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Abstract

The purpose of this study was to elucidate the role of extracellular matrix components such as aggrecan, fibronectin, and laminin in the extraocular muscle of patients with strabismus. Resected tissues of the medial rectus muscle of 47 patients with intermittent exotropia obtained during recession-resection surgery were frozen under liquid nitrogen and pulverized by a Freezer/Mill to solubilize the tissue for enzyme immunoassay. The total amounts of aggrecan, fibronectin, and laminin in the resected tissue were correlated with clinical data of patients such as age, exodeviation, and refractive error. The amount of aggrecan decreased significantly with the advance of age ($P < 0.0001$, Spearman rank correlation test), while the amount of laminin or fibronectin had no correlation with age. Patients with basic type intermittent exotropia showed larger, although not significantly, amounts of aggrecan than those with convergence insufficiency type ($P = 0.0538$, Mann-Whitney U-test). The amount of aggrecan may be related to motor aspects of intermittent exotropia.

KEYWORDS: extraocular muscle, aggrecan, laminin, fibronectin, intermittent extropia

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Original Article

Clinical Correlations of Aggrecan in the Resected Medial Rectus Muscle of Patients with Intermittent Exotropia

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The purpose of this study was to elucidate the role of extracellular matrix components such as aggrecan, fibronectin, and laminin in the extraocular muscle of patients with strabismus. Resected tissues of the medial rectus muscle of 47 patients with intermittent exotropia obtained during recession-resection surgery were frozen under liquid nitrogen and pulverized by a Freezer/Mill to solubilize the tissue for enzyme immunoassay. The total amounts of aggrecan, fibronectin, and laminin in the resected tissue were correlated with clinical data of patients such as age, exodeviation, and refractive error. The amount of aggrecan decreased significantly with the advance of age ($P <$ 0.0001, Spearman rank correlation test), while the amount of laminin or fibronectin had no correlation with age. Patients with basic type intermittent exotropia showed larger, although not significantly, amounts of aggrecan than those with convergence insufficiency type ($P = 0.0538$, Mann-Whitney U-test). The amount of aggrecan may be related to motor aspects of intermittent exotropia.

Key words: extraocular muscle, aggrecan, laminin, fibronectin, intermittent exotropia

The extraocular muscles and their innervation by cranial nerves play a crucial role in eye movement and eye alignment. The muscles consist of a muscular part and tendon part [1, 2]. They belong to skeletal muscles and contain a large amount of extracellular matrix, not only in the muscle sheath, but also in the connective tissue ensheathing each muscle fiber as intramuscular septa (basement membranes). The major components of the tendon are extracellular matrix molecules such as collagen, proteoglycan, laminin, and fibronectin. In rats, laminin and fibronectin have been localized to basement membranes ensheathing muscle fibers [3]. The extraocular muscles are unique in the fact that their tendon inserts directly into the sclera. In general, the extracellular matrix is important for elasticity and the tension of the tissue. Until now, no biochemical study elucidating the role of the extracellular matrix in the extraocular muscles has been performed. To understand the role of extracellular matrix components such as aggrecan, fibronectin, and laminin in the development of strabismus, we measured the amounts of these molecules in the human medial rectus muscle resected during strabismus surgery and studied their correlation with clinical data of the patients.

Materials and Methods

Resected tissue fragments of the medial rectus muscle were obtained from 47 patients with intermittent exotropia during recession-resection surgery (Fig. 1) at Okayama University Hospital from December 1997 to October 2001. The study conformed to the Declaration of Helsinki

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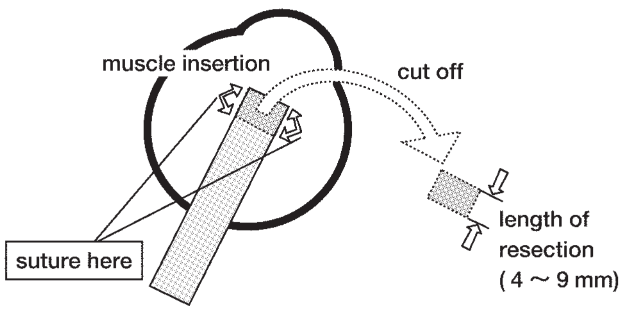

