Virchows Arch (1998) 432:357–361

ORIGINAL ARTICLE

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Thymic myoid cell turnover in myasthenia gravis patients and in normal controls

Received: 2 July 1997 / Accepted: 20 November 1997

Abstract Thymic myoid cells (TMC) are sparse muscle-like cells in the thymic medulla, which are believed to trigger the autoimmune response in myasthenia gravis (MG). Ultrastructural investigations have revealed mature, degenerating, and immature TMC, but the number of TMC in MG patients does not differ from that in controls. We examined the turnover of TMC at the subcellular level, performing an immunocytochemical study with muscle-specific anti-desmin labelling of 10 thymuses derived from MG patients with lymphofollicular hyperplasia and from 8 normal controls. All thymuses examined revealed mature, immature, and degenerating TMC. Mature TMC contained desmin filaments in between Z-discs provided the sarcomeres were arranged in register. Morphological features of degenerating TMC included hypercontracted sarcomeres, cytoplasmic granular debris, chromatin clumping and, occasionally, membrane-bound bodies. Macrophages were not involved in the process. Immature TMC were of small diameter and contained myofilaments not arranged in myofibrils. In an MG thymus, small immature TMC were found clustered with dying TMC. It may be that degeneration of TMC is a stimulus for the generation of new TMC with faster turnover. This mechanism may mean that more antigen is available in MG patients than in normal controls, despite constant TMC numbers

Key words Thymus · Myoid cells · Desmin · Myasthenia gravis · Immunoelectron microscopy

Introduction

Thymic myoid cells (TMC) are mononuclear cells that are sparsely distributed in the thymic medulla. They resemble skeletal muscle in that their cytoplasm is tightly packed with orderly or haphazardly arranged myofibrils. They express actin and muscle-specific myosin [5, 21], the muscle-specific proteins desmin [8, 11, 24], myogenin [8, 16, 20], creatine kinase specific for striated muscle, b-enolase [20], troponin, the fetal acetylcholine receptor [21], probably Myf-4 mRNA in normal murine [7] but not in myasthenic thymus [9], and myogenin mRNA in myasthenic thymus [12]. TMC are thought to trigger the autoimmunization in myasthenia gravis (MG) [14, 30]. A majority (65–80%) of MG patients exhibit thymitis with or without lymphofollicular hyperplasia [30], and thymectomy is frequently beneficial in MG patients [29].

Most previous studies on the role of TMC in MC were based upon light-microscopical investigations of numbers. However, TMC are a heterogeneous cell population when viewed at the ultrastructural level. The normal subtype is as described above and will be termed *mature* in this paper. In *immature* forms, actin and myosin filaments are distributed individually and not yet arranged in myofibrils. In *degenerating* TMC, the myofibrils are contracted and clumped together [3, 5, 18, 28].

We performed a pre-embedding immunohistochemical study on TMC with a monoclonal anti-desmin antibody. We examined 10 MG patients with lymphofollicular hyperplasia and 8 normal controls. The study was aimed at following the fate of degenerating TMC and finding whether differences existed between the TMC populations of MG patients and of normal controls at the subcellular level.

Materials and methods

Thymuses were surgically removed from 10 patients with MG and lymphofollicular hyperplasia. The patient's ages varied between 10 and 32 years (mean, 24.1 years). For diagnostic purposes, the major part of each specimen was fixed en bloc in formalin and embedded in paraffin. For immunoelectron microscopy, a small part of each thymus was chopped into pieces measuring 0.5 cm in diameter and immersion fixed in either 4% freshly prepared para-

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formaldehyde, or in Zamboni fixative [25] for 4 h at 5° C. The tissue particles were then stored in Millonig buffer with 0.1% sodium azide (Sigma). For comparison, 8 thymuses were obtained at open heart surgery from children varying in age between 7 days and 13 years (mean, 3.5 years), and these were worked up identically.

Sections 5 µm thick were cut from the paraffin blocks. They were stained with haematoxylin and eosin, perodic acid-Schiff, and Giemsa stains. Sections of at least one block were also immunostained with anti-desmin (Dako, D33, 1:20). The quantitative distribution of TMC was assessed by counting the number of desmin-positive TMC/mm^2 of thymic lymphoid tissue, using a square plane in the ocular level of the microscope.

