Increased Connective Tissue in Skeletal Muscles of Hypophysectomised Rats Treated *In Vivo* With Human Growth Hormone

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Abstract

Groups of hypophysectomised, adult, male rats were given subcutaneous injections of 60 mIU of human Growth Hormone (hGH, somatotropin) daily for 7 days. Assessment of the hind limb muscles by histological examination of transverse sections showed areas of excessive connective tissue deposition. Other unusual features seen in muscles from these rats were heterogeneity of muscle fibre size, centrally positioned nuclei, and increased number of active fibroblasts in the endomysial regions.

Groups of hypophysectomised, control (saline injected) rats and normal animals receiving hGH all appeared to have normal muscle morphology after 7 days treatment.

The GH-treated hypophysectomised rat may act as a suitable model for the study of progressive muscle fibrosis.

Key words: skeletal muscle, fibrosis, growth hormone, hypophysectomised rat, connective tissue, collagen.

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The preparation of therapeutic human growth hormone (hGH) from pituitary glands previously restricted its use to the treatment of hGH-deficient dwarfism [12]. With the availability of large amounts of recombinant DNA hGH, its supply no longer restricts its use [30]. The hormone is now for example being used in high doses for therapy in Turner's syndrome [22, 25] as well as in short but otherwise normal children [9, 23] and short children with chronic renal failure [11]. It is also being considered for the treatment of fractures and diseases of muscle wasting [26] as well as a variety of adult conditions such as ageing, obesity and osteoporosis (for review see Bauman and Silverman [6]).

Early work established an effect of treatment with hGH on increases in skeletal muscle weight [13, 14]. In rats bearing GH-secreting tumours, changes in muscle fibre diameter [21] and increases in number of nuclei and satellite cell nuclei per fibre, were observed [18]. In the human, it was found that in the *quadriceps* muscle of acromegalic patients, Type I fibres hypertrophied, while Type IIA fibres atrophied [19] and in hypopituitary dwarfs, hGH administration induced changes in muscle cell size and numbers [20]. In view of the likelihood of the expanding use of hGH, there is thus a need for detailed information on the changes in muscle fine architecture or biochemical characteristics after hGH administration.

We report here on abnormal local changes in the structure of skeletal muscles from hypophysectomised rats treated daily with hGH for seven days. Some preliminary changes in the ultrastructure [2, 3] and in collagen expression [31] have been reported.

Materials and Methods

Male Sprague Dawley rats (180-200 g, age approx 6 weeks) purchased from Interfauna, Huntingdon, Cambs., U.K. were hypophysectomised by the parapharangeal method and fed on maintenance diet (RM1) (BP Nutrition, Special Diet Services, Witham, Essex CM8 3AB) *ad libitum*. The injection regime was that used routinely for bioassay of hGH in the body weight gain assay using the hypophysectomised rat and was identical to that used in previous studies [2-4]. Completeness of hypophysectomy

was monitored by observing the absence of body weight increases and the maintenance of the immature fluffy coat. Commencing 14 days after hypophysectomy, the rats received 7 daily injections (60 mIU/day) of purified hGH (WHO International Standard [4], code no. 80/505, NIBSC) or recombinant hGH (code no. 88/624, NIBSC) in 0.5 ml diluent (phosphate-buffered saline with 0.1% w/v bovine serum albumin) or 0.5 ml diluent alone. The rats were injected subcutaneously in the nape of the neck and killed 16 hours after the last injection - the *gastrocnemius* and *soleus* muscles were then excised as quickly as possible (< 1 min) from the hind limbs.

Samples from the midpoint of each muscle were fixed in glutaraldehyde (4% v/v) and embedded in epoxyresin (Araldite). Transverse sections, 1 µm thickness, cut from the deep central regions and outer peripheral regions were stained with Toluidine Blue (0.1% in 0.1 M acetate buffer, pH 6.2) and viewed by light microscopy at low magnification (x60) and higher magnification (x500). Fields across the full muscle section were scanned and representative areas selected for photography. Cross-sectional areas of photomicrographs of muscle fibres were calculated by image analysis software using an MOP Digiplan (Kontron, Messgeraete GmbH, München, Germany) which comprised a magnetized tablet and stylus for circumscribing fibre perimeters.

