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Original Article

Age-related changes of mitochondrial transcription factor a expression in rotator cuff degeneration

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Abstract: One cause of rotator cuff tears is thought to be age-related degenerative changes occurring in the rotator cuff. Using Rat rotator cuff we determined age-related changes in mitochondrial transcription factor A (TFAM) expression in rotator cuff degeneration to clarify the presence/absence of mitochondrial stress. The materials used were rotator cuffs (supraspinatus) of 5-, 24-, 48-, and 100-week-old Wistar Rats (five animals each). Histopathological study revealed a 4-layer structure consisting of a bone layer, calcified cartilage layer, non-calcified cartilage layer, and tendinous component), with age-related narrowing of the non-calcified cartilage layer confirmed to be present. In an immunohistochemical TFAM study positive findings of the non-calcified cartilage layer were less prominent in the 100-week-old group. In an Enzyme-Linked Immunosorbent Assay (ELISA) study, these were more prominent in the 5-week-old to 24-week-old groups, and slightly less so in the 48-week-old group as compared to the 24-week-old one. In the 100-week-old group as compared to the 24-week-old one they were significantly less prominent ($p<0.05$). The non-calcified cartilage layer is a major site for the dispersion of mechanical energy, and the change in TFAM expression noted at the same site in the present study and the results of ELISA suggest that age-related changes in mitochondrial stress may be one cause of rotator cuff degeneration.

Keywords: Rotator cuff degeneration , supraspinatus (SSP), mitochondrial transcription factor A (TFAM), age-related change

Introduction

Precipitating factors of shoulder rotator cuff tears can be broadly divided into traumatic and atraumatic ones. Of these, the main causes of atraumatic rotator cuff tears are degeneration of the rotator cuff itself and those caused by the presence of subacromial spurs. With regard to the relation between subacromial spurs and rotator cuff tears, Ogawa et al. reported an association between the presence of ≥5 mm spurs and rotator cuff tears [1], while Bjornssonet et al. described that by performing subacromial decompression in shoulder impingement syndrome the incidence of rotator cuff tears is decreased [2].

On the other hand, various reports have suggested problems intrinsic to the rotator cuff itself, including age-related degenerative changes occurring in the rotator cuff muscles that are thought to induce tendinitis and tears. A torn rotator cuff almost never undergoes spontaneous healing, with this related to the presence of degenerative changes in the torn rotator cuff portion [3, 4].

The incidence of rotator cuff tears increases with age, with approximately 30% of cases aged ≥60 years [5]. This has been attributed to degenerative changes occurring in the rotator cuff with increasing age [6]. Factors promoting the development of rotator cuff degeneration have been reported to include thinning of collagen fibers, structural disorientation, decreased volume of fibrous cartilage at the site of attachment, decreased number of cartilage cells and blood vessels, and age-related changes in the generation of oxidative stress [7]. Oxidative stress induces a state in which the balance between in vivo oxidation and redox state is upset. Such a state has recently been implicat-

ed in the development and progression of various pathological conditions as well as age-related changes in tissue degeneration [8-12], with the mitochondria considered to be the main site of regulation and generation of this process [13, 14].

Also, of the studies hitherto focusing on the causes of rotator cuff tears, the majority have relied on rotator cuff tear models to examine repair or genetically modified models, whereas it also remains important to confirm the development of simple age-related changes in normal models without tears.

Furthermore, in most studies using rats, 'young' rats have usually been considered as being less than 10 weeks old, and 'old' or 'adult' ones as about 24 weeks old. However, about 24-week-old rats can be considered to correspond to young adulthood in humans, and so when examining tissue degeneration older rats also have to be assessed.

This prompted us to investigate age-related changes in mitochondrial transcription factor A (TFAM) expression in Wistar rat rotator cuff (supraspinatus). TFAM is an important factor in mitochondrial DNA preservation and stabilization that has been demonstrated to play multifunctional roles at both the molecular and cellular levels. Using rats raised until 5-100 weeks' old, we focused on the influence of mitochondria on rotator cuff degeneration.

