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THE EFFECTS OF HYPOXIA ON HUMAN SKIN, LUNG AND TENDON CELLS *IN VITRO*

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Summary.—Fibroblasts derived from human foetal tendon, skin and lung were cultured in media containing dissolved oxygen at concentrations of 20%, 10% and 2·7%, Total protein and collagen synthesis were reduced at 2·7% oxygen, tendon-derived cells proving significantly less sensitive than lung and skin fibroblasts. The effects of hypoxia were more marked in low-density cultures than in confluent cultures. Proliferation rates varied greatly between the 3 cell lines but were not affected by hypoxia. The implications of these results with respect to tendon degeneration and rupture are discussed.

TENDON DEGENERATION and rupture is a common problem, particularly in older subjects and in those embarking on over-ambitious athletic programmes. It has been suggested that degeneration of the Achilles tendon may be the result of ischaemia, since it is almost always found in the area least well served by blood supply (Burry and Pool, 1973) and, furthermore, is more common in older subjects in whom tendon blood flow is known to be reduced (Rothman and Parke, 1965). The great natural strength of the tendon would almost certainly be impaired if anoxia led to suppression of protein synthesis or cell death.

Deleterious effects of excessive, or insufficient, concentrations of oxygen on cell growth *in vitro* have been reported previously (McLimans *et al.*, 1968; McLimans, 1972; Balin *et al.*, 1976) and, in the study reported here, the effects of various oxygen tensions on cell proliferation, protein and, specifically, collagen synthesis were examined. Cell proliferation and total protein and collagen synthesis were quantified, and differences in the behaviour of fibroblastic cells derived from 3 different foetal tissues were sought, since the possibility that tendon cells might have different characteristics had not been examined previously.

METHODS

1. *Tissue culture.*—Tissue culture materials were obtained from Gibco Europe Ltd, Paisley. ^{3}H-labelled proline (TRK323, 29 Ci/mmol) was supplied by the Radiochemical Centre, Amersham. Scintillation chemicals were obtained from Packard Ltd, Reading. GF/C filter-paper discs were from Whatman Ltd, Maidstone. Disposable scintillation vials were from Sterilin Ltd, Teddington. Other chemicals were obtained from B.D.H. Ltd, Poole.

Human foetal lung, skin and Achilles-tendon samples were washed in tissue-culture medium (TCM) to which benzyl penicillin (2 mg/ml) and streptomycin sulphate (1 mg/ml) had been added, and then sliced into small (1 mm^3) pieces and placed in 25cm^2 tissue culture flasks. TCM was added to form a fine layer over the explants, not of sufficient depth to cause them to float from the plastic surface. The TCM consisted of Dulbecco's modified Eagle's minimal essential medium containing Earle's salts supplemented with the following:

Hepes buffer (N′-2-hydroxyethyl piperazine N2-ethane sulphonic acid), 20 mM
L-ascorbic acid, 50 μg/ml
Sodium bicarbonate, 40 μg/ml
Benzylpenicillin, 200 μg/ml
L-glutamine, 2 mM

Foetal calf serum was added to 30% (v/v) and the medium adjusted to pH 7·2 with a few drops of 4M sodium hydroxide. After 24 h incubation

at 37°, 5 ml of fresh TCM containing foetal calf serum to 10% (v/v) was added to each flask and the incubation continued. Subsequently the medium was changed twice a week. Once adequate cell migration and differentiation had been achieved from the explants (usually after 6–10 days) the explants were removed and the cell monolayer incubated for a further 3–7 days. Cell suspensions were then prepared by discarding the culture medium covering the cells, washing the cell monolayer with 2–3 ml of 0·25% trypsin in TCM containing no serum and incubating for 5–10 min at 25°. Fresh TCM containing foetal calf serum to 10% (v/v) was added and a single-cell suspension obtained by gently aspirating through a syringe fitted with a 19-gauge needle. The cells were normally subcultured twice weekly and were used experimentally between the third and ninth passages.

2. *Experimental cell culture.*—Cell suspensions were prepared and flasks sealed with approximately 10^5 cells in 5 ml of TCM containing foetal calf serum to 10% (v/v). "Sparse" cell monolayers were those used after 6 h incubation at 37° and "confluent" monolayers were those used after 24 h incubation. Replicate cultures were compared after a further 24 h at a normal atmospheric partial pressure of oxygen (150 mmHg, 20%) and at either 75 mmHg (10%) or 20 mmHg (2·7%) measured on a Radiometer PHM71 acid/base analyser.

