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Morphometric analysis of loading-induced changes in collagen-fibril populations in young tendons*

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Summary. This study was designed to gain more detailed morphological information on skeletal tendons in the course of adaptation to physical loading. The effect on collagen fibrils was investigated in 6-week-old mice by means of electron microscopy. Physical loading was performed on a treadmill 5 days a week for 1, 3, 5, 7 and 10 weeks. Morphometric analysis of collagen fibrils revealed the mean diameter, the diameter distribution, the number and the cross-sectional area. The principal observations included:

1. After one week of physical loading an increase in mean fibril diameter (30%, $p \leqq 0.01$), in number (15%, $p \leqq 0.05$), and in cross-sectional area (15%, $p \leqq 0.05$), as well as a change in mean fibril diameter distribution.

2. From the third to the seventh week a fall under the level of the controls in mean diameter (26%, $p \leqq 0.01$), in number (26%, $p \leqq 0.01$), and a reduced cross-sectional area (17%, $p \leqq 0.01$), accompanied by signs of splitting of individual collagen fibrils.

3. In the long-term study an increase in fibril number (29%, $p \leqq 0.01$), a fall in mean diameter from 189 nm in the controls to 179 nm ($p \leqq 0.05$) but no statistically significant change in the relative cross-sectional area (32%) per unit in comparison to unloaded tendons.

The possible physiological implications of the findings are discussed in the light of several regulatory mechanisms known to appear during the course of physical loading in connective tissues.

Key words: Tendon – Collagen fibrils – Morphometry – Ultrastructure – Loading – Mouse

The adaptation of biological systems can be realized by genetic specialization and/or environmental plasticity (Dobzhansky 1951; Miller 1976; Bonner 1982). The latter aspect may be defined by the qualitative and quantitative expression of the functional structures within the tissue.

Since collagen is involved in different structures and tissue formations of the human body (Penttinen et al. 1980) and contributes to the dynamics and biological functions of connective tissue (Fitton-Jackson 1968; Flint 1976; Miller 1980), it impinges on a wide range of other biomedical disciplines in addition to being a facet of structural studies. Thus, progress has also been made in studies of its metabolism as well as in the interactions between the extracellular components. Moreover, skeletal tendons provide for a variety of practical and conceptual reasons a superb vehicle for functional experimentation into aspects of environmental plasticity.

Whilst there is insufficient morphometric information on the peculiar relationship between structure and function of tendons, it is the purpose of this morphometric analysis to reveal the morphological changes in the organization of their extracellular components in the course of functional adaptation. It appears appropriate to collect more information on the tendon of the flexor digitorum longus muscle exposed to loading by an analysis of its collagen fibrils.

Materials and methods

1. Animals

Proximal portions of tendons of flexor digitorum longus muscles from 52 female NMRI-mice (6 weeks old) were investigated.

2. Experimental design

To produce a model of tendon loading animals were gradually adapted to running on a treadmill (Tittel and Otto 1970). The exercise was performed at a speed of 0.3 m/sec, 5 days a week, for 1, 3, 5, 7 and 10 weeks. The daily exercise time was progressively increased from 10 min on the first day up to 30 min after one week. At all times five mice were not subjected to loading and served as controls.

3. Preparative procedures for electron microscopy

At designated times from both groups, five animals were anesthetized 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. Proximal portions of tendon were excised near the muscle-tendon junction. Only samples from the right hindlimbs, positioned the same in each preparation, were used. Special care was taken to avoid mechanical damage. Tissue samples were further prepared for electron microscopy as previously de-

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scribed (Michna 1983). Sections with silver-gray interference colors were cut with the use of an LKB III ultramicrotome. The image contrast was enhanced with uranyl acetate and lead citrate.