Materials and methods

Animals

To appreciate the age-related changes, 5-week-old (5 w group), 24-week-old corresponding to young adult age in humans (24 w), 48-week-old (48 w) corresponding to middle age, and 100-week-old (100 w) corresponding to late middle-old age Wistar rats (five animals each) were used. They were studied at the Animal Center of Kanazawa Medical University. All rats were housed under standard laboratory conditions (temperature 24°C, 12-hour light/dark cycle) and were given food and water ad libitum.

This study was conducted in accordance with the guidelines of the Animal Research Committee of Kanazawa Medical University.

Tissue preparation

All rats were killed using an overdose of intraperitoneally injected sodium pentobarbital. After sacrifice, bilateral supraspinatus (SSP) muscles were obtained from the proximal humerus. The obtained right shoulder tissue was fixed in formalin, and the left side quick-frozen in liquid nitrogen. Then the tissues in the right shoulder were fixed for 1 week with 10% formalin -0.1 *M* phosphate buffer (pH 7.4). The samples were decalcified with 25% formic acid for 3 days. The specimens were embedded in paraffin, cut into 3 mm sections using a microtome, and stained with hematoxylin and eosin (H&E).

Histopathological study

After the obtained tissues were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, the 4-layer structure (bone tissue, calcified fibrous cartilage layer, non-calcified fibrous cartilage layer, tendinous tissue) at the supraspinatus tendinous-bone site of attachment was evaluated with a light microscope.

Immunohistochemistry

To examine the expression of TFAM in SSP, the SSP was stained immunohistochemically with anti-TFAM antibody. Briefly, after deparaffinization, the sections were treated with 0.3% H_2O_2 in methanol for 30 min at room temperature, and with 0.1% trypsin for 15 min at 37°C. Then the sections were reacted with TFAM monoclonal antibody (1:100) for 4° overnight, followed by incubation with Dako EnVision/HRP system (Dako, Tokyo, Japan) for 30 min at room temperature. Sections were then treated with DAB for 5 min, and counterstained with hematoxylin-eosin for 1 min.

Measurement of TFAM in SSP level by enzyme-linked immunosorbent assay (ELISA)

We weighed differences between each group to detect TFAM levels in SSP. TFAM levels were determined with a competitive ELISA kit. In this kit, TFAM concentrations were measured at an absorbance of 450 nm. The levels of total protein (mg/dl) in SSP samples were simultaneously measured for compensation of TFAM levels (pg/mg protein) in SSP.

