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Intracellular collagen fibrils: evidence of an intracellular source from experiments with tendon fibroblasts and fibroblastic tumour cells*

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INTRODUCTION

A central enigma in ultrastructural connective tissue research is the appearance of intracellular equivalents of collagen fibrils in collagen producing cells discovered by Ten Cate (1972). After the intracellular appearance of these mechanical structures of connective tissue, which in general is part of the extracellular matrix, it seems plausible to suppose a phagocytosis of extracellular collagen fibrils by fibroblasts, smooth muscle cells, epithelial cells and chondrocytes (Usuku & Gross, 1965; Glauert, Fell & Dingle, 1969; Brandes & Anton, 1969; Perez-Tamayo, 1970; Faure, Graf, De Kosak & Pouliquen, 1970; Cullen, 1972; Ten Cate & Syrbu, 1974; Staubesand, 1977; Postacchini, Ippolito, Pudda & De Martino, 1981; Kopf & Staubesand, 1984). Nevertheless in recent years, it has been hypothesised that these vacuoles could be merely narrow cytoplasmic recesses (Chaplin & Greenlee,1975; Garant & Cho, 1979; Marchi & Leblond, 1983; McBride, Hahn & Silver, 1985) for it is within these recesses that the collagen fibrillogenesis occurs (Trelstad & Hayashi, 1979; Birk & Trelstad, 1984, 1986).

With biochemical evidence of a possible intracellular degradation of newly processed procollagen (Bienkowski, Cowan, McDonald & Crystal, 1978; Bienkowski, Baum & Crystal, 1978; Bienkowski & Engels, 1981) before secretion in fibroblasts and the finding of crystallisation of procollagen into collagen fibrils with native periodicity *in vitro* (Schmitt, Gross & Highberger, 1955), a second source of collagen fibrils has to be taken into account. Thus, there is evidence from some peculiar results (Trelstad, 1971; Renteria & Ferrans, 1976; Fernandez-Madrid, Noonan & Riddle, 1981; Michna, 1983*a*, 1984; Zorn, Bevilacqua & Abrahamsohn, 1986) that, for the explanation of intracellular equivalents of collagen fibrils in collagen-producing cells, an inclusion of intracellular procollagen with a subsequent crystallisation into collagen fibrils seems more likely. Accepting the latter hypothesis an experimental stimulation of protein synthesis might induce the formation of intracellular collagen fibrils. Some of our experimental models for the study of connective tissue provide a superb vehicle for functional experimentation into these aspects of intracellular collagen and in this way fulfill the need of "a differentiated methodology...to arrive at a convincing interpretation of the intracellular collagen" (Staubesand, 1977). Therefore it seems appropriate to collect more information on tendon fibroblasts and on cells with an enhanced turnover, with tumours of mesenchymal origin. To produce models for

* This article is cordially dedicated to Professor Dr Johannes Lang, Würzburg in honour of his 65th birthday.

studying intracellular collagen, protein synthesis in tendon fibroblasts and of the tumour cells of mesenchymal origin, *in vivo* and *in vitro*, was experimentally enhanced.

MATERIALS AND METHODS

Tendon fibroblasts

Proximal portions of tendons of the flexor digitorum longus muscles from 6 weeks old female mice (SPF-NMRI) were investigated. To create a model of enhanced collagen synthesis in tendon fibroblasts, animals were adapted to running on a treadmill. Within this training period the speed of the treadmill running was increased to a maximum of 33 m/min at constant slope of 3% (for further details, see Tittel & Otto, 1970; Michna, 1983*b*). The exercise was performed five days a week, for a short term (1 week) and a long term (10 weeks) training period. At all times 10 mice were not subjected to this exercise and served as controls. Each of 5 mice from the experimental and control groups of animals received Methandienon (DIANABOL®, Ciba Geigy AG, Basel), given in a dose of 3·2 mg/kg/week i.m. The hormone was dissolved in sesame oil. This anabolic steroid, a testosterone derivative, was chosen because of its marked anabolic activity (Dorfman & Shipley, 1956) and its property of enhancing collagen synthesis in tendon fibroblasts as previously reported (Michna, 1986*a*, *b*). Another 5 mice from each group received 5 μl sesame oil per 10 g body weight and served as controls.

