Effect of hyperoxia on articular tissues in organ culture

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SUMMARY The effect of hyperoxia on pig articular tissue has been studied in organ culture. Hyperoxia (55% O₂) causes an increased release of hydroxyproline and collagenolytic activity from synovial tissue as compared with control explants in 20% O₂, but neither 55% nor 95% O₂ has any effect on the breakdown of isolated cartilage during 10 days in culture. When synovium and cartilage are cultured in contact, the breakdown of cartilage collagen is greater in the hyperoxic (55% O₂) group than in the controls (20% O₂), but the breakdown of proteoglycan is not increased. The enhanced collagenolytic action is due to an increase in the direct effect of the synovial tissue on the cartilage matrix. In 20% O₂ the synovium causes the chondrocytes to degrade the cartilage matrix, but this effect is inhibited by 55% O₂.

In osteoarthritis catabolic enzymes from the chondrocytes are thought to be involved in the destruction of the cartilage (Howell, 1975), and there is increasing evidence that the chondrocytes also play a role in the breakdown of articular cartilage in rheumatoid arthritis (Chaplin, 1971; Muirden *et al.*, 1974). In both these diseases raised levels of lysosomal enzymes have been found in the cartilage (Sapolsky *et al.*, 1973; Muirden *et al.*, 1974), and recently a collagenolytic agent also had been described in osteoarthrotic cartilage (Ehrlich *et al.*, 1977). The factors controlling the behaviour of the chondrocytes in these diseases are not known.

Hyperoxia has been shown to increase the lysosomal enzyme activity of HeLa and chick embryonic cells in cell culture (Allison, 1965). A similar effect was found by Sledge and Dingle in embryonic limb bone rudiments maintained in organ culture in 85% oxygen; after 8 days there was loss of metachromatic staining in the cartilage matrix associated with increased cathepsin D activity in the medium (Sledge and Dingle, 1965). Loss of proteoglycan from isolated articular cartilage in organ culture has been reported in rabbit knee cartilage cultivated in 90% oxygen (Brighton *et al.*, 1974) and in human cartilage from the femoral head in 95% oxygen (Jacoby and Jayson, 1975). At this level of oxygen,

however, three-quarters of the chondrocytes in the human cartilage were dead after 8 days.

Recently Fell and Jubb have studied the destructive effect of synovial tissue on pig articular cartilage in organ culture (Fell and Jubb, 1977). In this system the synovium caused the breakdown of the cartilage both by a direct presumably enzymatic action on the matrix, which required contact between the tissues, and by an indirect action mediated through the chondrocytes via the culture medium, which could operate even when the tissues were widely separated.

Although the level of oxygen is low in rheumatoid synovial cavities (Falchuk *et al.*, 1970), a 3-fold increase in blood flow and oxygen uptake by rheumatoid synovium has been reported (Goetzel *et al.*, 1971).

It was decided to study the effect of hyperoxia on pig articular cartilage and synovium and also on the interaction between the 2 tissues during cultivation in organ culture.

Materials and methods

TISSUE

The articular tissues were obtained from the metacarpophalangeal joints of pigs aged 6 to 9 months and weighing 50 to 75 kg, which were routinely killed at a local slaughterhouse.

Cartilage. Samples of cartilage were cut from the distal end of the third and fourth metacarpals. A

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strip was taken from the condylar ridge of each joint, the zone of calcified cartilage and the underlying marrow being avoided. Each strip was cut into 2 equal parts, one half acting as an experimental and the other as a control explant.

Synovial tissue. It was found to be technically impossible to obtain equivalent explants of intact synovium (see Fell and Jubb, 1977). To surmount this difficulty sheets of joint capsule were removed from a number of joints, and the synovial villi and some subsynovial tissue were cut off, finely divided, and the minced tissue from a number of joints was pooled. The pooled synovial mince was washed, well stirred, and measured amounts served as explants.

ORGAN CULTURE

Medium. The tissues were maintained in BGJ₅ with 15% heat-inactivated normal rabbit serum. BGJ₅ is the chemically defined medium BGJ (Biggers *et al.*, 1961) supplemented with 5 mg sodium acetate and 15 mg ascorbic acid per 100 ml. 1.5 ml medium was used for each culture and was changed every 48 h, the old medium being kept for analysis.