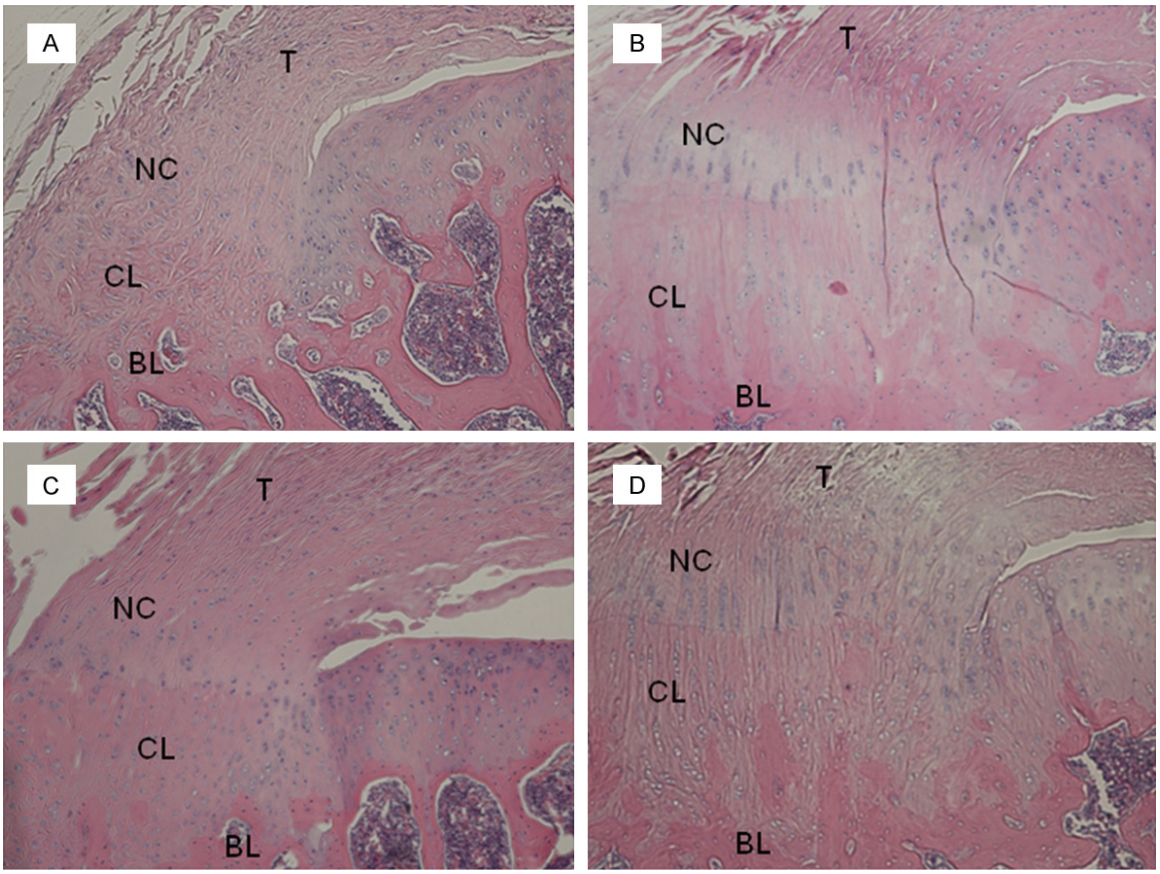

Figure 1. Histopathological study (×100). A: 5 w group, B: 24 w group, C: 48 w group, D: 100 w group. In each group a 4-layer structure consisting of a bone layer (BL), calcified cartilage layer (CL), non-calcified cartilage layer (NC), and tendinous component (T) was found. In the 100 w group narrowing of both the calcified and non-calcified cartilage layers was found, and was particularly marked in the non-calcified cartilage layer.

Statistical analysis

TFAM levels in SSP were expressed as the mean ± standard error. Statistical analysis was performed using the one way analysis of variance with Tukey's post-hoc test. *P* values less than 0.05 were considered significant. Statistical analysis was performed using StatView J-5.0 software (SAS Institute).

Results

Histopathological study

In the morphological study using HE staining, in SSP at the site of attachment a 4-layer structure consisting of a tendinous layer, calcified fibrous cartilage layer, non-calcified fibrous cartilage layer and bone tissue was found. In the comparison between each of the groups, in the 100 w group as compared to all groups up to the 48 w group, narrowing of the calcified and non-calcified cartilage layers was found, which was particularly marked in the non-calcified cartilage layer (**Figure 1**).

Immunohistochemical study

In the 5 w to 48 w groups, the expression of TFAM in all 4 layers was extremely positive. In contrast, in the 100 w group staining was decreased in all 4 layers, with a clear decrease in expression noted especially in the calcified fibrous cartilage layer and the non-calcified cartilage layer (**Figure 2**).