Electron microscopic technique

At designated times from all four groups five animals were chosen randomly, anaesthetised with Nembutal® and perfused through the left cardiac ventricle with a solution of 2·5% glutaraldehyde in 0·1 M cacodylate buffer at pH 7·4. After fixation, proximal portions of appropriately sized tendon segments were carefully excised near the muscle-tendon junction. Only samples from the right hindlimbs, positioned similarly in each preparation, were examined. Tissue samples were further prepared for electron microscopy. The tissues were postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon 812. The image contrast was enhanced with uranyl acetate and lead citrate (Venable & Coggeshall, 1965). Sections were examined in a Philips 400 T or a Zeiss EM 10 A electron microscope.

Tumour cells

A tumour of mesenchymal origin, the slowly growing S-180 sarcoma (Foley *et al.* 1960), was chosen and established *in vitro*. The cells were grown as monolayers in Eagle's minimal essential medium with Hank's salts, 10% heat-inactivated fetal calf serum, 100 units penicillin/ml, and 100 μg streptomycin/ml. Cells were subcultured at 5 day intervals by trypsinisation.

In vitro

In five independent groups 20000 or 150000 sarcoma cells were seeded into culture dishes. Viability of the cells was determined by the trypan blue exclusion test (Holmberg, 1961). In each experiment 12 cultures were incubated overnight. The monolayers were washed and then the complete medium, containing 6 μg anabolic steroid hormone/ml medium was added and incubated at 37 °C in a humid atmosphere for 24 hours. After a further 24 hours and three successive washings with phosphate-buffered sodium chloride solution the hormone-containing medium was exchanged with a corresponding hormone-free medium and finally incubated for 24

hours. Control experiments were carried out in complete medium without, as well as with, the solvent for the hormone (1·2 μl absolute ethyl alcohol/ml medium).

In vivo

Tumours were produced *in vivo* in female mice (6 weeks old, SPF-NMRI) by subcutaneous inoculation of 0·1 ml of S-180 cell suspension (1×10^6 cells/ml) into the interscapular region. After 1, 3 and 4 weeks of growth 5–8 tumours were pooled at each time interval. To stimulate collagen synthesis in tumours *in vivo*, mice were given a weekly intramuscular injection of 3 mg Dianabol®/kg body weight, dissolved in sesame oil. Controls received isovolumetric sesame oil into the posterior thigh. At designated times electron microscopy was carried out as described above to detect intracellular collagen fibrils in sarcoma cells *in vivo* and *in vitro*.

RESULTS

Tendon fibroblasts

The tendon fibroblasts were strikingly altered after treatment with the anabolic steroid hormone, displaying a remarkable differentiation of the cytoplasm. Increase in the amount of endoplasmic reticulum and the accumulation of intermediate vesicles and transitional elements of the rough endoplasmic reticulum and the Golgi complex is in line with the concepts concerning the functional basis of protein synthesis (Cho & Garant, 1981). In all tendons treated with the anabolic steroid the fibroblasts contained single or multiple membrane-bound smooth-walled bodies often lining the cell membrane and the endoplasmic reticulum (Fig. 1), the so-called zebra-bodies. These elongated bodies, enclosed by single membranes, reached a length of 300 nm in the longitudinal and 90 nm in the transverse axis. Their most conspicuous feature was the presence of large aggregates of parallel 4-6 nm microfibrils. In hormone-treated and simultaneously exercised tendons, these organelles were more frequently encountered than in tendons only treated with the hormone, but were rarely seen in the controls.

By far the most striking feature was the appearance of a new structure in highly activated tendon fibroblasts, namely the appearance of intracellular equivalents of possible collagen fibrils (Fig. 2). Their characteristic cross-banding pattern displayed a periodicity of mostly 55 nm and their diameters ranged between 50 and 120 nm. The usually spindle-shaped bodies were always membrane-bound and each body contained only one presumed collagen fibril (Fig. 3).

Of considerable interest was the finding that sometimes the orientation of these intracellular collagen segments did not follow the orientation of the parallel extracellular collagen fibrils. Collagen fibrils in intracellular vacuoles were seldom found in exercised tendons, more frequently found in hormone-treated tendons, but were not detected in fibroblasts of control tendons.