3. *Cell proliferation.*—After the 24 h incubation period at varying partial pressures of oxygen, a single-cell suspension was prepared in 10 ml of TCM and the number of cells per flask calculated by counting aliquots of each cell suspension in a Fuchs–Rosenthal haemocytometer.

4. *Protein synthesis.*—^{3}H-proline in TCM containing no serum was added to each of 4 replicate flasks to give a final concentration of 1 Ci/ml. After incubation for 24 h at 37°, the cells were scraped from the surface of the flask and dispersed by aspiration through a syringe and 19-gauge needle. Each sample was then divided equally, one fraction being utilized for total protein estimation and the other for collagen estimation. Cold (4°) trichloro-acetic acid (TCA) was added to a final concentration of 5% (w/v) and the suspension left at 4° for 3 h to allow complete protein precipitation. The precipitates were then collected by filtration under vacuum through 2·5cm-diameter glass-fibre filter discs. The discs were washed *in situ* with 10–20 ml of TCA (5% w/v) and subsequently with 10–20 ml of methanol. The filter papers were then transferred to plastic scintillation vials containing 10 ml of scintillation fluid (0·5% w/v Permablend III, 1% v/v Soluene 350 dissolved in toluene). Radioactivity was measured over a 5-min period in an LKB "ultrobeta" liquid scintillation counter with external standardization and expressed in disintegrations per minute (d.p.m.).

5. *Collagen synthesis.*—Native collagen synthesis was estimated using a scaled-up version of the method of Webster and Harvey (1979). This involved the addition of acetic acid to the fractions kept for collagen analysis to give concentrations of 0·5M before addition of pepsin to a concentration of 0·1 mg/ml. The samples were shaken gently at 4° for 16 h. All subsequent procedures were carried out at 4°. The samples were transferred to plastic centrifuge tubes and acid-soluble, foetal skin collagen (200 mg/ml) added to act as a carrier. This was prepared from human foetal skin by extraction in 0·5M acetic acid at 4°. The collagen was then purified using standard techniques of serial precipitation with sodium chloride at acid and neutral pH (Piez, 1967). Sodium chloride (5% w/v) was added to the supernatants to precipitate the radioactively labelled collagen together with the carrier collagen. The samples were centrifuged at 4000 *g* for 30 min and the supernatant discarded. The precipitate was redissolved in 0·15M sodium chloride in 0·05M tris (hydroxymethyl) amino-methane hydrochloride (Tris HCl), pH 7·5, and 20% w/v sodium chloride added to reprecipitate the collagen. After standing for 2 h at 4°, the samples were centrifuged at 4000 *g* for 30 min and the supernatant discarded. The collagen precipitates were washed by resuspending in 20% v/v ethanol in 0·05M Tris HCl, pH 7·5, and centrifuging at 4000 *g* for 20 min. The final precipitates were each dissolved in acetic acid (0·5M) and transferred to plastic scintillation vial inserts containing 2 ml of scintillation fluid (0·5% w/v Permablend III, 1% v/v Soluene 350 dissolved in 33% v/v TritonX100 in toluene). Radioactivity was measured over a 5-min period with external standardization and expressed in d.p.m.

RESULTS

The results are shown in summary in Tables I–III. Changes in proliferation rate or protein synthesis when concentrations of oxygen of 10% or 2·7% were employed rather than oxygen at atmospheric pressure are shown in parentheses as percentages of the values obtained from paired cultures in 20% oxygen. Except where indicated, they are the means and standard deviations of 4 recordings.

Table I demonstrates that there was no significant change in proliferation rate in the cultures gassed with sub-atmospheric oxygen concentrations, either in "sparse"

TABLE I.—*Cell proliferation*

	Cells/flask × 10^5			
Confluent	20%	10%	20%	2·7%
Skin	3·8±0·2	3·7±0·2 (−2·6%)	3·9±0·1	3·6±0·2 (−7·7%)
Lung	8·1±0·4	8·4±0·4 (+3·7%)	8·0±0·4	7·8±0·5 (−2·5%)
Tendon	7·9±0·4	7·5±0·4 (−5·1%)	7·8±0·3	7·3±0·3 (−6·4%)
Sparse				
Skin	2·0±0·1	2·1±0·2 (+5·0%)	2·1±0·2	2·0±0·2 (−4·8%)
Lung	4·7±0·4	4·6±0·3 (−2·1%)	4·8±0·2	4·4±0·2 (−8·3%)
Tendon	4·7±0·2	4·9±0·2 (+4·3%)	4·4±0·3	4·5±0·3 (+2·3%)

