultures
were randomised and a group selected to determine initial numbers. The remainder were treated with test or control media, sealed and incubated at 37°C for predetermined periods. For final counting, cultures were rinsed with HBSS or PBS and trypsinised (0·5% trypsin in PBS). Cell numbers were determined with a Coulter electronic cell counter.

ENZYMES
N-acetyl-β-D-glucosaminidase (NAGase). Activity was determined in supernatants after centrifugation (1400 g, 15 min) and in cellular extracts prepared from cells in trypsin-saline suspension after counting. The method of assay was as described previously (Le Marshall, et al., 1977).

Lactic acid dehydrogenase (LDH). This was measured in culture supernatants with the facilities of the Department of Biochemistry, Royal Melbourne Hospital, by courtesy of Dr D. Campbell.

HYALURONIC ACID
Tritiated sodium acetate (Radiochemical Centre, Amersham, UK; 25 μCi/ml) was added to control and carrageenin-treated synovial cells in 20% FCS. After 72 h at 37°C supernatants were aspirated and frozen. Cells were counted with a Coulter counter. Hyaluronic acid was separated from proteins by density gradient ultracentrifugation in caesium chloride (density × 1·51 g/ml) by adapting the method of Silpananta et al. (1967) to tissue culture medium (Baxter et al., unpublished).

Results

UPTAKE OF CARRAGEENIN
Control and treated synovial cells were fixed in methanol and stained for 30 min with 0·1% alcian blue in 0·025 M acetate-HCl buffer (pH 5·7) with 0·7 M MgCl₂; alternatively with 0·1% toluidine blue in 30% methanol. Stained particulate matter was observed inside many of the cells, indicating that carrageenin was ingested.

MORPHOLOGY
Synovial cells in carrageenin-treated cultures spread more widely on the culture surface and developed a fenestrated appearance (Fig. 1). This seemed to be due to condensation of the cytoplasm into a pattern of ridges. The onset of the phenomenon depended in part on carrageenin concentration but required at least 16 h. The effect occurred in fetal calf serum, human serum, or mixtures or both, whether fresh or heat-inactivated. The proportions of cells affected varied with individual cell lines and ranged from 10–80% of the total, the maximum effect occurring from 48–96 h. After removal of carrageenin the effect usually persisted for 24 h, but most cells resumed normal morphology within the next 24 h. Carrageenin concentrations from 5 μg/ml–2 mg/ml had no effect on the number or size of cytoplasmic granules seen in synovial cells by phase contrast. At the highest concentrations (1 and 2 mg/ml) synovial cells often developed irregular margins and sometimes clustered into groups but remained fully extended on the culture surface.

Human embryonic fibroblasts (MRC 5) showed no morphological responses to carrageenin (50–500 μg/ml).

HYALURONIC ACID
Hyaluronic acid secretion was strongly inhibited by carrageenin (Table 1). A slight reduction occurred in total istopically labelled protein in the medium.
Table 1  Effect of carrageenin on production of hyaluronic acid by human synovial cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hyaluronic acid (c.p.m./cell, mean ± SD)</th>
<th>Protein (c.p.m./cell, mean ± SD)</th>
<th>Ratio HA/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.69 ± 0.41</td>
<td>4.41 ± 0.62</td>
<td>1.77</td>
</tr>
<tr>
<td>Carrageenin</td>
<td>1.83 ± 0.19</td>
<td>3.58 ± 0.23</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Cells were grown for 72 h in EBM with 20% fetal calf serum. Cell numbers were determined with a Coulter electronic cell counter.

compared with untreated cells, possibly due to precipitation with carrageenin.

GROWTH
The effects of carrageenin on the multiplication of synovial cells were not statistically significant from untreated controls (Table 2).

SUPRAVITAL STAINING
As seen previously (Le Marshall et al., 1977), sucrose (0.08 M) caused an increase in intracellular organelles, which fluoresced bright red with acridine orange. These particles have been identified as lysosomes (Allison and Young, 1964, 1969). However, carrageenin produced no differences from untreated controls.

N-ACETYL-β-D-GLUCOSAMINIDASE
Sucrose (0.08 M) and carrageenin up to 500 µg/ml did not interfere with the enzyme assay. At higher concentrations carrageenin became inhibitory. Fetal calf serum possessed high background activity, but other available animal sera had inconsistent growth-promoting properties. Heat-inactivated human serum was therefore used throughout this part of the study.