Tumour cells in vivo

After inoculation of tumour cells into the interscapular region, in the cells of the control groups no intracellular collagen fibrils were found, although numerous bodies with intracellular collagen were present following anabolic steroid administration (Fig. 4). Sometimes the fibrillar and microfibrillar material possessed a rectangular orientation. Often the fibrillar material was striated, and most of it displayed a collagen banding pattern. In contrast to tendon fibroblasts, the collagen-containing vacuoles of sarcoma cells mostly accumulated more than one collagen fibril.

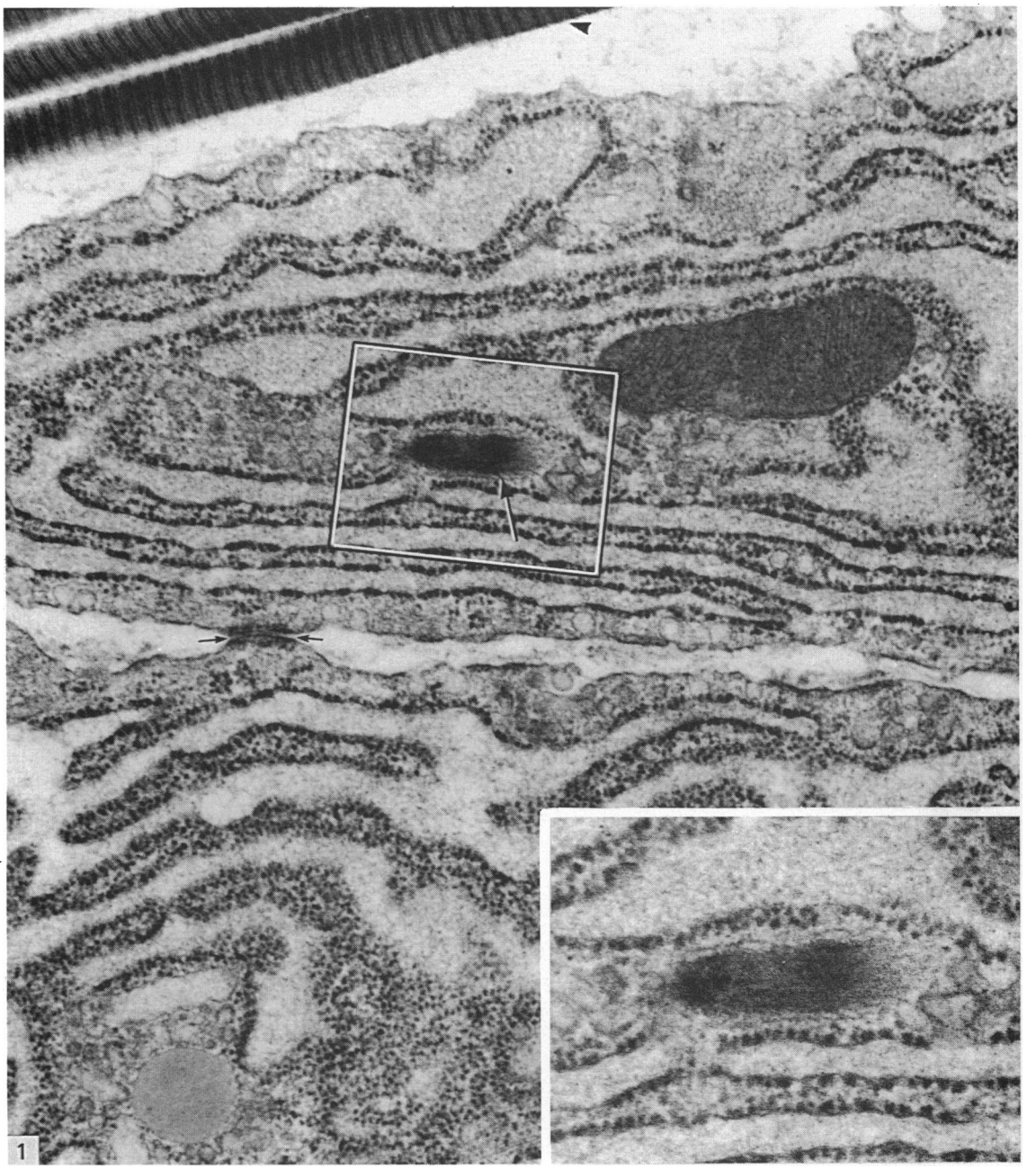

Fig. 1. Portions of two adjacent tendon fibroblasts one week after anabolic steroid hormone administration, illustrating prominent endoplasmic reticulum, tightly packed vesicles at the cell surface in the neighbourhood of a tight junction (→ ←). The cell surface is associated with aligned collagen fibrils (▶); note the zebra body (→) between profiles of rough endoplasmic reticulum containing microfibrils. × 43750. Inset × 80000.