For immunoelectron microscopy, fixed tissue sections 50–100 µm thick were cut with a vibratome and stored in Millonig buffer with 0.1% sodium azide added. Sections were immersed free floating in the diluted primary antibody. Anti-desmin was purchased from Dako (D33), and all other chemicals from Sigma. The primary antibody was diluted (1:20) in Millonig buffer containing 0.1% sodium azide, 1% bovine serum albumin, and 0.01% saponin. The rinsing buffer did not contain bovine serum albumin. The vibratome sections were incubated for 16 h at room temperature. The reaction product was made visible by the biotin–avidin-peroxidase method. The sections were incubated in biotinylated rabbit anti-mouse immunoglobulins (1:100) for 2 h, followed by 2 h in peroxidase-conjugated avidin (1:100). After this step, pure Millonig buffer was used for rinsing. For the reaction product, 0.5% diaminobenzidine was dissolved in distilled water. H_2O_2 was added to a final concentration of 0.0003%.

The sections were postfixed in osmium tetroxide, dehydrated in ethanol and embedded in Spurr resin according to standard procedures. For light microscopy, sections 2 µm thick were cut with a glass knife. From areas of interest, thin sections were cut from the same block, using a diamond knife. If was thus possible to view identical areas by both light and electron microscopy.

Results

There was a high variation in the individual values for TMC number among both the normal controls and the MG patients. In MG patients, the number of TMC/mm^2 ranged between 0.1 and 5.4; in normal controls, between 0.4 and 5.7 TMC/mm^2.

TMC were confined almost exclusively to the thymic medulla (not shown). They were either round, measuring approximately 10 µm in diameter, or elongated (Fig. 1a). A few TMC (less than 1%) revealed cross-striation (Fig. 1). The reaction product in these cells was localized between myofibrils at the Z-disc level (Fig. 1b). Most TMC, however, revealed diffuse staining with whole mount viewing, which resulted in a peripheral rim in thick sections (not shown).

Many TMC, however, had entered the degenerative process. In these cells, the sarcomeres were clotted together and sarcomeres were no longer discernible. The sarcolemma was intact at the beginning (Fig. 2a). With the degenerative process advancing, the sarcomeres shrank further. Desmin filaments were localized at the periphery of the clotted and shrunken sarcomeres. Still more to the periphery, organelles and granular cytoplasmic material tended to drop off the TMC and to be expelled into the intercellular space (Fig. 2b). By then, the plasma membrane had disappeared. The granular cytoplasmic material sometimes condensed to form structures without a membrane (Fig. 2c).