Expression of TFAM determined by ELISA

The values of TFAM were 118.5±20.3 pg/mg protein in group 5 w, 176.5±45.3 pg/mg protein in group 24 w, 155.4±19.2 pg/mg protein in group 48 w and 74.1±18.3 pg/mg protein in

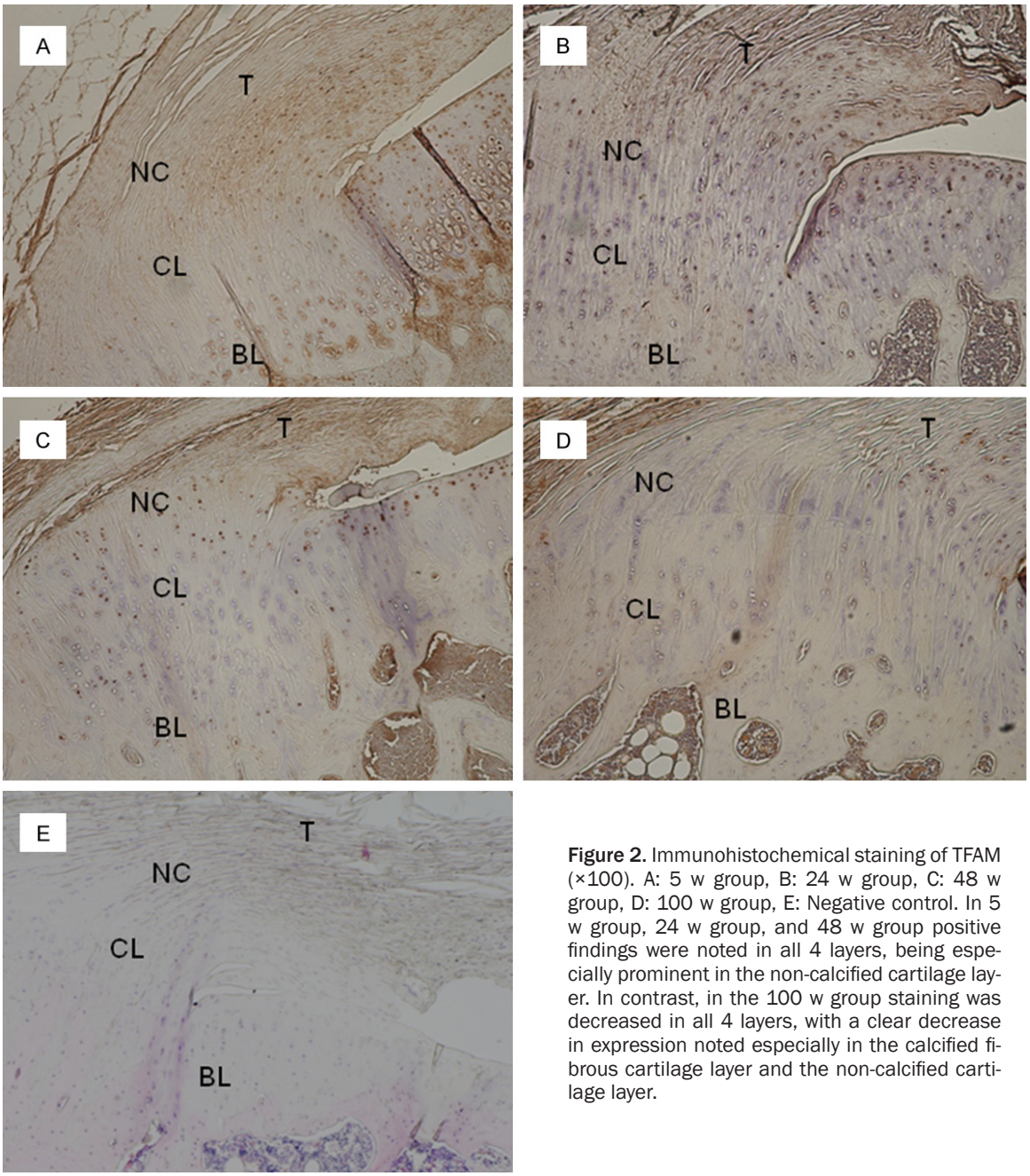

Figure 2. Immunohistochemical staining of TFAM (×100). A: 5 w group, B: 24 w group, C: 48 w group, D: 100 w group, E: Negative control. In 5 w group, 24 w group, and 48 w group positive findings were noted in all 4 layers, being especially prominent in the non-calcified cartilage layer. In contrast, in the 100 w group staining was decreased in all 4 layers, with a clear decrease in expression noted especially in the calcified fibrous cartilage layer and the non-calcified cartilage layer.

group 100 w. A significant decrease was found in the 100 w group as compared to 24 w group ($p<0.05$) (**Table 1**).