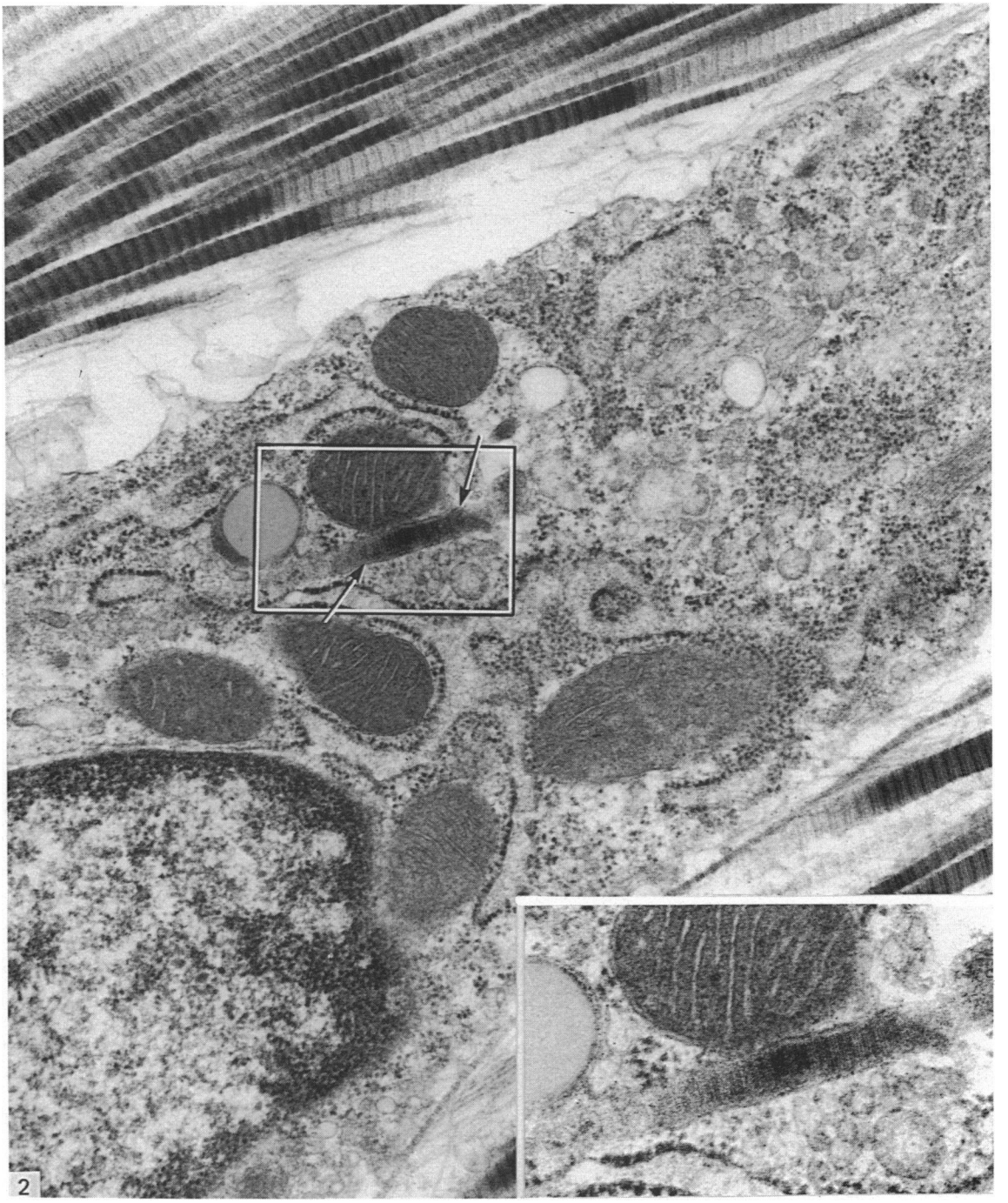

Fig. 2. Tendon fibroblast one week after anabolic steroid hormone administration showing a fibrillar aggregate with collagen-like banding. Parts of the limiting membrane (→) are visualised. × 33600. Inset × 62400.