Fig. 1 Schematic representation of resection of the medial rectus muscle during recession-resection surgery for intermittent exotropia. The length of resection (mm) was used for normalizing the quantities of extracellular matrix components in the resected tissue.

and was approved by the University Ethical Committee. Informed consent was obtained from each patient. The patients were 21 males and 26 females with ages ranging from 5 to 74 (mean, 23) years. Preoperative exodeviation at 5 m measured by alternate prism and cover test ranged from 14 to 78 (median, 30) prism diopters. Resected length of the medial rectus muscle ranged from 4 to 9 (mean, 6) mm, based on a calculation of 1 mm recession and 1 mm resection for each 5 prism diopters of exodeviation with a maximum limit of 9 mm. After the surgery, all patients except for 1 had binocular peripheral fusion on the Bagolini striated glasses test, while all patients except for 10 had stereoacuity of 120 sec or better as determined by the TNO Test for Stereoscopic Vision, Tenth Edition (Lameris Ootech, Veenendaal, The Netherlands).

Immediately after removal, the muscle fragments were frozen in liquid nitrogen and stored at $-135\,^{\circ}C$ until use, at which point they were pulverized by No. 6700 Freezer/Mill (Spex Industries, Edison, NJ, USA). The powdered tissue was then suspended in 750 μL of phosphate-buffered saline and centrifuged to obtain the supernatant.

For aggrecan measurement with Human Aggrecan (Proteoglycan) EASIA ELISA Kit (BioSource International Inc., Camarillo, CA, USA), 50 μL of tissue extracts or standards mixed with 100 μL incubation buffer were added to microtiter wells coated with a monoclonal antibody against aggrecan, and incubated for 2 h at room temperature. After being washed 3 times, wells were incubated for 1 h at room temperature with 200 μL of another anti-aggrecan monoclonal antibody conjugated with horseradish peroxidase, and washed again. The color was developed by tetramethylbenzidine and hydrogen peroxide, and read at 450 nm by a microplate reader (EIA Reader, Model 550, Bio-Rad Laboratories Japan, Tokyo, Japan).

Fibronectin and laminin were measured by competitive binding assay using QuantiMatrix Human Fibronectin ELISA Kit and QuantiMatrix Human Laminin ELISA Kit, respectively (Chemicon International Inc., Temecula, CA, USA). Fifty μL of tissue extracts or standards was mixed with an equal volume of anti-human fibronectin or laminin rabbit antibody and incubated for 15 min at room temperature. The mixture was then added to microtiter wells coated with human fibronectin or laminin, and incubated for 1 h. After being washed 4 times, wells were incubated for 30 min at room temperature with 100 μL of anti-rabbit IgG goat antibody conjugated with horseradish peroxidase, and washed again. The color was developed by tetramethylbenzidine and hydrogen peroxide, and read at 450 nm by a microplate reader.

Medical charts were reviewed to obtain clinical data for each patient, including resected length of the medial rectus muscle, gender, age at surgery, refractive error of operated-on eyes expressed as spherical equivalents, exodeviation at 5 m determined by alternate prism and cover test before and after surgery, peripheral fusion on Bagolini striated glasses test, stereoacuity before and after surgery at the final visit, the extent of recurrent exodeviation in one month after the surgery, the presence of family history of strabismus, and the presence of abnormalities in pregnancy and delivery [4]. The patients were also divided into basic type (32 patients), convergence insufficiency type (15 patients), or divergence excess type (none), based on a difference of 15 or larger prism diopters between the near and distant exodeviations. The total amounts of aggrecan, fibronectin, and laminin were calculated from the concentrations in the solubilized resected muscle tissue and normalized by the length of resection. The amounts in unit length (mm) of the resected muscle (Fig. 1) were used for statistical analysis to find the relationship with clinical data.