Culture system. The culture system was that previously described by Fell and Barratt (1973). Two flat-bottomed glass vessels were enclosed in a Petri dish containing some isotonic saline, which acted as a moist chamber. The explants were placed on a millipore filter (13 mm diameter; pore size 8.0 µm) and supported in the vessels by a table of stainless steel gauze. A measured volume of synovial tissue (0.025 ml) was deposited on the millipore substrate, and a cartilage explant was placed either on the synovium with the articular surface downwards or at a distance from the synovium. Cultures were also made of isolated cartilage or isolated synovial tissue only.

The Petri dishes were stacked in a modified Fildes Mackintosh jar and gassed with one of the following mixtures: 20% O₂+5% CO₂+75% N₂; 55% O₂+5% CO₂+40% N₂; 95% O₂+5% CO₂. The cultures were maintained for 10 days at 38.5 C (body temperature of the pig is 39.3 C).

HISTOLOGY

After 10 days' cultivation the explants were fixed as previously described (Fell and Barratt, 1973). Serial sections were cut at 6 µm and stained with either toluidine blue or the sequence celestine blue, Carazzi's haematoxylin and van Gieson's stain. Toluidine blue stains proteoglycan metachromatically a deep purple, and the van Gieson colours collagen red, with the haematoxylin giving good nuclear detail.

BIOCHEMICAL ASSAYS

The used culture medium was collected at 2-day intervals. Each experimental group consisted of 4 explants, and the medium from these cultures was pooled before analysis. It has already been shown that there is good correspondence in the behaviour of paired cartilage explants when they are cultured under identical conditions (Fell and Jubb, 1977). The analytical methods used have been described in detail elsewhere, and references to them are given in the text.

Proteoglycan. Proteoglycan released into the culture medium was estimated by a modification of Whiteman's alcian blue assay (Whiteman, 1973; Dingle *et al.*, 1975), which measures polyanionic molecules. In view of the interference with the assay by medium from synovial cultures, the samples were routinely treated with trypsin (10 mg/ml) before assay (Fell and Jubb, 1977). The standard used for reference was chondroitin sulphate (ex-shark's fin, Koch-Light).

Collagen. The amount of hydroxyproline in the medium was determined as an indication of collagen release. It was estimated after acid hydrolysis by the method described by Woessner (Woessner, 1961; Burleigh *et al.*, 1974).

Collagenolytic activity. This was measured by the breakdown of reconstituted collagen fibrils (Werr and Burleigh, 1974). Purified skin collagen (rat, labelled with ¹⁴C-glycine was used as substrate and clostridial collagenase (Worthington) as a standard; the results were expressed as units of bacterial collagenase. The samples were pretreated with potassium thiocyanate to dissociate enzyme inhibitor complexes (Nagai *et al.*, 1975).

Results

ISOLATED ARTICULAR CARTILAGE

Explants of cartilage were cultured in 20%, 55%, or 95% O₂. After 10 days histological examination showed no sign of depletion of either proteoglycan or collagen in the 2 hyperoxic groups as compared with their paired controls in 20% O₂ (Table 1, Fig. 1). In some of the explants exposed to 95% O₂ pyknotic nuclei were present among the superficial cells in immediate contact with the atmosphere, but otherwise the cells of the hyperoxic cartilage appeared viable.

The cumulative release of proteoglycan into the medium was slightly lower in the hyperoxic group, but this was not statistically significant, and there was no release of hydroxyproline. Collagenolytic activity was not detected (Table 2).

ISOLATED SYNOVIUM

The synovial explants maintained in 55% O₂

appeared macroscopically smaller than the controls in 20% O₂, but no histological difference could be seen. The explants were a jumble of fibrous tissue, synovial lining cells, and fragments of blood vessels. In both groups the cells were viable, with many

invading the millipore substrate, and mitotic figures were common.

Analysis of the used medium (Table 2) showed significantly more proteoglycan and hydroxyproline in the hyperoxic than in the control cultures. Only a small amount of material precipitable with alcian blue was released from either set, and its exact nature has not been determined. Latent collagenolytic activity was present in the medium of both hyperoxic and control cultures; it was 36% higher in the hyperoxic group.