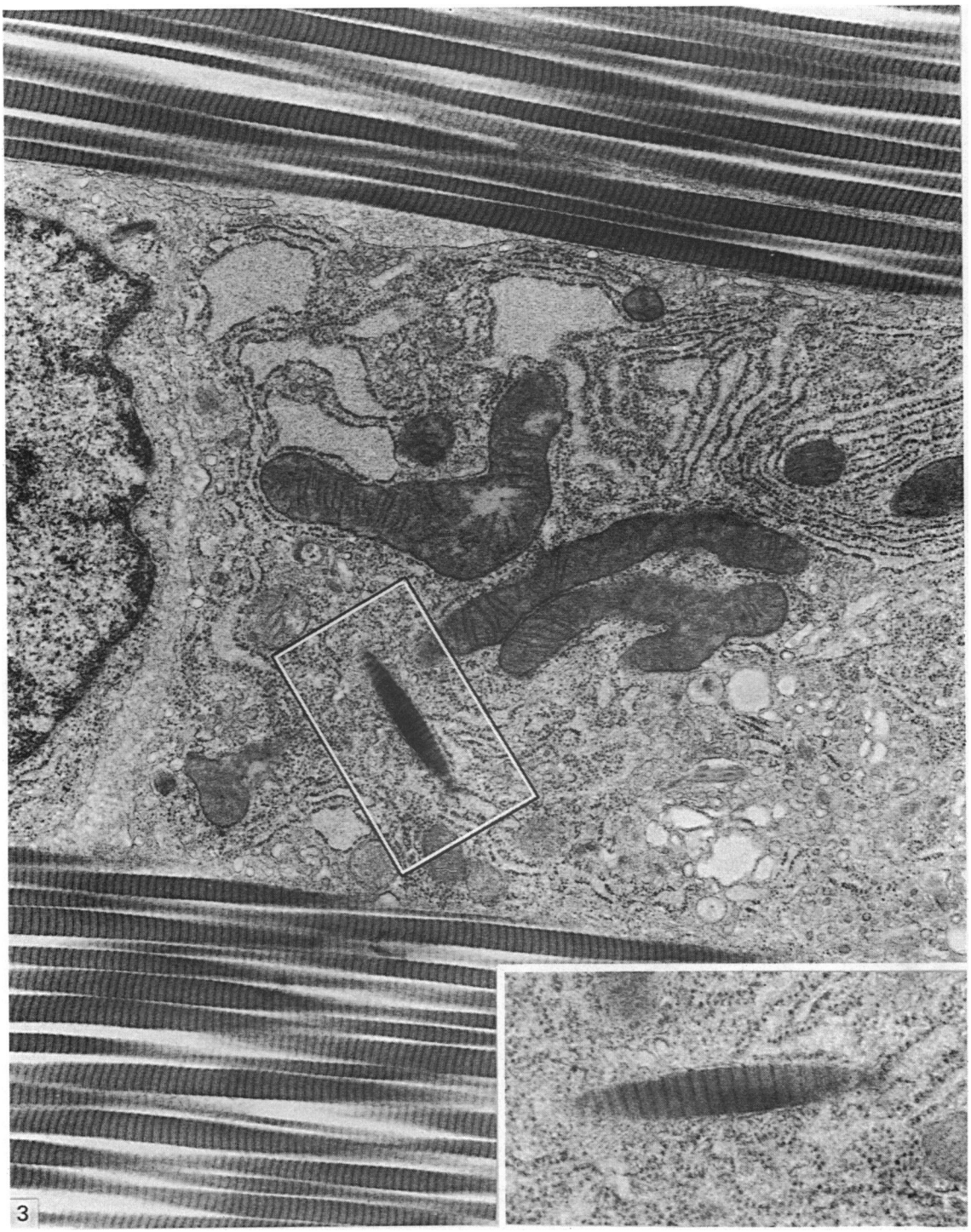

Fig. 3. Tendon fibroblast 7 weeks after anabolic steroid hormone administration and exercise, displaying a typical membrane-limited and spindle-shaped cytoplasmic body in the midst of a very active endoplasmic reticulum that contains a well-stained collagen fibril. × 31 250. Inset × 57 500.