Results

The amount of aggrecan normalized by unit length of resected muscles decreased significantly with the advance of age ($P < 0.0001$, Spearman rank correlation test, Fig. 2), while the amount of fibronectin or laminin did not correlate with age (Fig. 3 and Fig. 4). No correlation was found among the amounts of aggrecan, fibronectin,

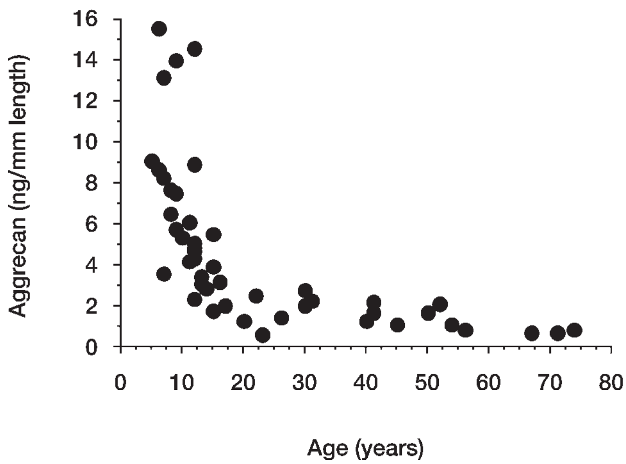

Fig. 2 The amount of aggrecan in the resected medial rectus muscle of patients with intermittent exotropia normalized by unit length (mm) of resection. The amount decreases significantly with age ($P < 0.0001$, Spearman rank correlation test).

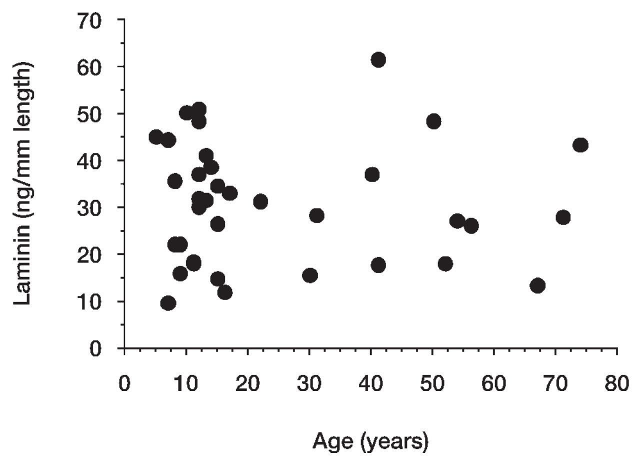

Fig. 4 The amount of laminin in the resected medial rectus muscle of patients with intermittent exotropia normalized by unit length (mm) of resection. Note no correlation between the amount and age.

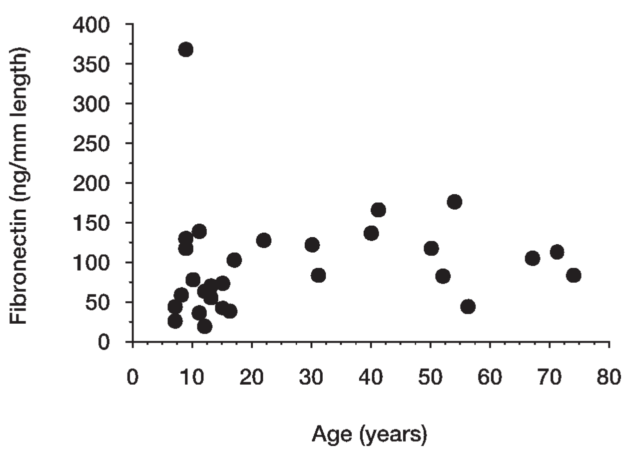

Fig. 3 The amount of fibronectin in the resected medial rectus muscle of patients with intermittent exotropia normalized by unit length (mm) of resection. Note no correlation between the amount and age.

and laminin in the resected tissue of the medial rectus muscle ($P = 0.5195$ for aggrecan versus laminin, $P = 0.3911$ for aggrecan versus fibronectin, and $P = 0.7609$ for laminin versus fibronectin, Spearman rank correlation test).

Patients with basic type intermittent exotropia had larger, although not significantly, amounts of aggrecan than those with convergence insufficiency type ($P = 0.0538$, Mann-Whitney U-test, Fig. 5, Table 1). Patients with the basic type were also younger, although not significantly, than those with the convergence insufficiency type ($P = 0.0708$, Mann-Whitney U-test, Table 1). The amounts of aggrecan, fibronectin, and laminin did not correlate with patients' gender, refractive errors, exodeviation, peripheral fusion, stereoacuity, recurrent exodeviation, family history of strabismus, or abnormalities in pregnancy and delivery (Table 2).