Ultrathin sections (< 100 nm thickness) were also cut from the same areas of the muscles, stained with lead citrate and uranyl nitrate and viewed by transmission electron microscopy (magnification X 9000).

Observations

The appearance of normal rat *gastrocnemius* muscle is typified by the 1 µm thickness transverse section stained with Toluidine Blue shown in Fig. 1A. The fibres seem of relatively uniform cross section, closely packed, separated by a thin basement membrane and each fibre appears to be in close contact with several capillaries. The elongated nuclei are visible at the periphery of the fibres just inside the sarcolemma membrane. Table 1 gives the median fibre areas and shows that there is a preponderance of large (> 1200 μm^2) fibres.

Transverse sections of *gastrocnemius* muscle from normal rats treated with pituitary hGH (Fig. 1B) appear to be similar to those derived from normal rats. Muscle sections from hypophysectomised control rats treated with saline (Fig. 1C) also have an appearance comparable to those of normal *gastrocnemius*, with a similar proportion of medium and large fibres (Table 1). Four sections from rats (n = 5) were examined for each of the treatment groups shown in Figs 1A-C. Typical light micrographs from a rat from each group are shown; little variation was evident in the different samples from within each group.

In contrast, among the group of hypophysectomised rats treated with pituitary hGH, (n = 6) or recombinant hGH (n = 6) all of the transverse muscle sections of gastrocnemius examined showed some degree of extra connective tissue

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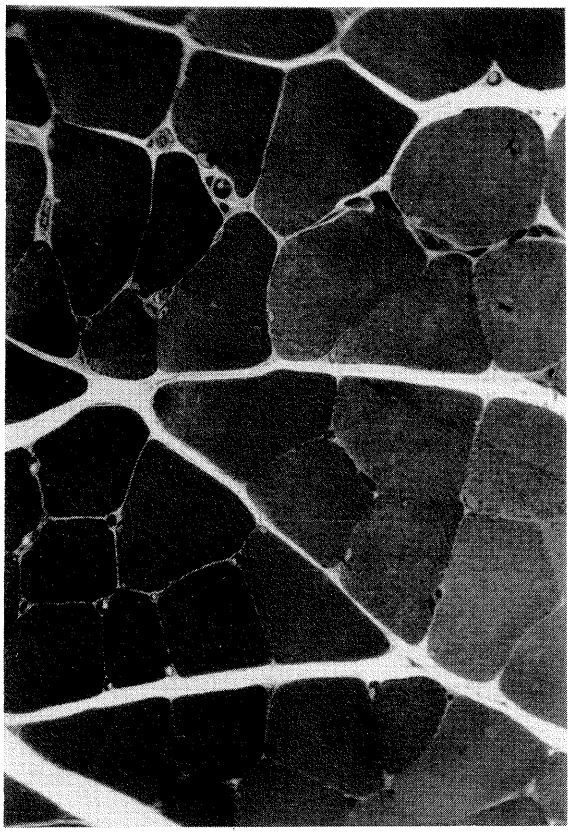

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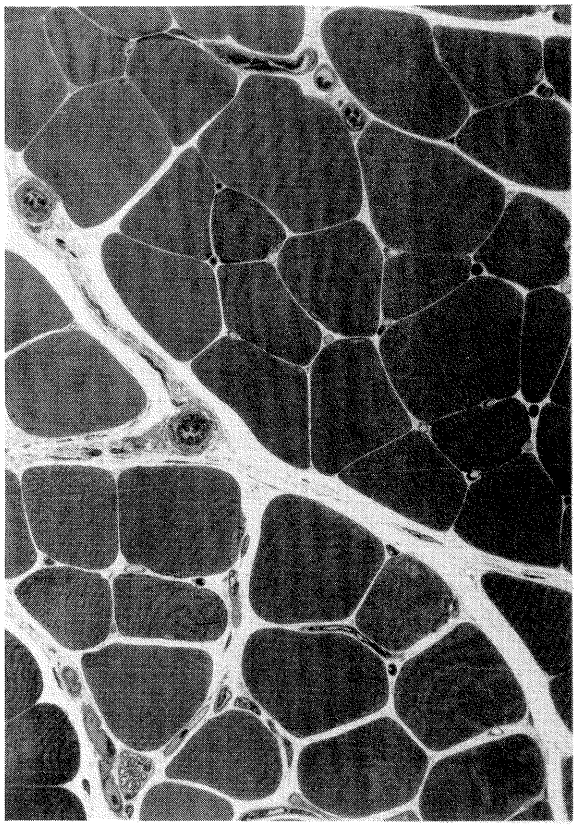