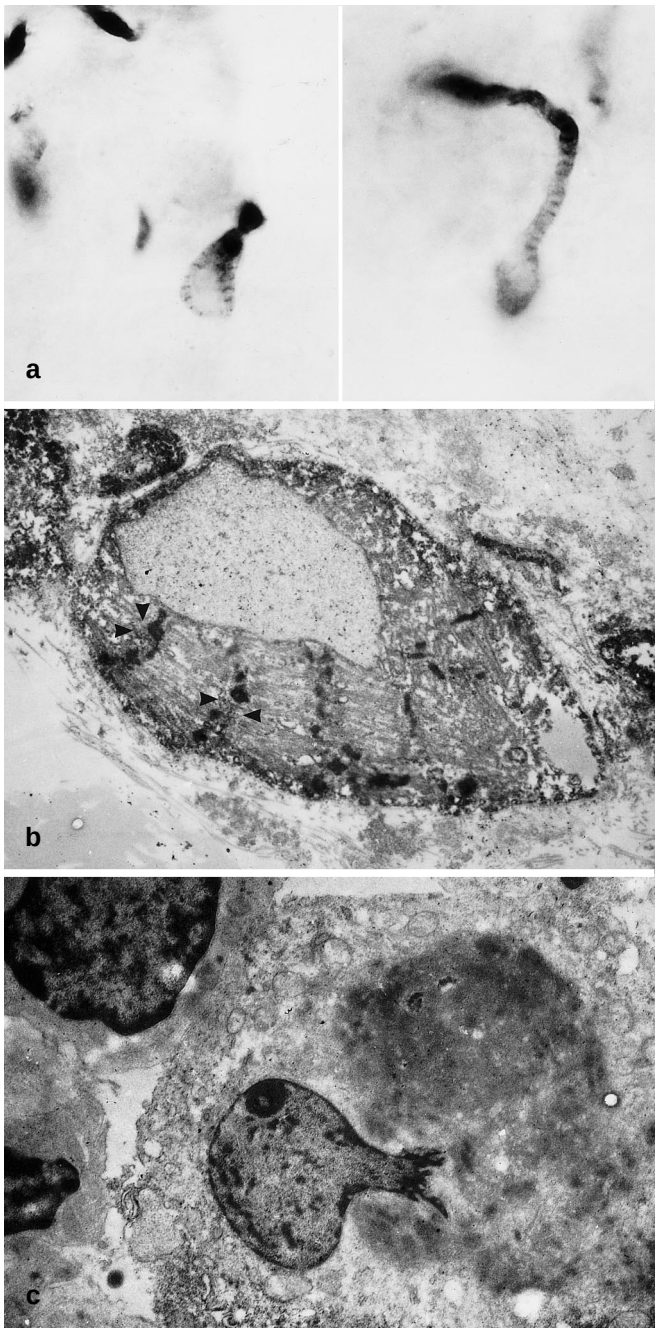

Fig. 1a–c Mature thymic myoid cells (TMC). Anti-desmin staining. **a** Light microscopy. Intact TMC in vibratome sections. Round and elongated TMC displaying cross-striation ×950. **b** Electron microscopy. The sarcomeres are precisely aligned. The reaction product is localized between the Z-discs (*arrowheads*). ×8550. **c** Negative control. ×11000

Occasionally, small immature TMC were observed. They contained few myofilaments not arranged in myofibrils (Fig. 3a). As maturation advanced, the TMC sometimes extended pseudopodial cytoplasmic processes into invaginations of the wall of a neighbouring TMC (Fig. 3b).

In a myasthenic patient, but not in normal controls, clusters containing both immature TMC, which were in

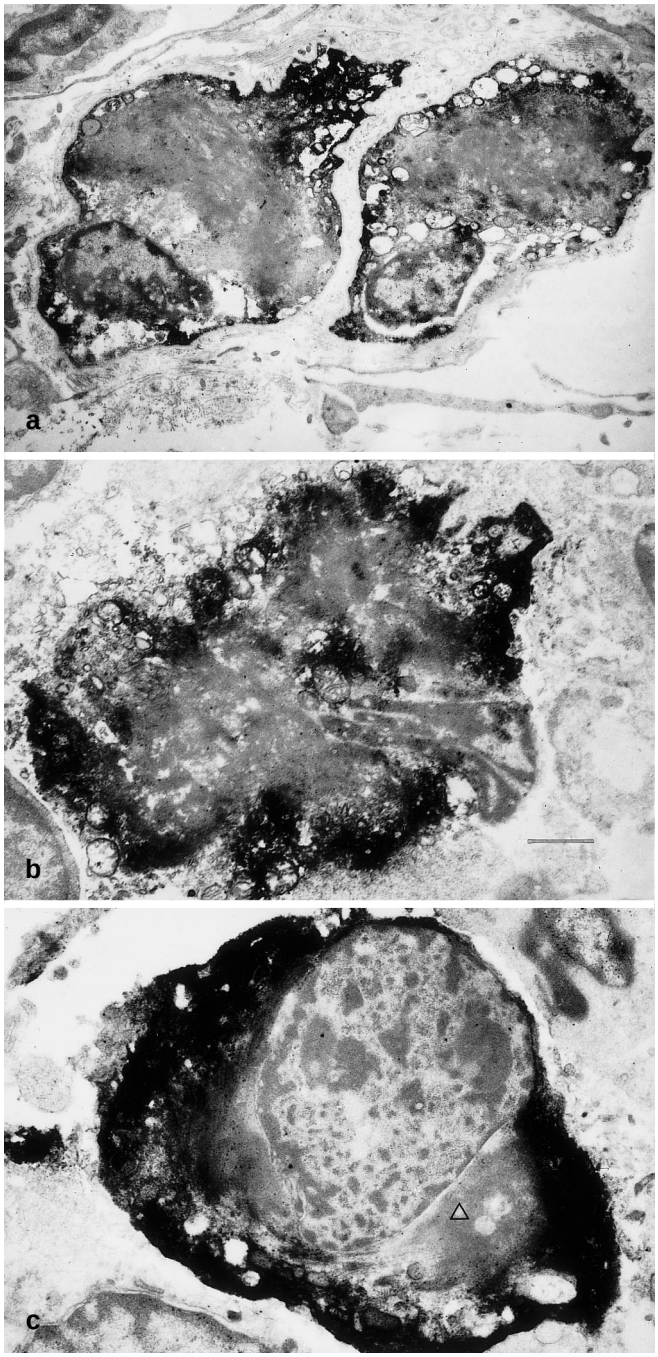

Fig. 2a–c Degenerating TMC. Anti-desmin staining. Electron microscopy. **a** Two TMC. The sarcomeres are shrunken; individual sarcomeres are not discernible. The nucleus shows clumped chromatin. The plasmalemma is intact (not seen at this magnfication) ×6100. **b**, **c** TMC in advanced stages of disintegration. **b** The nucleus, showing clumped chromatin, is in the process of being extruded from the cell. As in **a**, the sarcomeres are shrunken and surrounded by a rim of desmin-positive material. In the periphery, cellular organelles and granular material are localized and starting to drop off the TMC. The plasma membrane is lost, but the cellular outline is still discernible. ×11000. **c** The sarcomeres are condensed to form a minute mass (*triangle*). The granular material is also condensed. The outlines of the cell are no longer discernible. In fact, this structure would hardly be recognized as a myoid cell if the anti-desmin staining did not hint at it ×12200