Discussion

Since mitochondria account for ≥80% of ATP synthesis in cells, mitochondrial genome abnormalities create major impediments to cellular survival and function. The mitochondrial electron transport system in general accounts for ≥90% of cellular oxygen consumption, 1-5% of which is converted to active oxygen species, and is considered to be the major source of intracellular active oxygen generation. For this reason, mtDNA as compared to nuclear DNA is thought to be subjected to more severe oxidation injury, and is considered as the main site of oxidative stress generation [13, 14]. Michikawa et al. investigated mtDNA mutations at various ages in man and noted frequent and marked heteroplasmy in elderly subjects aged ≥65

Table 1. SSP TFAM expression determined by ELISA

Group	TFAM (pg/mg)
5 w	118.5±20.27
24 w	176.5±45.29
48 w	155.4±19.15
100 w	74.1±18.31*

Note: TFAM expression gradually increased from 5 w to 24 w. At 48 w a tendency to decreased expression was noted. At 100 w as compared to 24 w TFAM expression was significantly decreased. Data are shown as mean ± SE. *, $p<0.05$ vs. 24 group.

years [15]. Also, it has been reported with mtDNA using a next-generation DNA sequencer, namely ultra deep sequencing, that the accumulation of age-related changes in mutations is not due to the development of new mutations, but rather to the clonal increase of mutations originally present [16]. In any case, such reports document that mtDNA needed for tissue preservation and somatic cell mtDNA mutations accumulate at a high rate with increasing age [17]. Namely, mitochondria themselves are a source of latent oxidative stress (being the in vivo site of stress generation), with the delicate and precise control of their function and amount important for their normal survival.

The mitochondrial transcription factor A (mitochondrial A: TFAM) focused on in this study is the first mtDNA transcription factor to be purified and cloned [18, 19]. Various biochemical experiments have clarified that almost all See comment in PubMed Commons be.

TFAM is bound to mitochondrial DNA, and its localization is consistent with that of mitochondrial DNA [20]. It has been reported that serial changes in the expression of TFAM and mitochondrial DNA run largely parallel [21]. In contrast, when overexpression of TFAM is induced the amount of mitochondrial DNA also increases in proportion to the amount of the former [21]. Accordingly, TFAM is considered essential for the stable presence of mitochondrial DNA.

This study clarified that TFAM increased in the process of maturation until 24 weeks of age and subsequently decreased, suggesting that in the rotator cuff as well (consistent with reports on other organs) mitochondrial function becomes less stable with increasing age. As mentioned above, TFAM by enhancing mitochondrial function and stability is thought to be important in preserving cellular function and number. In fact, in the present study too at 100 weeks, the presence of tissue structural derangements was confirmed, and TFAM levels at 100 weeks were found to be significantly decreased. Furthermore, the non-calcified fibrous cartilage layer was noted to show a softer physical property than tendinous tissue, with this possibly contributing to the dispersion of mechanical stress [22]. In the present study TFAM expression was especially prominent at this site until 48 weeks, while in the 100-week-old group an age-related decrease in expression was detected. This suggests that the increased dysfunction and instability of mitochondria associated with aging as well as tissue degeneration interfere with the dispersion of various stresses, including mechanical stress at the rotator cuff, thereby predisposing it to injury.

The results of this study suggest that in vivo stress resulting from decreased mitochondrial function promotes rotator cuff degeneration, namely that mitochondrial stress is involved in this process.

Hitherto a number of reports have demonstrated that TFAM administration may be useful in preventing cellular and tissue degeneration, as well as the development of various pathological conditions [23, 24]. Future investigations will also need to focus on issues such as the inhibitory effect of TFAM administration on rotator cuff degeneration.

Disclosure of conflict of interest

None.

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