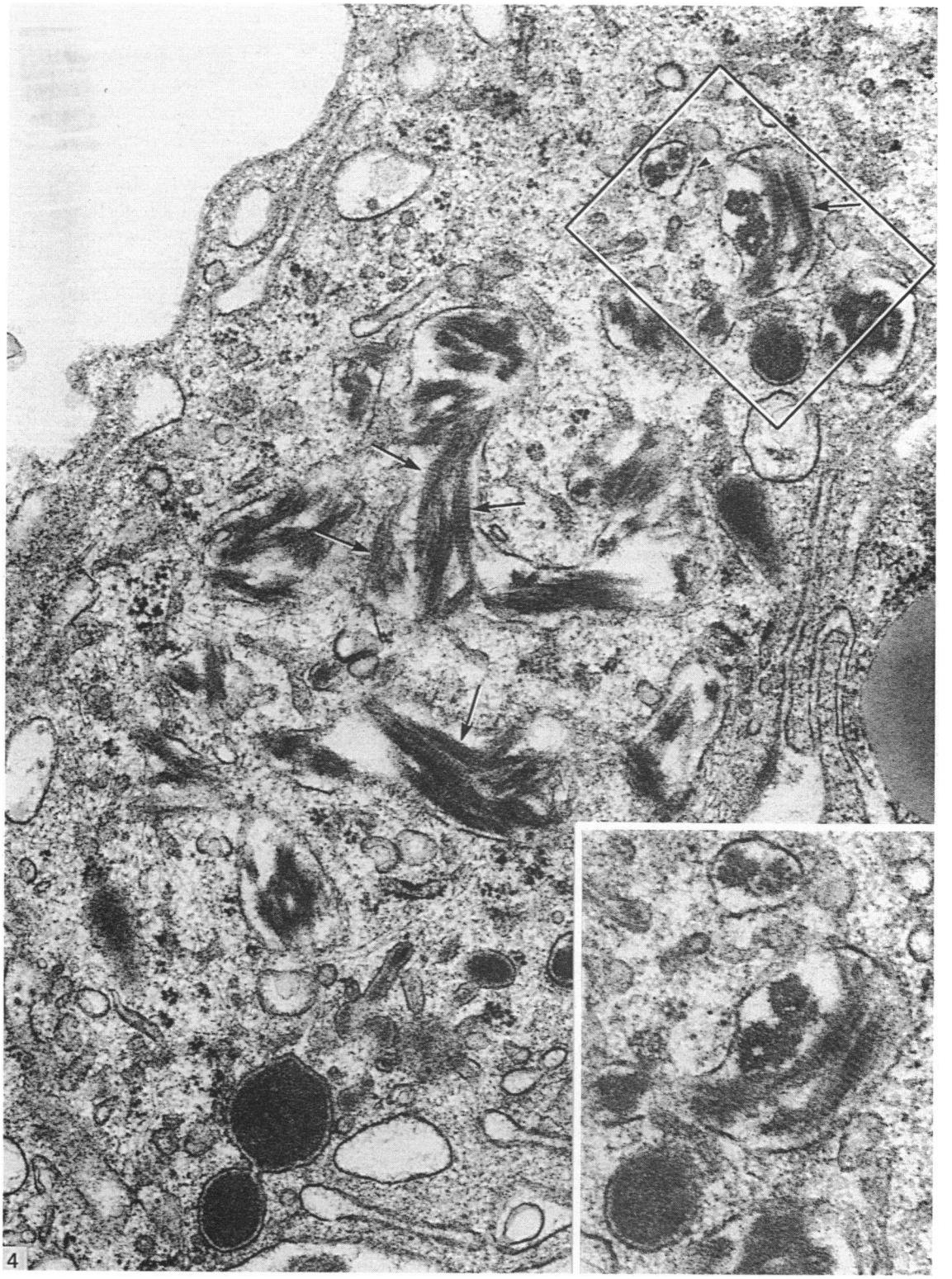

Fig. 4. Part of a sarcoma cell *in vivo* one week after anabolic steroid hormone treatment which is filled with collagen fibrils (→) and membrane- limited bodies containing fibrillar material with circular profiles of sizes corresponding to the diameters of collagen fibrils (▶). ×44200. Inset × 68000.

Tumour cells in vitro

In all experiments with sarcoma cells done in tissue culture after administration of anabolic steroids no intracellular collagen fibrils were found.

DISCUSSION

The present experimental work with tendon fibroblasts and sarcoma cells indicates that the appearance of intracellular equivalents of collagen fibrils has a connection with the activated synthesis of intracellular collagen precursors. Furthermore, because of the mostly distinctive banding pattern of intracellular collagen fibrils one may argue that these are not the result of phagocytosis of extracellular collagen fibrils although their intravacuolar location would be consistent with this idea. Such an explanation is generally accepted for the presence of intracellular collagen fibrils in macrophages of tissues that are undergoing rapid remodelling (Parakkal, 1969*a*, *b*; Michna, 1984). In the present study, the appearance of intracellular collagen fibres could easily be explained as an autophagic process (De Duve & Wattiaux, 1966) of tropocollagen macromolecules; as a result, these macromolecules may aggregate within the intracellular vacuoles as microfibrils and cross-striated collagen fibrils. Therefore, the formation of collagen fibrils may be attributed entirely to the action of an enzyme system or, more probably, a specific procollagenase acting on newly synthesised procollagen whereby aggregatable tropocollagen macromolecules are formed.