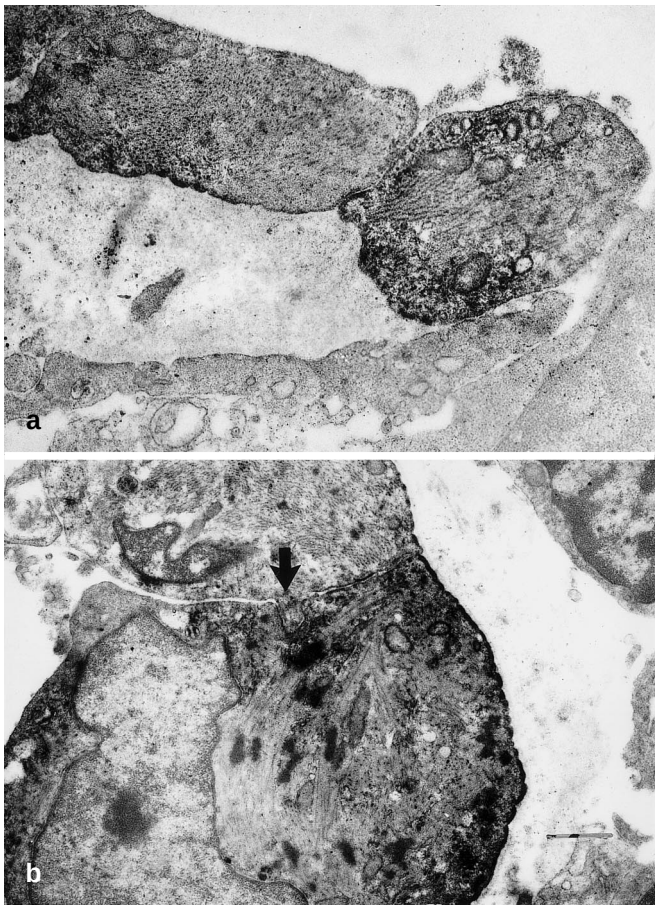

Fig. 3a, b Immature TMC. Electron micrographs. **a** Nascent TMC display myofilaments that have not yet formed myofibrils. ×24000 **b** A young TMC protrudes a pseudopodial process (*arrow*) into an invagination of the wall of a neighbouring TMC ×24000

different states of maturation, and degenerating TMC were encountered (Fig. 4). The cytoplasm of the small immature cells was interspersed with myofilaments that had not yet formed myofibrils. They were diffusely labelled by anti-desmin staining (Fig. 4).

Discussion

The pattern of anti-desmin staining was contingent on the degree of sarcomere organization in the cytoplasm. In TMC with well-arranged sarcomeres, desmin was localized between the myofibrils at the level of the Z-discs (Fig. 1), corresponding to the normal distribution of desmin in adult skeletal muscle fibres [19, 27]. This was true for less than 1% of the TMC. In most TMC, the sarcomeres were arranged in a whorl-like pattern with anti-desmin staining confined to the periphery (not shown). In skeletal muscle, lateral alignment of the sarcomeres precedes desmin confinement to the Z band [26]. The same seems to be true for the localization of desmin in TMC: that is to say that, in TMC in which

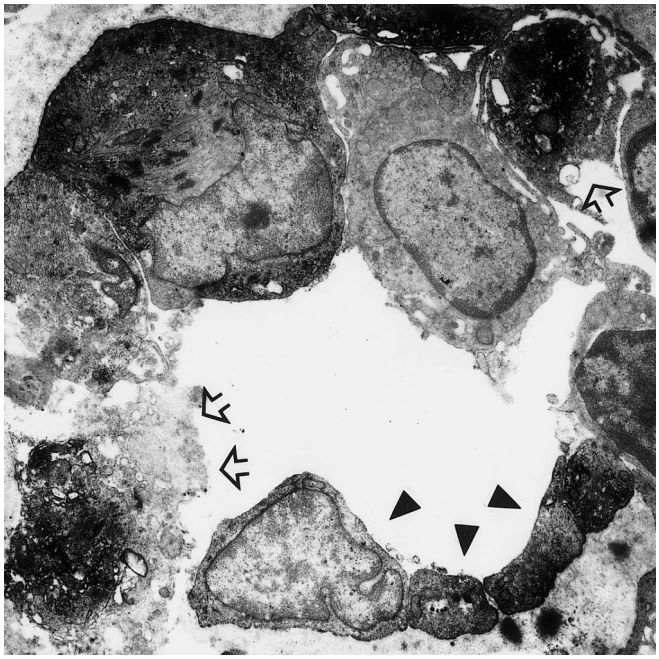

Fig. 4 Cluster of immature and degenerating TMC. Electron micrograph. Several degenerating (*open arrows*) and four immature (*arrowheads*) TMC are arranged in a semi-circle ×8000