4. *Morphometry and statistics*

Always two transverse sections were picked up on successive grids and thus widely spaced (Weibel 1969) to make sure that this selection was random. Specimens were examined using a Zeiss EM 10A electron microscope, which was calibrated from micrographs of diffraction gratings. It must be taken into account that due to shrinkage of collagen fibrils during the procedures for electron microscopy the measurements may not be absolute (Bowes and Cater 1968; Hickey and Hukins 1979; Eikenberry et al. 1982). Micrographs of a constant magnification of $\times 20000$ were taken from randomly selected areas of tendon, displaying circular, correctly cross-sectioned collagen fibrils. With the use of a semi-automatic CONTRON MOP AM 02 image analysis system equipped with an HP 9825 A desk computer, measurements were made at a final magnification of 180000 on a tracing board with a light stylus. Diameters were measured in x- and y-axes to control inaccurancy in measuring, caused by unfavourable planes of sectioning. To circumvent this problem, it is expedient to tolerate only those measurements in which differences in diameters in both axes do not reach 10%. Measurements were done in two squares for 1 μm^2 tissue surface per picture. According to the experience of Parry et al. (1978) and Parry and Craig (1977), sufficient measurements (2500–5700 from each experimental group) had to be collected to obtain a reliable analysis of the fibril population for broad multimodal distributions. Thus, in this series of experiments there is a significant correlation ($p \leqq 0.01$) between the results of repeated estimates made by two observers. According to Dyer and Enna (1976), there was no significant difference in the analysis of collagen fibril diameters across the tendon from different levels.

In particular, the following aspects of the analysis of transverse sections through collagen fibrils will be described: 1) the mean diameter and the diameter distribution, 2) the number per unit area, and 3) the cross-sectional area.

Statistical comparisons between the distributions of diameters of collagen fibrils were made by using the Kolmogorov-Smirnov two-sample test (Sachs 1978). Lord tests were used to evaluate the statistical significance of differences between the means. This test is performed for small samples and is analogous to Student's "t" test (see Sachs 1978). The correlation between the means of collagen-fibril diameters and age was tested with the Pearson correlation coefficient (r). Correlation coefficient and slope were calculated by using equations for simple linear correlation and regression (Woodward 1972).[1]

Results

The morphometric analyses of mean fibril diameters are summarized in Fig. 1a, b. In the maturing tendon tissue of the control mice, there was a considerable monotonical increase in the mean fibril diameter during the experiments, which proved to correlate significantly ($r = 0.87$, $p \leqq 0.001$, Fig. 1a).

Already after one week, morphometric changes are recorded in loaded tendons: An increase in mean fibril diameter (30%, $p < 0.01$, Fig. 1b), in number (15%, $p \leqq 0.05$, Fig. 2) and in cross-sectional area (15%, $p \leqq 0.05$, Fig. 3) of collagen fibrils per unit area. Correspondingly, in the