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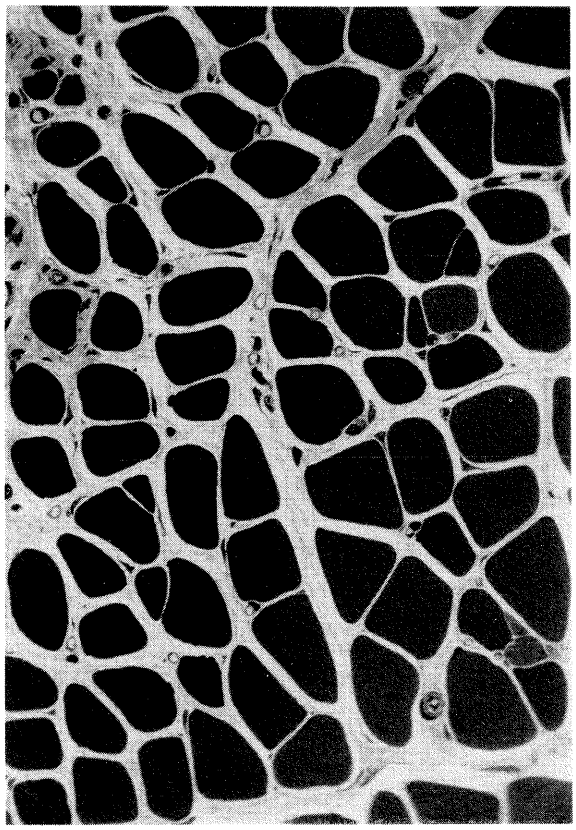

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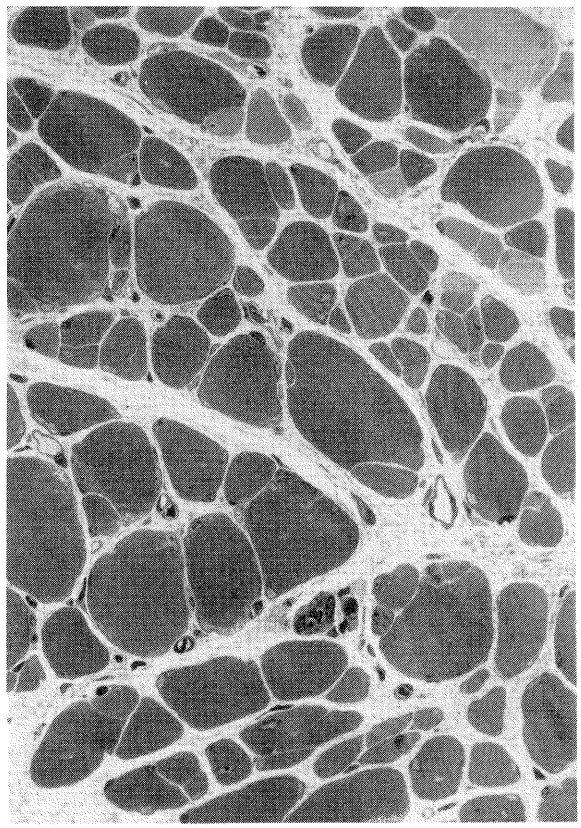