It has already been pointed out that this concept is based on the assumption that the synthesis of tropocollagen macromolecules may exceed the transport capacity of the cell. This is, perhaps, indicated by the fact that intracellular collagen fibrils could be detected in tendon fibroblasts and sarcoma cells after experimental activation of protein synthesis. Although other possible explanations of a limited transport capacity of connective tissue cells during the process of exocytosis are possible, the appearance of intracellular collagen appears to be due to a limited translocation capacity for intracellular vesicles. This is supported by a radioautographic study of collagen secretion in ligament fibroblasts (Cho & Garant, 1981) and electron microscopic studies in osteoblasts (Scherft & Heersche, 1975) and fibroblasts (Fernandez-Madrid *et al.* 1980) after administration of colchicine. These studies demonstrated that the absence of microtubules in colchicine-treated cells may account for the accumulation of intermediate vesicles and the formation of larger smooth-walled vesicles and segments of long crystallites. Indeed it has been stated that some of the spindle-shaped 'zebra-bodies' (Voelz, 1964; Movat & Fernando, 1962) during this period of secretory inhibition contained striated fibrillar inclusions which could be labelled as collagen precursors undergoing polymerisation. These observations could as well support the concept of autophagocytosis, although limitations in translocation capacity have actually been demonstrated only in cells which have been treated with colchicine. In addition, the existence of lysosomal enzyme activity within the collagen-containing vacuoles (Deporter & Ten Cate, 1973; Ten Cate & Syrbu, 1974; Garant, 1976; Yajima, 1976; Soames & Davies, 1977; Yee, 1979; Rose, Yajima & Mahan, 1980; Yajima, 1986), which is believed to confirm the theory of phagocytosis, could as well represent the need for lysosomal activity during autophagocytosis.

In summary, the findings reflect that only highly metabolising modified fibroblasts and sarcoma cells accumulate collagen precursors exceeding the secretory transport capacity of the cell and inducing compensative autophagocytic reactions, thus producing the second source of intracellular collagen fibrils.