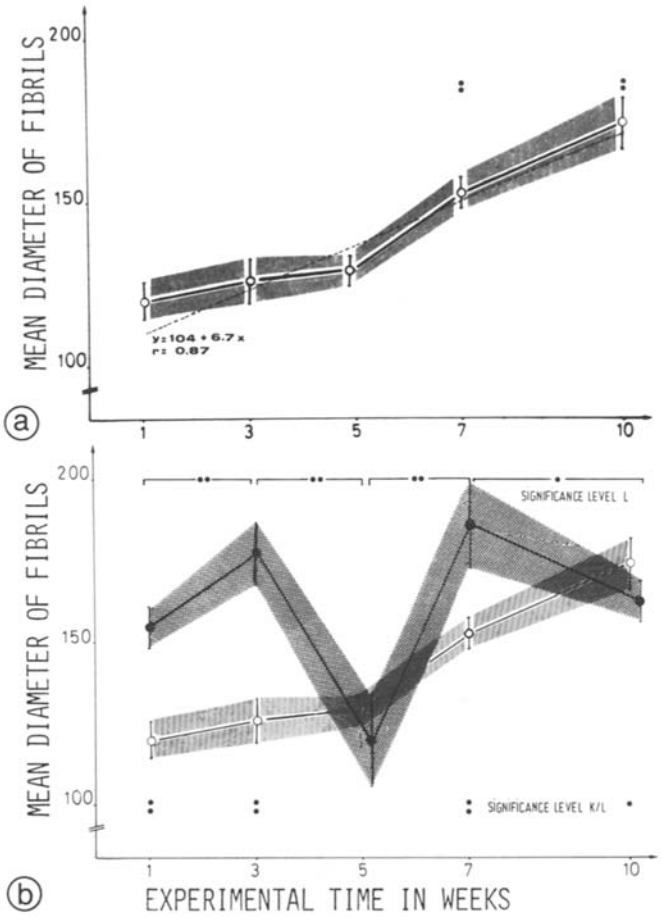

Fig. 1a, b. Development of mean collagen fibril diameter in unloaded (o—o, *K* Fig. 1a) and loaded (●—●, *L* Fig. 1b) tendons. Statistical significance (probability level): **p* 0.05; ***p* 0.01

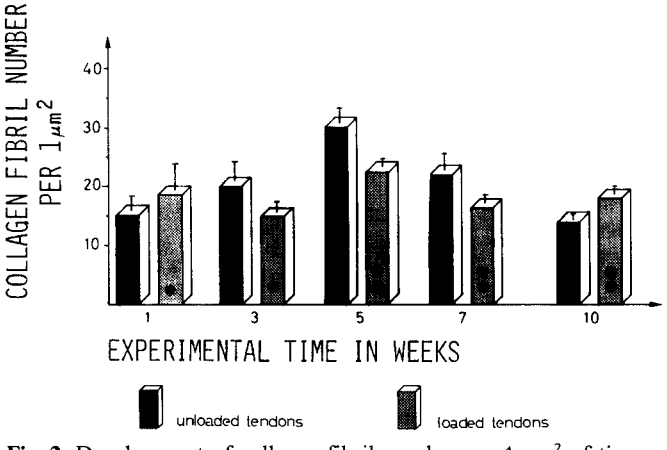

Fig. 2. Development of collagen fibril number per 1 μm^2 of tissue surface in loaded and unloaded tendons. For explanation of statistical symbols, see Fig. 1

1 The author is indebted to Professor Dr. Herbert Haug, Lübeck, for bringing his attention to the fact that, according to the stereological principle of Delesse (see Weibel 1979), measuring of the relative area of collagen fibrils can be related to volume density

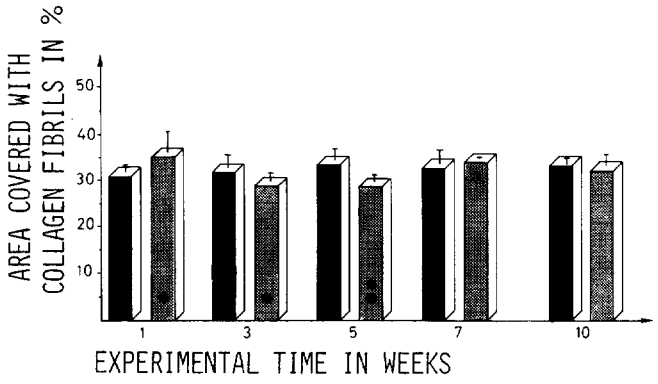

Fig. 3. Development of percentage of cross-sectional area covered with collagen fibrils in loaded and unloaded tendons. For explanation of symbols, see Figs. 1 and 2