Figure 1. Transverse sections (1 μm thick) of rat gastrocnemius muscle stained with toluidine blue. Sections were viewed in the light microscope at X500 magnification.
Gastrocnemius muscle from:
A) a normal rat (untreated)
B) a normal rat treated s.c. with 7 daily injections of pit. hGH (60 mIU/day)
C) hypophysectomised (control) rat treated with saline
D) hypophysectomised rat treated with 7 daily injections of pit. hGH (60 mIU/day).
E) a different hypophysectomised rat treated with 7 daily injections of pituitary hGH (60 mIU/day).

in the endomysial regions between the muscle fibres. A typical *gastrocnemius* muscle section is shown in Fig. 1D. The marked increase in the amount of extracellular connective material is clearly seen between the fibres; several muscle fibres appeared to be completely isolated from the surrounding capillary network by this disorganised connective material. Another distinctive feature seen in at least 3 of the hGH-treated animals was the great variability in size and shape of muscle fibres in these regions with excess collagen (Fig. 1E). This fibre size heterogeneity is summarised in Table 1, which lists the proportion of fibres of different sizes in normal, GH-treated normal, hypophysectomised control and GH-treated hypophysectomised rats and shows that small (0-400 μm^2) fibres are the most abundant in the latter group. The data in Table 1 also shows that hGH-treatment markedly diminishes the median fibre area for hypophysectomised rats.

In *gastrocnemius* these clusters of small fibres may arise by fibre splitting. Analysis of consecutive serial sections have revealed the appearance of longitudinal splits in the fibres [2].

Some similarities were evident in *soleus* muscles from hGH-treated rats. Light micrographs are shown of transverse sections from normal (Fig. 2A), saline-treated hypophysectomised (Fig. 2B) and pituitary hGH-treated hypophysectomised (Fig. 2C) rats. Abnormal features (extra connective tissue and small fibres) were seen only in the hGH-treated hypophysectomised animals.

Histological assessment of transverse sections of both *gastrocnemius* and *soleus* muscles by the Van Gieson and Gomori stains established the extracellular material to be mostly collagen. This was also evident from the typical banded appearance of the fibrils seen by electron microscopy (Fig. 3). Other characteristics seen at the high magnification used for these transverse sections (Fig. 3) are the central nuclei in many of the small fibres and the large numbers of active fibroblasts in the perimysial regions and in the endomysial spaces between the muscle fibres. These fibroblasts can be identified by their irregular shape, their multiple processes and their high content of rough endoplasmic reticulum.

Discussion

Our observations in the light microscope indicate that treatment of hypophysectomised rats with hGH for 7 days results in local areas of abnormal muscle architecture in animals of this group, while no changes were observed in muscles from any of the normal rats treated with hGH or of the hypophysectomised rats treated with saline. The main abnormal features seen (Figs. 1-3) were areas of extra collagen deposition and heterogeneity of muscle fibre size with clusters of small, central-nucleated cells. This fibre size heterogeneity may be due to either fibre degeneration or splitting. These possibilities are under further investigation by examining earlier and later time points of GH administration.

The time course of collagen production is also being studied by assessment of levels of mRNA for collagen I and III using *in situ* hybridization. Preliminary studies show hGH-induced increases in mRNA for collagen I and III in focal regions across the muscle sections [32].

If the fibrosis becomes excessive it could eventually give rise to an ischaemic environment for the muscle fibres which may result in impaired muscle function or fibre atrophy. In addition, local areas of longitudinal fibre splitting may outstrip the rate at which new fibres could be innervated and could also result in atrophy of selective fibres.

Table 1. Muscle fibre areas calculated from the gastrocnemius sections shown in Fig. 1.

Gastrocnemius from treated or untreated rats	Median Fibre Area μm^2 (n = Total fibre number)	% fibres of areas a, b and c (n = number of fibres of areas a, b or c)**		
		0-400 μm^2 a	401-1200 μm^2 b	> 1200 μm^2 c
Normal (Fig. 1A)	1740 (n = 29)	0 (n = 0)	21 (n = 6)	79 (n = 23)
Normal + Pit. hGH (Fig. 1B)	1110 (n = 50)	0 (n = 0)	62 (n = 31)	38 (n = 19)
Hypox + Saline (Fig. 1C)	1436 (n = 35)	0 (n = 0)	37 (n = 13)	63 (n = 22)
Hypox + Pit. hGH (Fig. 1E)	180 (n = 110)	75 (n = 82)	20 (n = 22)	5 (n = 6)

**Chi-square statistic, to test independence of treatment for areas a, b and c, was computed for Hypox + saline group versus Hypox + pit. hGH group. The resulting chi-squared statistic was highly significant ($p < 0.001$).