Discussion

The resected tissue of the medial rectus muscle contained both tendon and muscular portions since the average length of tendon at its insertion in the medial rectus muscle is 3.5–3.7 mm [1, 2]. The hardness of these tendon and muscle tissues prevent their solubilization, which is a first step in biochemical studies. Pulverization of frozen tissues in liquid nitrogen, as performed in this study, allowed for efficient solubilization of the human extraocular muscle. For the first time, we could demonstrate the presence of aggrecan in the human medial rectus muscle. In addition, we discovered that the amount of aggrecan decreases with the advance of age, while the amounts of fibronectin and laminin have no such correlation with age.

In this study, the total amounts of extracellular matrix components in the resected muscle tissue were normalized by the length of resection at the surgery. Ideally, the total amounts of extracellular matrix components would be

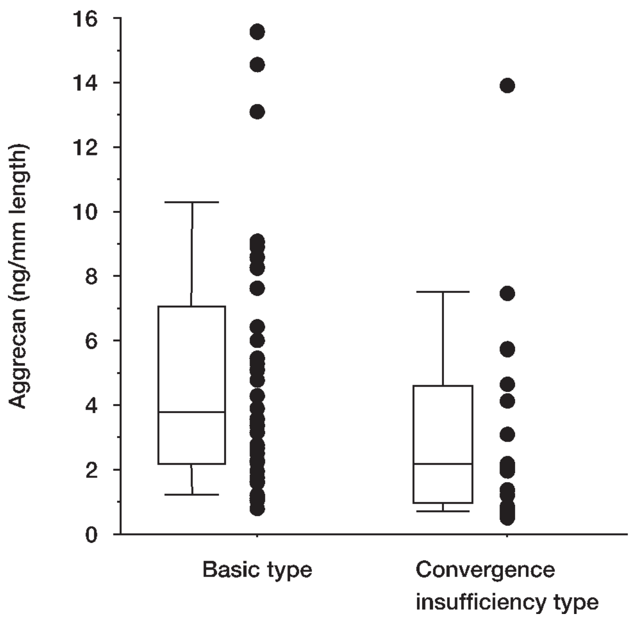

Fig. 5 The amount of aggrecan in the resected medial rectus muscle of patients with intermittent exotropia normalized by unit length (mm) of resection. Patients with basic type intermittent exotropia show larger amounts of aggrecan than those with convergence insufficiency type ($P = 0.0538$, Mann-Whitney U-test). In the box plot, the lower T-bar, lower and upper lines of the box, and upper T-bar indicate 10, 25, 75, and 90 percentiles of the sample, respectively. The line inside the box indicates the median.

normalized by the weight of the resected tissue or the total amounts of protein. Since the resected muscle tissues were too small to weigh accurately or to use for total protein measurement, the length of resection was instead used for normalization.

Aggrecan is a main constituent of cartilage [5] and has also been detected in serum [6]. The amount of aggrecan in serum also decreases with age. The influence of contamination with blood aggrecan would be negligible in the measurements of the present study since resected muscle tissues contained few blood vessels. Aggrecan is also present in the human sclera [7] as well as in the tendons of other human skeletal muscles [8-10]. Concurrently with this study, aggrecan was immunohistochemically detected in the tendon of the superior oblique muscle [11]. Based on these facts, aggrecan could be present in the tendon portion of the medial rectus muscle. An immunohistochemical study to localize aggrecan in the medial rectus muscle was not performed since the main aim of this study was to correlate the amounts of aggrecan with clinical features of strabismus.

The enzyme immunoassay kit for aggrecan is based on a sandwich method using 2 kinds of monoclonal antibodies directed against glycosaminoglycan. In the anterior portion of the human sclera, the content of the core protein of aggrecan did not change with age, while the content of glycosaminoglycan decreased with age [12]. The

Table 1 The amounts of aggrecan, fibronectin, and laminin in the resected medial rectus muscle tissues of patients with convergence insufficiency type intermittent exotropia and basic type intermittent exotropia