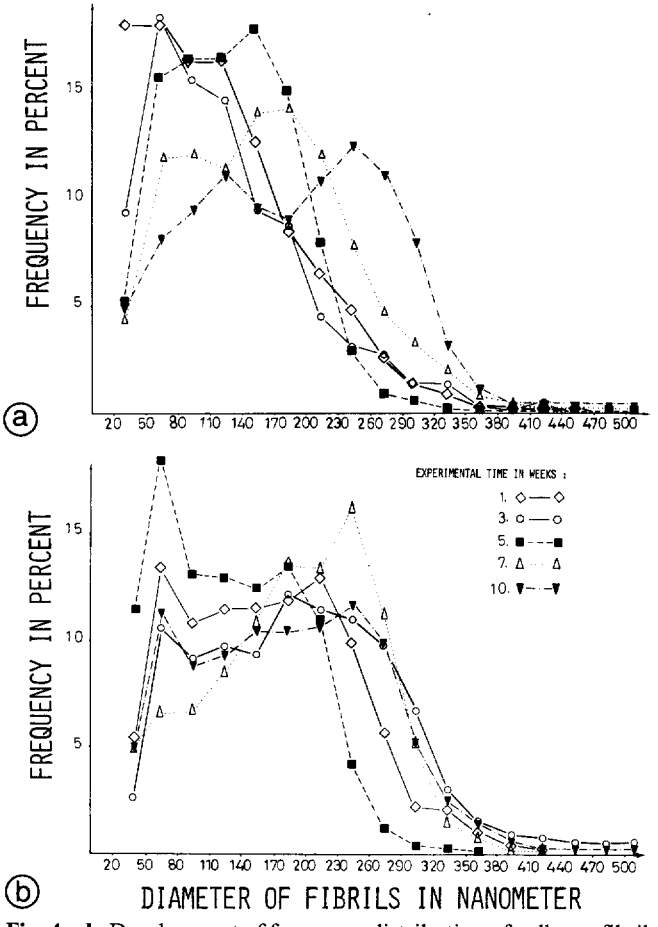

Fig. 4a, b. Development of frequency distribution of collagen fibrillar diameters in unloaded (Fig. 4a) and loaded (Fig. 4b) tendons

analysis of histograms, the diameter distributions of loaded tendons have altered significantly ($p \leqq 0.001$) and have a dextroverted form. They have already become bimodal (Fig. 4b).

Furthermore, one may note that a dramatic change of mean fibril diameter in loaded tendons occurs from the third to the seventh week of loading. There is a fall (26%, $p \leqq 0.01$, Fig. 1b) under the level of the controls in mean diameter accompanied by a significant reduction in the number of constituent fibrils (26%, $p \leqq 0.01$, Fig. 2). The reductions are due to the accumulation of smaller fibrils measuring approximately 50 nm (Fig. 4b). There are only a few larger fibrils, and those over 380 nm have disappeared. Simultaneously, the cross-sectional area was found to be decreased (17%, $p \leqq 0.01$, Fig. 3).

During this period another effect of loading on the morphology of collagen fibrils was detected. As can be seen in Fig. 5, ultrastructural studies revealed that individual collagen fibrils appear to undergo a splitting or a confluence. These parts of the collagen fibrils show no obvious alteration in banding patterns and demonstrate a diameter of about 50 nm.

Finally, a marked bimodal form of the distribution of collagen fibril diameters becomes re-established after seven weeks (Fig. 4b). At ten weeks, the distribution approximately resembles that obtained from unloaded tendons (Fig. 4a, b). It is particularly noteworthy that in loaded tendons the thickest collagen fibrils could be detected. However, simultaneously they contain very thin fibrils and as a consequence attain broader diameter distributions compared to the controls. Thus, there is a slight fall in mean diameter from 189 ± 7 nm in unloaded to 179 ± 9 nm in loaded tendons ($p \leqq 0.05$).

In addition, in the long-term study no morphometrically significant change of cross-sectional area occurs in loaded tendons ($32 \pm 2\%$, Fig. 3). However, physical loading of tendons induced a significant increase in the number of their fibrils (29%, $p \leqq 0.01$, Fig. 2) per unit area.

Discussion

The caliber of collagen fibrils of tendons is known to increase steadily from birth to maturity (see Parry and Craig 1977, 1978; Parry et al. 1978a, b; Eikenberry et al. 1982). Statistical analysis of the measurements indicates a significant correlation with age.