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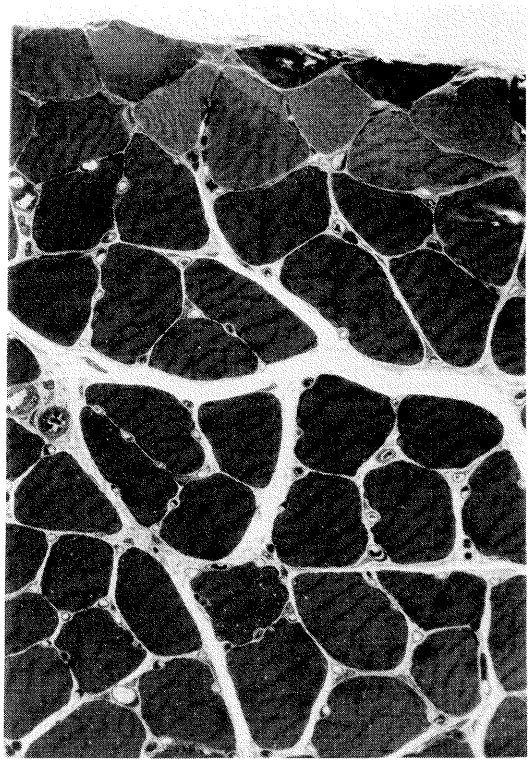

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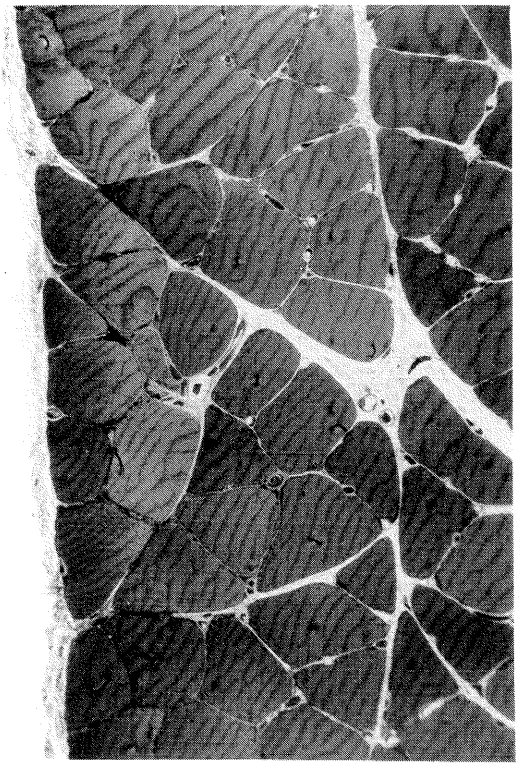

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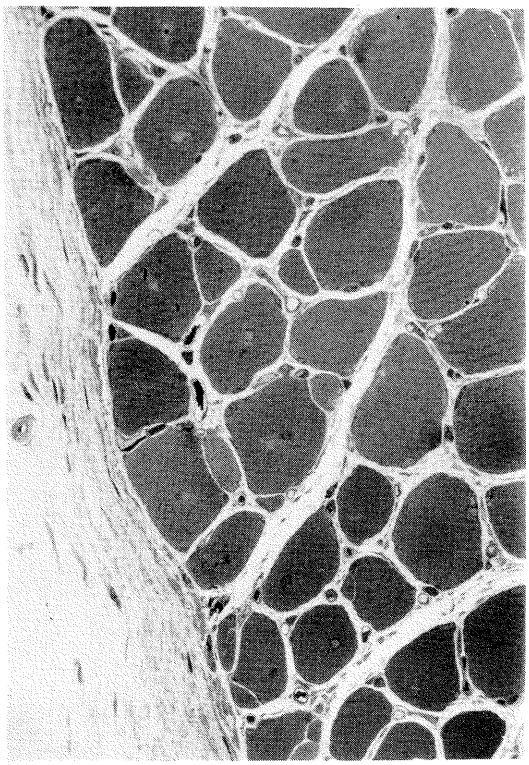