As observed previously (Le Marshall et al., 1977), sucrose produced increased lysosomal enzyme activity in synovial cells but did not cause release into the medium (Table 3). Carrageenin did not produce any consistent difference in intra- or extracellular levels of NAGase during 72 h or longer periods of treatment.

In all of the above studies the responses of non-rheumatoid and rheumatoid synovial cells to carrageenin were identical.

Discussion
The morphological response of synovial cells to carrageenin was similar to the effect of dibutyryl cyclic AMP (DB cAMP) and other agents, which cause increase in endogenous cAMP (Fraser et al., 1979). Phase contrast and scanning electron microscopic observations in that study indicated that the apparent fenestration of cells was due to a pattern of cytoplasmic ridging. Electron microscopy of other cell types after cAMP shows aggregation of microfibrils around the microtubules (Willingham and Pastan, 1975), and the effect of carrageenin might therefore be due to alteration in the cytoskeletal structure related in some ways to change in endogenous cAMP levels.

Morphological changes in synovial cells induced by DB cAMP were accompanied by increased hyaluronic acid secretion (Fraser et al., 1979). Uptake of undegradable saccharides is usually followed by morphological and enzymic evidence of activation in the lysosomal system (Le Marshall et al., 1977). It therefore appears that subsequent events in both segments of cellular activity—endocytosis and lysosomal activation on the one hand and the specific

Table 2  Growth of synovial cells in carrageenin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of observations</th>
<th>Ratio final count/initial count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>1.41—2.90</td>
</tr>
<tr>
<td>Carrageenin 50 µg/ml</td>
<td>8</td>
<td>1.38—2.69</td>
</tr>
<tr>
<td>Carrageenin 500 µg/ml</td>
<td>7</td>
<td>1.74—2.61</td>
</tr>
</tbody>
</table>

Experiments were for 72 h in EBM with 20% (v/v) heat-inactivated human serum. *Not statistically significant (Wilcoxon matched-pairs signed-ranks test).

Table 3  Effect of carrageenin and sucrose on N-acetyl-β-D-glucosaminidase activity of human synovial cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of observations</th>
<th>N-acetyl-β-D-glucosaminidase**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intracellular Mean ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extracellular Mean ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proportion excreted %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>176 ± 79.6</td>
</tr>
<tr>
<td>Carrageenin 50 µg/ml</td>
<td>7</td>
<td>179 ± 78.5</td>
</tr>
<tr>
<td>Carrageenin 500 µg/ml</td>
<td>7</td>
<td>182 ± 53.7</td>
</tr>
<tr>
<td>Sucrose (0.08 M)</td>
<td>7</td>
<td>239 ± 93.5</td>
</tr>
</tbody>
</table>

Experiments were for 72 h in EBM with 20% (v/v) heat-inactivated human serum. *Statistically significant (P<0.01, Wilcoxon matched-pairs signed-rank test). **µg p-nitrophenol released from p-nitrophenol-2-acetamide-2-deoxy-D-glucopyranose per cell per h, 35°C.
cytoplasmic reorganisation associated with enhanced hyaluronic acid secretion on the other—are blocked in the initial stages of response after exposure to carrageenin. It is also possible that the apparent inhibition of cellular response does not extend to the secretion of neutral proteases, since other studies indicate that the lysosomal and neutral protease enzyme systems may respond independently after uptake of certain agents (Web and Reynolds, 1974). Experiments are being considered to test these hypotheses.

The in-vitro results raise questions on the synovitis in carrageenin-induced arthritis in animals. The increased lysosomal enzymes in synovial tissue (Muirden and Peace, 1969) might arise specifically from the action of carrageenin on macrophages in the synovium (Allison et al., 1966) or be secondary to inflammation triggered by carrageenin in some other way. Carrageenin was found in this and other studies (Turner and Magnusson, 1962) to precipitate with some serum proteins and would be expected to come out of solution in synovial fluid. Inflammation might be initiated by phagocytosis of carrageenin-protein complex by leucocytes in the synovial fluid, with subsequent discharge of degradative enzymes (Weissmann et al., 1972; Oronsky et al., 1973). It is also possible that aggregated protein in the complexes might evoke an autoimmune response. Rheumatoid-factor like antibodies have not been sought in carrageenin-treated animals, but the synovial infiltration is characterised by lymphocytes and plasma cells similar to the pattern in rheumatoid arthritis (Muirden and Peace, 1969).

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References