Since in the annulus fibrosus of the intervertebral disc the mean fibril diameter is not correlated with age (Hickey and Hukins 1981, 1982) and in contrast to tendons contains a higher proportion of type-II collagen (Eyre and Muir 1974, 1976; Herbert et al. 1975), these findings might reflect a variation in extracellular matrices after compensatory hy-

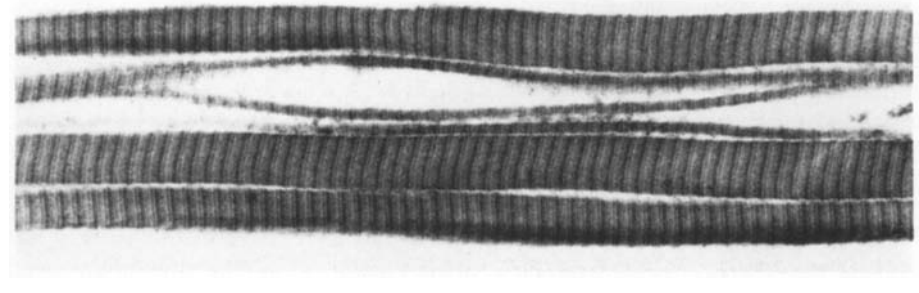

Fig. 5. Collagen fibrils of loaded tendon (5 weeks). Individual collagen fibrils appear to undergo a splitting or a confluence. × 36250

pertrophy necessary for the different functional requirements of both connective tissues. Interestingly a similar function of age as for tendon collagen fibrils has also been noted for the mean diameter of skeletal muscle fibers (Rowe 1969).

Although the cross-sectional area of collagen fibrils remains fairly constant, which is in agreement with similar studies (Torp et al. 1975; Parry et al. 1978a), our material shows evidence of varying fibril numbers as a function of age per unit area (Fig. 2).

It is worth noting that in loaded tendons just after one week of training statistical analysis reveals an increase in mean diameter, number and cross-sectional area of collagen fibrils compared to the controls. The applied physical loading may also determine the diameter distribution, which has become bimodal with a predominance of thicker fibrils. In particular, it has been postulated that thicker fibrils are predicted to withstand greater tensional forces than thinner fibrils due to more intrafibrillar covalent cross links (Parry et al. 1978b, 1980). This is in good agreement with previous studies of size distributions of collagen-fibril diameters in mature animals (see Parry and Craig 1977; Parry et al. 1978a) and in tendons subjected to tensional and additional compressive forces (Merrilees and Flint 1980).

However, altered fibril diameters are not the only morphological adaptations occurring in response to changes in loading. At the same time, the present study demonstrates that there is an increase in number and, as a consequence, there is an increase in major cross-sectional area. The data thus might suggest that loading could enhance collagen synthesis. Other local environmental factors, which indicate physical feedback mechanisms, can enhance collagen synthesis as well (for review, see Merrilees and Flint 1980). More recently, a similar approach has been adopted in vitro. An increase in production of type-I and -II collagen was observed in smooth-muscle cells when subjected to repeated cyclic stretching (Leung et al. 1977a, b). In addition, further evidence that the metabolism of collagen is accelerated by physical stress in several connective tissues is given by the finding of increased hydroxyproline incorporation in mice (Suominen and Heikkinen 1975a; Suominen et al. 1980) and humans (Suominen and Heikkinen 1975b; Suominen et al. 1977). Since the observed changes in structural organization of tendons were rapid and quantitatively very dramatic just after one week of loading, it seems problematic to classify this tissue as a "bradytrophic" one (cf. Merker and Barrach 1982). Among the results presented, the fall of the measured parameters below those of the representative controls in the course of physical stress merit particular attention. The morphometric analysis of collagen-fibril populations demonstrates that there is a disappearance and reappearance of thick instead of thin fibrils from the third to the fifth week (Fig. 4b). Since no necrotic collagen fibrils were revealed, the disappearance of thicker fibrils with the consequence of an increase of thinner fibrils should be sought in the form of the individual fibril. By far the most interesting observation is the presence of individual fibrils appearing to undergo deformations which in the cross sections for quantitative analysis would be counted more than once (Fig. 5).

In conclusion, if the changes in morphology of collagen fibrils are interpreted as a splitting, then not only the variations in collagen-fibril populations, but also the development of mean fibril diameters are recognized as well. This statement is in line with the opinion of Parry and Craig (1977) who conclude that fibril fusions do not generally seem very likely.