There was no significant difference in cell numbers cultured in 10% oxygen compared with controls in 20% oxygen or in 2·7% oxygen compared with controls in 20% oxygen (Student's t test where $n=8$).

or "confluent" cultures. Skin fibroblast proliferation rate was, however, considerably less than that of lung or tendon fibroblasts in both "confluent" and "sparse" cultures. This may be caused, in part, by the different cell-type sizes imposing contact inhibition of proliferation at varying cell numbers. Indeed, the lung and tendon fibroblasts used were considerably smaller than the skin fibroblasts.

Tables II and III indicate that at 2·7% oxygen there is a substantial decrease in both collagen and total protein synthesis in all 3 cell lines, the changes being greater in cultures of fibroblasts derived from skin and lung, than in tendon-cell cultures. Sparse cultures were always more sensitive to hypoxia than confluent cultures.

At 10% oxygen both confluent and sparse cultures of skin fibroblasts showed substantial depression of both total protein and collagen synthesis, lung fibroblasts were less affected and tendon cells showed no significant change in activity. The relative amounts of protein and collagen synthesized varied markedly between the 3 cell types, probably affected

TABLE II.—*Protein synthesis*

	DPM/flask			
Confluent	20%	10%	20%	2·7%
Skin	7065±632	5845±265 (−17·3%—$P<0·01$)	*6801±382	5350±377 (−2·31%—$P<0·005$)
Lung	9035±474	8113±345 (−10·2%—$P<0·02$)	9250±526	5877±495 (−36·5%—$P<0·001$)
Tendon	5874±1129	5266±730 (−10·4%—N.S.)	4895±476	4173±167 (−14·8%—$P<0·05$)
Sparse				
Skin	3682±183	2703±319 (−26·6%—$P<0·002$)	2722±329	1662±368 (−40·0%—$P<0·005$)
Lung	6213±452	*4823±696 (−22·4%—$P<0·02$)	5825±516	3118±206 (−46·5%—$P<0·001$)
Tendon	2612±384	2637±387 (+1·0%—N.S.)	2704±266	1757±374 (−35·0%—$P<0·005$)

The expression of statistical significance, P, is obtained from a Student's t test where n usually equals 8 (N.S.—not significant).

* Mean of 3 recordings, $n=7$.

TABLE III.—*Collagen synthesis*

	DPM/flask			
Confluent	20%	10%	20%	2·7%
Skin	1282 ± 191	970 ± 83	*1266 ± 206	932 ± 86
	(−24·3%—$P < 0·02$)		(−26·4%—$P < 0·05$)	
Lung	1572 ± 238	1383 ± 56	1566 ± 200	*1058 ± 140
	(−12·0—N.S.)		(−32·4%—$P < 0·01$)	
Tendon	990 ± 109	1301 ± 84	888 ± 178	751 ± 97
	(+4·1%—N.S.)		(−15·4%—N.S.)	
Sparse				
Skin	696 ± 125	498 ± 16	532 ± 99	327 ± 95
	(−28·5%—$P < 0·02$)		(−38·5%—$P < 0·05$)	
Lung	1166 ± 146	*933 ± 119	1020 ± 706	550 ± 64
	(20·0%—N.S.)		(46·1%—$P < 0·005$)	
Tendon	515 ± 48	479 ± 110	451 ± 64	325 ± 61
	(−7·0%—N.S.)		(27·9%—$P < 0·05$)	

The expression of statistical significance, P, is obtained from a Student's t test where n usually equals 8, (N.S.—not significant).

* Mean of 3 recordings, $n = 7$.

in part, by the differences in proliferation rates exhibited by these cells.