Table 1 *Effect of oxygen on isolated cartilage in organ culture for 10 days. Analysis of histology**

No. of explants	% oxygen	Loss of proteoglycan (toluidine blue)						
		++++	+++	++	+	±	0	
12	20					2	4	6
12	55					3	9	
9	95					3	6	

*The degree of depletion of the proteoglycan and collagen of the matrix, as shown by their staining reactions, is expressed by the + signs. Proteoglycan +++++=total absence of metachromasia; 0=no effect. Collagen +++++=complete disappearance of all intercellular material; 0=no effect. Intermediate changes in both components are represented by +++, ++, +, ±. The number of explants in each group is shown. (In the legends for the photographs, each specimen illustrated is graded according to the above scale.)

CARTILAGE IN CONTACT WITH SYNOVIUM

After 10 days' cultivation in contact with synovial tissue metachromasia was often completely absent in both the hyperoxic (55% O₂) and the control explants (Fig. 2, Table 3), but breakdown of collagen was greater in the hyperoxic cartilage (Table 3). In sections stained with van Gieson's stain it was seen that the degradation of the collagen began at the

Fig. 1 (Upper) Section of isolated cartilage maintained in organ culture with 55% O₂ for 10 days. No loss of metachromasia (grade 0, see Table 1). Toluidine blue, ×35. (Lower) Section of paired control cartilage maintained in 20% oxygen for 10 days. No loss of metachromasia (grade 0). Toluidine blue, ×35

Table 2 *Analysis of culture medium. Each value is the mean from at least 5 separate experiments*

	Cumulative release of proteoglycan			Cumulative release of hydroxyproline			Cumulative release of collagenolytic activity		
	20% oxygen µg/ml	*55% oxygen	†P value	20% oxygen µg/ml	55% oxygen	P value	20% oxygen units/ml	55% oxygen	P value
Isolated cartilage	86 (13)**	−14% (12)	NS	0	0		0	0	
Isolated synovium	23 (8)	+50% (34)	<0.05	50 (3)	+45% (13)	<0.005	3.17 (3.5)	+36% (19)	<0.01
Cartilage + synovium in contact	208 (102)	+5% (7)	NS	56 (16)	+62% (24)	<0.001	4.0 (1.9)	+58% (38)	<0.005
Cartilage + synovium separated	180 (25)	−20% (19)	NS	86 (24)	+24% (12)	<0.02	1.33 (0.75)	+51% (22)	<0.01

*Results in 55% oxygen expressed as a percentage of their paired controls in 20% oxygen. †Paired explants analysed by paired *t* test. NS not significant. **Standard deviation.

Fig. 2 (Upper) Section of cartilage and synovium in contact after 10 days in 55% O_2 . Complete loss of metachromasia (grade +++++). C=cartilage, S=synovium, M=millipore. Toluidine blue, $\times 35$. (Lower) Section of paired control explant in 20% O_2 for 10 days. Complete loss of metachromasia (grade +++++). Toluidine blue, $\times 35$.

Table 3 Effect of oxygen on cartilage in contact with synovium in organ culture for 10 days. Analysis of histology*

No of explants	% oxygen	Loss of proteoglycan (toluidine blue)						Loss of collagen (van Gieson)					
		++++	+++	++	+	$\pm$	0	++++	+++	++	+	$\pm$	0
13	20	6	5	1	1				1	1	10	1	
15	55	8	3	4				4	4	6	1		
not significant						$\dagger p < 0.001$							

*The degree of depletion is described in Table 1.

$\dagger$ Paired explants analysed by paired *t* test.

margin of the articular zone, the surface of which was in contact with the synovial tissue. There was considerable variation in the degree of breakdown in different pairs of explants, and in some the matrix had almost disappeared in the specimens cultured in high oxygen (Fig. 3).

The biochemical findings confirmed the histological observations. There was little difference either in the amount of proteoglycan liberated by the 2 groups after 10 days or in the pattern of its release (Fig. 4). In the hyperoxic cultures, however, the collagenolytic activity of the used medium was about 50% higher than that of the controls and about 60% more hydroxyproline was released (Table 2).