The foregoing discussion raises certain questions concerning the present concepts of molecular stabilization of the collagen fibril. Although no definite explanation can be offered for the splitting of collagen fibrils, it seems probable that the relative amounts and the nature of the intermolecular cross links of collagen (Light and Bailey 1979, 1980) and interactions with the interfibrillar matrix (Steven 1967; Finlay et al. 1971; Evans and Barbenel 1975), and thus the tensile strength, would be expected to be not yet sufficiently adapted to loading. That rapid growth may render collagen fibrils less stable was already pointed out by Udén (1980). The assumption fits well with current knowledge; indeed, there is firm evidence from some peculiar results (Byrd 1973; Viidik 1973) that there can be a reduced amount of cross-links in the course of loading. These findings are also well supported by biomechanical data indicating vulnerable metaplastic processes with a predisposition to tendon rupture (Rollhäuser 1954). Nevertheless, it may be added that, if this argument is accepted, there remains a lack of understanding why no collagen fibrils could be found here with the typical features of fibrils "exploded" into sub- and microfibrils (Steven et al. 1975; Torp et al. 1975; Parry and Craig 1977). The observed marked reductions in number and cross-sectional area may have general implications for a reduced tensile strength.

Together with the study of the fine structure, this does tend to favor the hypothesis that some changes in serum enzyme activities after physical stress and an increased immunological defense (Liesen et al. 1977) may be derived from a hitherto presumed damage of collagen fibrils (Franzblau et al. 1976; Liesen et al. 1977). It seems likely from the findings presented that there actually are some morphological changes.

Finally, the progressive alterations of collagen organization in tendons in the course of loading result in the long-term study into a conservation of the genetically regulated proportions of collagen fibrils and interfibrillar substance. The fact that thinner fibrils are present may be explained by suggesting that in this way tissue is designed to return to its original form after tension due to a causal relationship between an increased surface area per unit mass of the fibrils and the number of electrostatic interactions between the collagen fibrils (Parry et al. 1978). Environmental plasticity in the need of biological adaptation of tendons is achieved in the quantitative organization of collagen packing and is in good agreement with the recent observations by Oakes et al. (1982) on rat anterior cruciate ligaments. Although there are some marked differences in absolute data of matrix organization (cf. Oakes et al. 1982), it is tempting to assume a close morphological resemblance after functional adaptation in the qualitative and relative quantitative data of matrix organization indicating similar regulatory activities in collagenous tissues subjected to probably similar physical forces. Furthermore, in the light of the similarity of both tissue activities, it is suggested that these variations are functional adaptations with regulatory significance.

Although various sources are adding to our understanding of the mechanism of the functional adaptation of collagenous tissue, the question, which structure primarily gives sign to changes in cell metabolism, remains enigmatic. The

fascinating idea that electrochemical potentials of collagen fibrils play a role in exerting an influence (Fukuda and Yasuda 1957; Bassett and Becker 1962; Fukuda and Yasuda 1964; Anderson and Eriksson 1968; Bassett 1971a, b; Bassett and Pawluk 1972; Pollack et al. 1977; Roth and Freund 1981a, b, 1982) sheds some light on this problem. It has already been suggested that the amount of surface charge on collagen may be one of the likely mechanisms to regulate cell metabolism (Gillard et al. 1979; Merrilees and Flint 1980). The hypothesis of a regulatory role of collagen on cellular activities in connective tissue is further reinforced by the finding that fibroblasts of tendons (this study) and ligament (Oakes et al. 1982) increase the number of thinner collagen fibrils when subjected to physical loading. It is to be expected that in this way an increase in electrochemical potentials is realized, which finally raises the possibility to contribute to the control of cell metabolism in the need of environmental plasticity of mature tissue. Nevertheless, it was speculated that alterations in the composition of glycosaminoglycans precede changes in collagen-fibril assembly and growth also in vivo (see Parry et al. 1982).

These arguments are of great interest in that they support the theory of "the control or influence of the extracellular matrix on gene expression" (Slavkin and Greulich 1975; Caplan 1981) and may be implicated in the etiology of some congenital diseases (Aureli et al. 1981).

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