sarcomeres do not align but remain unorganized, desmin is not arranged between myofibrils.

Our findings confirm previous reports stating that in TMC disorganized myofibrils were more often found than precisely aligned ones and that cellular organelles, such as t-tubules and sarcoplasmic reticulum, were mostly confined to the periphery of the TMC rather than being located between myofibrils, as in normal skeletal muscle [5]. This has been compared to skeletal muscle after denervation or various other pathologic conditions [5]. However, after denervation organelles are still seen between myofibrils [6]. Aneural skeletal muscle develops a normal ultrastructure before it eventually disappears, if no innervation takes place [22].

We observed several degenerating TMC in each vibratome section examined at the ultrastructural level. The amount of dying TMC may be the reason for the failure to detect the muscle-specific transcription factor Myf-4 mRNA in myasthenic thymuses [9]. The degenerative process of TMC included hypercontraction and shrinkage of myofibrils, clumping of nuclear chromatin, relative preservation of cell organelles, expulsion of the nucleus, and occasional formation of membrane-bound particles with smooth unstructured material in them (Fig. 2). The eventual fate of TMC remains unexplained. Typical apoptotic bodies have never been observed. Macrophages were not seen to be involved in the process.

It is not known why TMC degenerate. They are not innervated, and skeletal muscle that is developing without innervation or has been denervated degenerates and eventually disappears [1, 23]. Alternatively, the thymic microenvironment may not contain the appropriate growth factors that are mandatory for muscle cells [13].

Immature TMC are small cells, with scarce cytoplasm in which myofilaments are diffusely distributed (Fig. 3). With ongoing maturation they sometimes send out pseudopodial processes into invaginations of the wall of a neighbouring TMC (Fig. 3b), as between neighbouring primary and secondary myotubes during fetal myogenesis [10]. Previously, immature TMC were identified by their loosely arranged cytoplasm [5, 18, 28]. *Small* immature TMC have not been described before. With their scanty cytoplasm, they bear a resemblance to stem cells [17]; however, with their scattered myofilaments they are definitely committed to the myogenic lineage.

The stimulus for new TMC to arise remains to be elucidated. In skeletal muscle, crushed muscle extract is a stimulus for satellite cells to proliferate and to form new muscle fibres [2] and by analogy, degenerating TMC might be a stimulus for the generation of new TMC. However, the source of new TMC is also not clear. In tissue culture studies, cross-striated myotubes arose 8 days after thymic tissue was placed in culture medium. It was concluded from this lag period that cross-striated myotubes arose from undifferentiated stem cells [31].

Several workers have suggested that TMC stimulate the autoimmune response that leads to the production of auto-antibodies against AChR and, eventually, to the destruction of the motor end-plate in MG [11, 30]. Other investigators, however, have raised some doubt about the unique role of TMC in intrathymic autosensitization in MG. Their points included the finding that the number of TMC in MG patients was not different from that in normal controls, and that there was no correlation between the number of TMC and age or duration of illness [5, 21], although others found depletion of TMC in MG [8] and an involvement of the thymus in an age-dependent involution process both in MG patients and in controls [15]. In our study, we found no significant difference between myasthenic patients and normal controls, with one exception (see below). In both groups there was a wide range of numbers of TMC and normal, degenerating, and immature TMC were encountered to a similar degree. Quantitative measurements have not been performed on electron micrographs

We found that in 1 MG patient, but not in controls, small groups of immature TMC formed clusters with degenerating TMC (Fig. 4). In this case, the turnover of TMC may be accelerated and more antigen may be available although the number of TMC is not increased. The microenvironment of TMC in myasthenic thymuses contains significantly more antigen-presenting cells than that in normal controls [11].

Acknowledgements We wish to thank R. Lisner for expert technical assistance and K. Herbig for photographical help. We are grateful to Professor von der Emde and his colleagues from the Department of Cardiac Surgery, University of Erlangen, for providing human thymic tissue. Parts of the results have already been published in abstract form [4].

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