CARTILAGE SEPARATED FROM SYNOVIUM

From the above results it was not clear whether the hyperoxia accelerated the breakdown of cartilage in contact with synovium by enhancing the direct enzymatic action of the synovium on the cartilage

matrix, by increasing its indirect effect mediated through the chondrocytes, or by stimulating both processes (Fell and Jubb, 1977). The experiments were therefore repeated with the cartilage and synovium grown on the same Millipore but at a distance from each other; this system would exclude the direct action of the synovium, for which contact between the 2 tissues is required.

Histological examination of the cartilage explants after 10 days' incubation under these conditions showed that, far from increasing the indirect action of the synovium, the hyperoxia actually diminished the loss of both metachromatic material and collagen from the matrix (Fig. 5, Table 4). The cartilage was placed on the millipore with the articular surface upwards; in the controls (20% O_2) the area immediately above the millipore, which included the cells of the columnar zone, showed depletion of the matrix and the transformation of the chondrocytes into basophilic cells of irregular shape, often

Fig. 3 (Left) High power of same explant as in Fig. 2 (upper). Cartilage in contact with synovium after 10 days in 55% O_2 . Loss of intercellular collagen from the cartilage with viable chondrocytes. C=cartilage, S=synovium. van Gieson, $\times 250$. (Right) High power of paired explant after 10 days in 20% O_2 . Collagenous component of cartilage matrix intact. van Gieson, $\times 250$

Fig. 4 Cumulative release of proteoglycan (PG) from explants of cartilage in contact with synovium. ●=explants maintained in 55% O_2 , ○=explants maintained in 20% O_2 . Graphs represent the mean values from four paired explants

in active mitosis (Fig. 6). These changes, though not entirely suppressed, were less obvious in the cultures exposed to 55% O_2 .

Biochemical assay of the used medium showed that the hyperoxic tissues released less proteoglycan into the medium than the controls in 20% oxygen (Table 2). Virtually all of this material is derived from the cartilage, as the synovium liberates very little.

The hydroxyproline in the used medium, which reflects the breakdown of collagen comes from both the cartilage and the synovium, and in these experiments it was only 24% above that in the used control medium, in contrast to a difference of 60% when the 2 tissues were in contact. The latent collagenolytic activity, which is produced entirely by the synovial explant, was about 50% higher in the hyperoxic group.

These results imply that the increased breakdown of cartilage matrix induced by hyperoxia (55%) when the cartilage is in contact with the synovial tissue is due to an enhancement of the direct enzymatic action of the synovium on the matrix. The release of proteoglycan is unaffected, and the increased destruction of the matrix is due to breakdown of collagen, which correlates with an increased release of collagenolytic activity from the synovium. This effect is seen despite inhibition of the collagenolytic action of the chondrocytes.

Fig. 5 (Upper) Section of cartilage maintained at a distance from synovial tissue in organ culture. Ten days' culture in 55% O₂. Patchy loss of metachromasia (grade +). Toluidine blue, ×35. (Lower) Section of paired control explant maintained in 20% O₂ for 10 days. Zone of loss of metachromasia above millipore (grade ++). Toluidine blue, ×35

Table 4 Effect of oxygen on cartilage separated from synovium in organ culture for 10 days. Analysis of histology*

No of explants	% oxygen	Loss of proteoglycan (toluidine blue)						Loss of collagen (van Gieson)					
		++++	+++	++	+	±	0	++++	+++	++	+	±	0
16	20			2	8	5	1			2	10	4	
15	55			2	7	5	1				4	5	6
		†p<0.005						†p<0.001					

*The degree of depletion is described in Table 1. †Paired explants analysed by paired t test.