Figure 2. Transverse sections (1 μm thick) of rat soleus muscle stained with toluidine blue. Sections were viewed in the light microscope at X500 magnification. Soleus muscle from a group of rats different from those examined in Fig. 1:
A) a normal rat (untreated)
B) hypophysectomised (control) rat treated with saline
C) hypophysectomised rat treated with 7 daily injections of pit. hGH (60 mIU/day).

It should be noted that the hypophysectomised group of animals is also deficient in all other pituitary hormones, notably TSH, LH and ACTH. These animals will therefore be markedly hypothyroid. Lack of thyroid hormone in humans has been shown to result in muscle weakness and mild features of disturbed muscle architecture eg. ring fibres, atrophy and some increased interstitial connective tissue [10]. However, none of these abnormal features were seen in the hypophysectomised control group of rats, and we therefore conclude that the changes seen in the treated group were a specific hGH effect.

Since abnormal connective tissue was not seen in any of the normal rats treated with GH, it is possible that lack of thyroid hormone (or any of the other pituitary hormones) has sensitized the animals to GH. This point is under investigation.

This enhanced hGH-sensitivity of muscles of hypophysectomised rats may have implications for hGH-treatment of those patients with established myopathy or multiple hormone deficiencies. The dose of GH generally used for the management of hypopituitarism in children