		Convergence insufficiency type n = 15	Basic type n = 32	Mann-Whitney U-test P value
Age (years)	mean	32.5	18.8	0.0708
	median	26	12.5	
	range	9～74	5～56	
Aggrecan (ng/mm length)	mean	3.39	5.05	0.0538
	median	2.12	3.76	
	range	0.55～13.97	0.82～15.62	
Fibronectin (ng/mm length)	mean	120.23	83.33	0.2319
	median	110.16	75.63	
	range	21.06～369.29	26.5～177.5	
Laminin (ng/mm length)	mean	30.6	31.02	0.8234
	median	27.94	31.5	
	range	13.44～61.75	9.9～50.88	
Male /Female		8 / 7	13 / 19	

Table 2 Statistical correlation between clinical factors and the amounts of aggrecan, fi-bronectin, or laminin in the resected medial rectus muscle tissues in 47 patients with intermittent exotropia

Clinical Factor	*P* value			Statistical test
	Aggrecan	Fibronectin	Laminin	
Gender	0.7563	0.2949	0.8121	Mann-Whitney *U*-test
Age (years)	< 0.0001	0.1328	0.6270	Spearman rank correlation test
Refractive errors	0.8333	0.5569	0.7485	Spearman rank correlation test
Deviation at 5 m before surgery	0.3323	0.7017	0.7284	Spearman rank correlation test
Deviation at 5 m after surgery	0.4487	0.7800	0.0828	Spearman rank correlation test
Peripheral fusion at 5 m before surgery	0.3998	0.8633	0.5316	Mann-Whitney *U*-test
TNO stereoacuity before surgery	0.4946	0.6207	0.8746	Spearman rank correlation test
TNO stereoacuity after surgery	0.5723	0.7338	0.6835	Spearman rank correlation test
Recurrent exodeviation	0.2754	0.2002	0.9966	Spearman rank correlation test
Convergence insufficiency type versus basic type	0.0538	0.2319	0.8234	Mann-Whitney *U*-test
Family history of strabismus	0.4566	0.1573	0.7572	Mann-Whitney *U*-test
Abnormalities in pregnancy and delivery	0.3651	0.3759	0.1501	Mann-Whitney *U*-test

amounts of aggrecan in the anterior portion of the sclera and in the tendon of the medial rectus muscle, thus show a similar change during the aging process.

In this study, patients with basic type intermittent exotropia had larger amounts of aggrecan than those with convergence insufficiency type. Since the patients with the basic type were also younger than those with the convergence insufficiency type, the difference might have been caused primarily by age. Other extracellular matrix components, laminin and fibronectin, did not change with age. In previous studies, age-related decreases in aggrecan were also found in human articular cartilage [13, 14]. Such age-related decreases in aggrecan in the extraocular muscle may underlie motor aspects of intermittent exotropia manifested clinically as the difference between convergence insufficiency type and basic type. It remains unknown whether the difference in aggrecan amounts is one of the causes for clinical types of intermittent exotropia or rather the result of prolonged muscle contraction or tension.

Until now, the muscle tension of the extraocular muscle has been measured in terms of passive and active force using a forceps or an electric strain gauge to assess the motor aspects of strabismus [15–18]. Not only the characteristics of muscle fibers, but also the content and characteristics of the connective tissue of the muscle such as tendon, muscle sheath, and basement membrane of muscle fibers, could play an important role in muscle tension or elasticity. Furthermore, the gross anatomy of the extraocular muscle, including the orbital layer and the global layer, also influences active and passive muscle forces [19, 20]. Compressive or tensile loading has been shown to influence synthesis and turnover of aggrecan in bovine tendons and rabbit ligaments in culture [21, 22]. The amount of aggrecan in the extraocular muscle might therefore be influenced by eye movement or alignment, and might underlie muscle tension measured clinically as muscle force.

Strabismus surgery is unique in that the tendon of the extraocular muscle is cut at the insertion to the sclera and then sutured again to the sclera at another site for recession or at the original site after the tendon and muscle have been resected. Proper healing of the wound is crucial for the function of the extraocular muscle after strabismus surgery [23–27]. Excess reaction to the surgery in healing might damage the functional results of strabismus surgery. Since aggrecan is known to be involved in fracture healing [28], the content of extracellular matrix components such as aggrecan could also affect the process of wound healing in strabismus surgery.

In conclusion, we showed that aggrecan is present in the resected tissue of the medial rectus muscle of patients with intermittent exotropia and that the amount of aggrecan decreases with age. Aggrecan levels may be related to motor aspects of intermittent exotropia.

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