DISCUSSION

As an essential respiratory gas, oxygen dissolved in culture medium must be sufficient to sustain a normal respiration rate but must not be at a partial pressure such that the cell is damaged (Swartz, 1973). Generally cell cultures in monolayer are grown routinely with a gas phase containing an atmospheric oxygen concentration of 16–20%, depending on altitude. However, at low concentrations of 1–3% oxygen-enhanced proliferation has been observed (Richter, Sanford and Evans, 1972; Taylor, Camalier and Sanford, 1974). Similarly, at this concentration increased chromosomal stability has been reported (Parshad and Sanford, 1971; Parshad *et al.*, 1977). *In vitro* experiments with granulation tissue have demonstrated a linear increase in collagen synthesis with increasing mean tissue oxygen tension (Erlich, Grislis and Hunt, 1972) and some investigators have suggested a similar relationship *in vivo* (Hunt and Pai, 1972; Hunt *et al.*, 1973). Further studies of the effects of oxygen concentration on the growth of mammalian fibroblasts in culture have demonstrated a density-dependent effect, in that low-density cultures of fibroblasts are stimulated to proliferate by low oxygen tension, whereas at confluence oxygen tension has no effect on proliferation (Taylor *et al.*, 1978). Our studies have not been able to confirm this effect in the 3 cell types examined.

The factors which control proliferation of fibroblasts are poorly understood (Ristow, Holler and Messmer, 1978) and the comparative importance of stimulants such as somatomedin B and C, insulin and glucocorticoids, as opposed to the supposed inhibitory effect of chalones, remains in dispute. Similarly the effect of oxygen concentration is also disputed. Taylor and others (1978) reported enhanced proliferation rates in foetal fibroblasts when sparse cultures were gassed with 1% oxygen, but a similar effect could not be demonstrated with confluent cultures. Kittlick (1977) had previously reported that hypoxia suppressed cell division, particularly when the culture was maintained at a low pH. The pH of 6·6 used in Kittlick's experiments was possibly too low to enable normal cellular metabolism to proceed. In the studies reported by Taylor and others (1978), cultures with a final pH of 6·8–6·9 produced high proliferation rates. It is

possible that fall in pH may enhance cell division down to a certain critical level below which suppression of growth occurs.

In our studies pH was held constant by the addition of Hepes buffer to the medium, and this may account for the lack of effect of low oxygen tension on cell proliferation. Metabolic studies of the effects of oxygen on fibroblasts have shown that at low concentrations in low-density cultures glucose uptake and lactate production are enhanced (Sanford and Westfall, 1969; Taylor *et al.*, 1978). This may well be the result of anaerobic metabolism resulting from low oxygen tension. Alternatively then, in our studies, the oxygen tension employed (2·7%) may not have been sufficiently low to force the cells into partially anaerobic metabolism and cause lactate accumulation. Although we could not show any effects of oxygen tension on cell proliferation we were able to demonstrate a marked variation in culture characteristics between cells derived from the 3 different tissues and this finding demonstrates the potential error of extrapolating results from one tissue to another. When tendon is being investigated *in vitro* it is clear that tendon-derived cells should be studied.

When the lesions of central degeneration of the Achilles tendon are examined (Burry and Pool, 1973), 3 separate zones can be identified: normal tendon, necrotic areas where the few remaining fibroblast nuclei are shrunken and the collagen fibres have lost their normal structure, and an intermediate zone where fibroblastic proliferation is taking place but the collagen fibres are few in number and are not arranged in the usual orderly parallel fashion. These zones could correlate with a gradation from well oxygenated tissue through a hypoxic zone to areas where nutritional demands cannot be met and cell death results. These appearances would fit with the theory that tendon degeneration and rupture may be the consequence of hypoxic conditions (Burry, 1975). In our experiments, tendon-derived cells appeared less sensitive to anoxia than other fibroblast lines, apparently reflecting the known low oxygen requirements of tendons. Nevertheless, at low oxygen tensions cellular metabolism was depressed, with reduction in both total protein and collagen production. The total protein output is primarily made up of collagen and proteoglycan. Such a fall in collagen synthesis *in vivo* would almost certainly cause reduction in the tensile strength of a tendon. Furthermore, disturbance of proteoglycan secretion may well alter diffusion characteristics within the tendon, further impairing cell nutrition.

The increased frequency of tendon degeneration and rupture in older athletes may be explained by the fact that blood flow in tendons is known to be lower in older subjects (Rothman and Parke, 1965). Blood flow is also known to be impaired by tension (Schatzker and Branemark, 1969) so that the apparently higher incidence of Achilles-tendon degeneration in athletes using low-heeled running footwear might be accounted for by the low heels causing greater stretch in the tendon, thereby impairing blood flow. Further investigations are required to confirm the effects of hypoxia and pH change on fibroblast activity *in vitro*, and measurement of intra-tendinous oxygen tensions, pH and lactate levels during exercise might shed further light on the role of hypoxia in the genesis of tendon lesions.

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