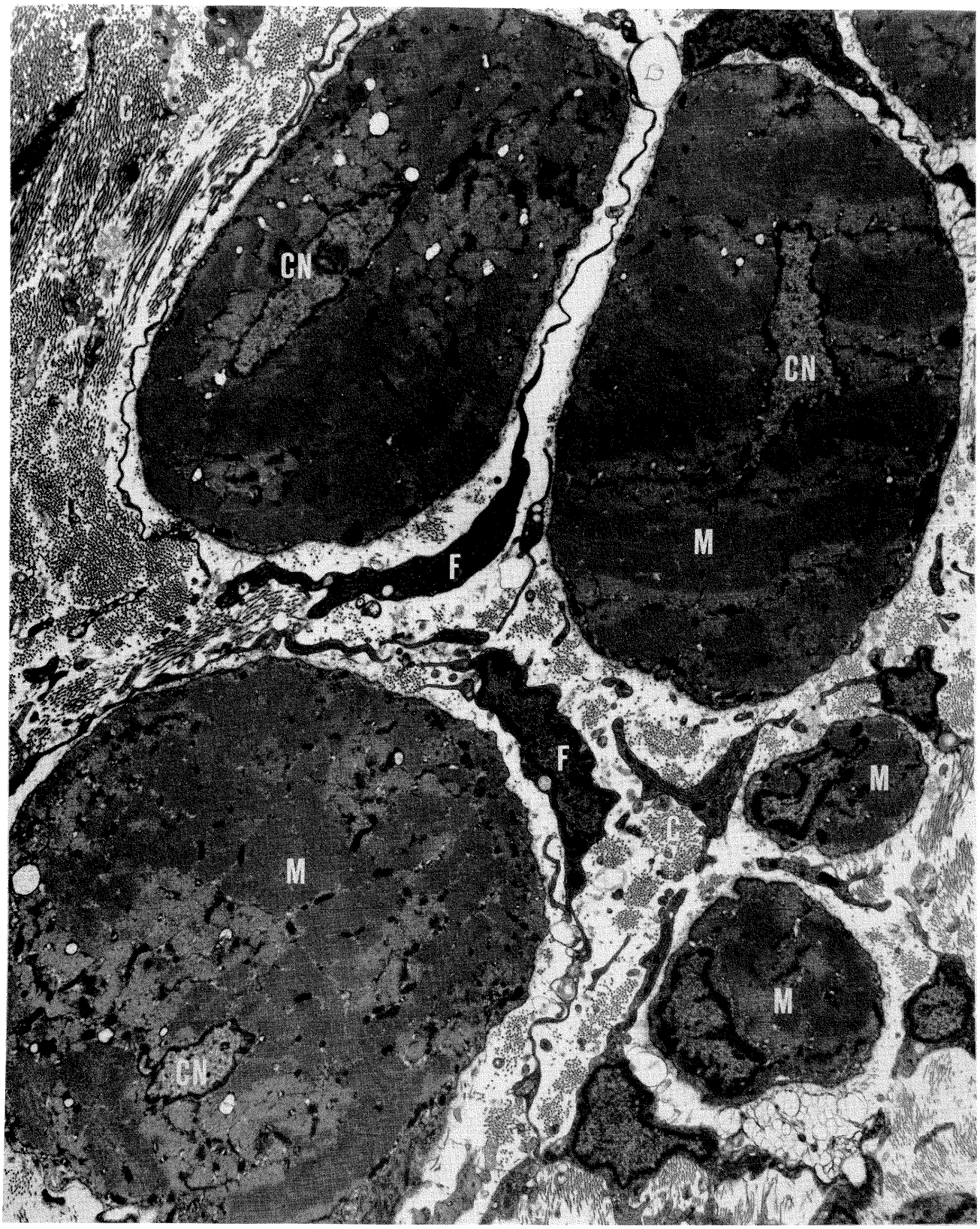

Fig 3. Electron micrograph of ultrathin (< 100 nm thick) muscle sections, stained with lead citrate and uranyl nitrate. Transverse sections (enlargement of x9000) of gastrocnemius muscle from Fig 1E).

during the last decade was 80-800 mIU hGH/kg (3-7 times weekly for several years) [24, 29, 30]. This compares with the 300 mIU hGH/kg (daily for 7 days) used in the present study with hypophysectomised rats.

It is of interest that the localized abnormal features in the muscles of hypophysectomised hGH-treated rats, closely resemble those seen in muscle biopsies from patients at the early preclinical stages of Duchenne Muscular Dystrophy (DMD) [1, 8]. These include: a) heterogeneity of fibre size, b) centrally positioned nuclei, c) extensive proliferation of extracellular material and d) loss of contact between fibres and the vascular network.

The inherited disorder of Duchenne Muscular Dystrophy has been shown to be associated with absence of the cystoskeletal protein, dystrophin [15]. However the biochemical links between the lack of dystrophin and the subsequent events of fibre necrosis and regeneration and development of local areas of fibrosis in the muscles of affected children are not well defined [7] and there is much evidence to support the hypothesis of an accompanying defect in muscle membrane permeability.

The abnormal features reported above were observed in the muscles of hypophysectomised rats treated with hGH for 7 days. These observations lead us to suggest that there may be a common mechanism for the development of fibrosis in this animal model and in the very early stages of muscles of DMD-affected individuals. This could be due to a defect in the control mechanisms by which hGH regulates its action on muscle tissue, either because of altered characteristics of GH receptors on the muscle membrane or altered IGF-1 (or IGF-binding protein) response. This would be consistent with observations reporting that reduced levels of circulating GH are associated with amelioration of the severity of the muscle weakness in affected DMD patients [33, 34].

In two other (non-dystrophin related) animal models of dystrophy it has been reported that reduced levels of endogenous GH resulting either from hypophysectomy or by cross-breeding with a genetic dwarf strain reduced the severity of the muscle abnormality [16, 28]. There have been previous reports of an effect of growth hormone in causing increased collagen production [35]. Nagulosparen *et al.* [19] also reported observations of excessive connective tissue in the skeletal muscles of acromegalic patients.

In conclusion, the GH-treated hypophysectomised rat might act as a suitable animal model for the study of progressive muscle fibrosis in the dystrophic myopathies and will be useful for further studies on the effects of hGH on muscle collagen synthesis.

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Key

C: Increased collagen deposition between the muscle fibres
CN: Central Nuclei (only peripheral in normal muscle)
M: Muscle fibres of very different diameters
F: Fibroblasts - extensive endoplasmic reticulum and typical long processes.

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