Fig. 6 (Left) High power of same explant as in Fig. 5. (Upper) Cartilage at a distance from synovium, after 10 days culture in 55% O₂. Minimal depletion of collagen. van Gieson, ×250. (Right) High power of paired explant after 10 days in 20% O₂. Loss of intercellular collagen with proliferation of chondrocytes. M=millipore, mi=mitosis. Van Gieson, ×250

Discussion

In the organ cultures of isolated articular cartilage from pig hyperoxia (55% and 95% oxygen) neither caused loss of metachromasia nor increased the amount of proteoglycan shed into the medium; indeed slightly less was released than by the controls in 20% oxygen. There was no evidence of loss of collagen. Jacoby noted some loss of proteoglycan from adult human articular cartilage after 8 days' cultivation at an oxygen tension of 95%, but more than three-quarters of the nuclei were pyknotic, which raises the possibility that the degradation of proteoglycan might have been due to the liberation of autolytic enzymes from these necrotic cells (Jacoby and Jayson, 1975).

In the present experiments isolated synovial tissue released a collagenolytic agent into the medium. The synthesis of collagenase by synovial tissue *in vitro* has been reported by others (Evanson *et al.*, 1967; Werb and Reynolds, 1975), and raised levels have been found in cultures of rheumatoid synovium (Harris *et al.*, 1969). The exact nature of the collagenolytic agent produced by synovial mince in culture is not known, nor are the factors that control its release understood. Its synthesis may be a response to the trauma inflicted on the tissue during preparation and possibly to the abnormal environment to which it is exposed in an organ culture. Although some was found in the medium at normal (20%) oxygen tension, the amount was increased by hyperoxia. Williams (1973) found that the optimum concentrations of oxygen for maintaining human synovium in organ culture were between 20% and 50%. Probably the tissue, which has an aerobic metabolism (Bywaters, 1937), is physiologically more active at the higher level.

In earlier work Fell and Jubb (1977) found that, when cartilage was cultivated in contact with synovium in 20% oxygen, its matrix was severely depleted though the chondrocytes survived. This was shown to be caused by 2 mechanisms: a direct, probably enzymatic effect of the synovial cells on the matrix and an indirect action mediated through the chondrocytes via the culture medium. In the present study hyperoxia greatly enhanced the breakdown of cartilage in contact with synovium, apparently by stimulating the direct action of the synovium on the matrix. When the direct effect was eliminated by cultivating the cartilage at a distance from the synovial explant, the partial breakdown of the matrix seen at 20% oxygen was reduced by exposure to 55% oxygen.

Thus the indirect action of the synovial cells on the matrix, in contrast to their direct action, was inhibited by the raised oxygen. It is possible that either the

hyperoxia prevented the synovial tissue from producing the changes in the culture medium that stimulate the chondrocytes to destroy their matrix, or that it inhibited the chondrocytes from reacting to the stimulus. The latter seems the more probable explanation. Unlike the synovium, cartilage has a largely anaerobic metabolism (Bywaters, 1937). That hyperoxia has an inhibitory effect on at least one synthetic activity of the chondrocytes has been shown by Lemperg *et al.* (1975), who found that exposure to 50% oxygen reduced the uptake of $^{35}\text{SO}_4$ into calf articular cartilage in organ culture. It may well be that the synthesis of the enzymes responsible for the degradation of the matrix is similarly inhibited in living cartilage.

Sledge and Dingle (1965) cultivated the cartilaginous limb-bone rudiments of the chick in an atmosphere of 85% oxygen and found that the matrix was severely depleted of proteoglycan. In the present experiments, however, hyperoxia failed to cause the breakdown of isolated articular cartilage. This apparent discrepancy between the two sets of results may be attributable to the fact that the limb-bone rudiments were enclosed in a sheath of connective tissue. It may well be that Sledge and Dingle's experimental system corresponded to the affronted explants of synovium and articular cartilage in which hyperoxia caused severe breakdown, rather than to the explants of isolated articular cartilage devoid of soft connective tissue. Alternatively, the chondrocytes of embryonic limb bones may respond differently to hyperoxia in organ culture.

It remains to be seen whether the action of hyperoxia on synovial tissue and cartilage in organ culture is related to the pathogenesis of inflammatory arthritis. In culture raised levels of synovial collagenolytic activity may be associated with an increased destruction of cartilage collagen provided the tissues are in contact. In rheumatoid erosion of cartilage matrix the synovium is known to synthesise collagenase (Evanson *et al.*, 1967), and during episodes of acute inflammation it becomes hyperaemic, with a consequent increase in the availability of oxygen. Whether this accelerates the destruction of the articular cartilage is not known.

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