The foregoing discussion raises the question why it was not possible to stimulate such an autophagic control mechanism to induce the formation of intracellular collagen fibrils *in vitro*. Although no definite explanation can be offered, one possibility is that the capacity of the intact microtubular network for the organised transport of collagen precursors and the exocytosis of collagen secretory granules *in vitro* may not be a limiting factor. The cells may not have been cultured long enough to produce extracellular collagen fibrils to be phagocytosed and tropocollagen molecules may not linger in the pericellular vicinity.

The possible existence of such a control mechanism would shed some light upon the early idea of phagocytosis of collagen fibrils in regenerating tendon (Postacchini *et al.* 1978; Postacchini *et al.* 1981). In addition, these considerations contribute to a better understanding of previous findings of intracellular collagen in regenerating cartilage and during uterine involution (Brandes & Anton, 1969; Dyer & Peppler, 1977; Zorn *et al.* 1986), during tissue remodelling (Gona, 1969; Seifert, 1971; Trelstad, 1971; Fox, 1972; Gronioski & Walski, 1975; Mendoza, 1979), in some mesenchymal tumours (Welsh & Meyer, 1967; Allegra & Broderick, 1969; Levine, Reddick & Triche, 1978) as well as their appearance in connective tissue of patients with inherited disorders of collagen metabolism (Renteria & Ferrans, 1976). Finally, the fact that, instead of normal collagen fibrils, the vacuoles mostly contain twisted ribbons and amorphous material may be explained by bearing in mind that the conversion of procollagen to collagen with fibril- and cross-link formation requires a number of specialised biochemical reactions (Bornstein, 1980).

These processes within the vacuole may be of paramount importance in the formation of intracellular collagen fibrils, and the question of whether or not intracellular collagen fibrils are formed may hinge on whether the biochemical environment for their production is reached within the autophagocytic vacuoles by chance. Nevertheless, the bizarre appearance of the intravacuolar material could as well be due to degradative mechanisms during phagocytosis.

On the basis of these considerations it is clear that this process does not always exist in autophagocytic vacuoles so that procollagen does not always crystallise to collagen fibrils. In addition, the findings of fibrillary collagenous aggregates and amorphous material may represent further steps of autophagocytosis of collagen fibrils. Since in connective tissue cells intracellular collagen fibrils can only be detected during metabolic reaction patterns and not during catabolic reactions (Michna, 1984), the idea of an extracellular source of intracellular collagen and a phagocytotic reaction of collagen-producing connective tissue cells is in doubt although the above arguments do not exclude the concept of an extracellular compartmentalisation during collagen fibrillogenesis (Birk & Trelstad, 1986).

The occurrence of filaments in the inclusions is a very interesting finding; on the one hand it may speak for an insufficient effect of procollagenase with subsequent aggregation inhibition and, on the other, for an activation of lysosomal enzymes since the increased occurrence of intracellular collagen fibrils after hormone treatment may be due to an activation or synthesis of procollagenase and/or to an inactivation or synthesis inhibition of lysosomal enzymes.

Finally, this would tend to favour the classical (Metchnikoff, 1892) as well as the modern (Van Furth *et al.* 1972) concept of a differentiation between collagen-producing connective tissue cells and cells of the collagen-degrading mononuclear phagocyte system.

SUMMARY

This study was designed to substantiate one or both of the two hypotheses for the explanation of intracellular collagen fibrils in collagen-producing cells. The more obvious is the phagocytosis of extracellular collagen fibrils by the cell and the other is a form of autophagocytosis of newly synthesised collagenous products. Information was collected on fibroblasts from murine tendons after exercise and simultaneously stimulating collagen synthesis by treatment with an anabolic steroid hormone. Moreover, *in vivo* and *in vitro* fibroblastic tumour cells which demonstrate enhanced protein synthesis were also treated with the anabolic steroid. The findings of intracellular collagen fibrils in tendon fibroblasts and the sarcoma cells after experimentally stimulating collagen synthesis are discussed in the light of the hypothesis that the findings may represent steps of autophagocytosis of newly synthesised collagenous products in the absence of a control mechanism to remove collagenous products which cannot be secreted.

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