Localization of nerve endings in relation to cholinesterase deposits in normal human eye muscles

J. Reimer Wolter and N. Thomas O'Keefe

Nerve endings and deposits of cholinesterase were stained in frozen sections of five normal striated human eye muscles with a stain that combines the nerve fiber technique of Hortega and the method of Koelle and Friedenwald for the demonstration of cholinesterase. Cholinesterase deposits are found at the base of the motor end plates. Dense cholinesterase deposits can also be found around thin nerves which form simple ring- or buttonshaped endings on muscle fibers. No cholinesterase is seen at the endings of the other type of thin (sympathetic?) nerves which also supply the muscle fibers and have networklike end formations as well as at all types of sensory (afferent) nerve endings in human eye muscle.

The anatomy of the terminal nerve apparatus in external human eye muscle is known from studies mainly with silver techniques.1-10 By staining the cholinesterase deposits in these muscles with the method of Koelle and Friedenwald,11 additional anatomical details have recently been reported.12 In the present study the results of a combined stain for nerve endings and cholinesterase deposits in the same sections are demonstrated in normal human extraocular muscle.

Material and methods

Both superior rectus muscles were obtained within 3 to 11 hours after death from 6 patients between 11 and 71 years of age. Two died shortly after head trauma, 1 of a myocardial infarct, 1 of leukemia, and the remaining 2 patients of heart failure. After fixation in formalin, frozen sections of the muscles were stained for cholinesterase according to the technique of Koelle and Friedenwald, modified by Conteaux.11 This modification calls for 15 to 45 minutes of incubation in copper glycine and acetylthiocholine at pH 5.3. It was found in this study that for a complete cholinesterase stain in human eye muscle, an incubation of about 60 minutes at 37° C. in a controlled temperature water bath shaker at pH 5.3 was essential. Some of the sections were mounted after the Koelle-Friedenwald stain, and cholinesterase deposits in a pattern similar to that seen in the photographs of Kupfer1- were observed. Most of the sections were transferred back into formalin and the nerve fiber stain of Hortega13 was added. This resulted in a good demonstration of the cholinesterase deposits as well as of the terminal nerve apparatus in the extraocular muscle. The photomicrographs of this article are unretouched.

Histologic description

The findings in the eye muscles of the different cases are very similar. Thus, the following description applies to all the muscles studied.

Fig. 1 shows the result of a cholinesterase stain of normal human eye muscle done according to the technique used by Conteaux and Kupfer. The dark spots in the
middle of the muscle are cholinesterase deposits. The muscle fibers are unstained and only their outlines can be recognized.

The additional application of the Hortega stain allows for a demonstration of the nerves and nerve endings in sections such as the one in Fig. 1. However, it then becomes obvious that the cholinesterase stain is not complete in all our sections stained exactly as outlined by Coers and Woolf\(^1\) and Kupfer.\(^2\) The motor end plates show only small areas of positive stain (Fig. 2) or no cholinesterase at all (Fig. 3). There are many small deposits on muscle fibers, on the other hand, which do not seem to have any relation to nerve endings (Fig. 3).

The thin nerves which form simple button- or ringshaped endings on human eye muscle fibers always show dense accumulation of stained cholinesterase, even in the sections with incomplete staining (Fig. 4). It has been shown in earlier papers\(^b, c, d\) that thin fibers of this type are seen as second nerve elements in addition to motor end plates (Fig. 5) on common human eye muscle fibers or as the only nerve supply on a special thin muscle fiber type.

Longer incubation in a controlled temper-
Fig. 3. For legend see opposite page.

Figs. 4 and 5. For legends see opposite page.
Cholinesterase deposits in normal human eye muscles

Fig. 6. Complete cholinesterase stain of human eye muscle after longer incubation. Frozen section; Koelle-Friedenwald stain; photomicrograph. (×50.)

Fig. 7. A motor end plate of human eye muscle surrounded by extensive cholinesterase deposit (arrow). Frozen section; combined stain for nerves and cholinesterase; photomicrograph. (×800.)

Fig. 3. Incomplete stain shows no cholinesterase at motor end plate (m) as well as deposits of cholinesterase on other muscle fibers without relations to nerves (arrow). Frozen section; combined stain for nerves and cholinesterase; photomicrograph. (×800.)

Fig. 4. Dense accumulation of cholinesterase around simple ending of thin nerve (arrow) on eye muscle fiber. Frozen section; combined stain for nerves and cholinesterase; photomicrograph. (×800.)

Fig. 5. So-called double innervation of human eye muscle fiber with motor end plate of thick nerve (m) and end button of thin nerve (b). Frozen section; Hortega nerve stain; photomicrograph. (×800.)
Fig. 8. A and B. Cholinesterase deposits around simple endings of thin nerves on human eye muscle fibers (arrows). Frozen sections; combined stain for nerves and cholinesterase; photomicrographs. (×800.)
Fig. 9, A, Sensory spinal and B, Brushlike sensory ending of human eye muscle show no cholinesterase deposits. Frozen sections; combined stain for nerves and cholinesterase; photomicrographs. (×800.)
Examples of such sensory (afferent) nerve endings, a spiral and a brushlike formation, after cholinesterase stain are seen in Fig. 9. The second type of thin (autonomic?) nerves which are known to originate from blood vessels and end on human eye muscle fibers with a delicate nerve fiber network also never show any relation to cholinesterase deposits (Fig. 10).

Discussion

Combined staining of the nerve endings and cholinesterase deposits in normal human extraocular muscle reveals the following facts:

1. The Koelle-Friedenwald stain for cholinesterase gives, in our hands, a complete stain only with longer incubation as used by Conteaux, Coers and Woolf, and Kupfer as well as with constant agitation and controlled temperature and pH.

2. Cholinesterase deposits are found at the motor end plates and at the endings of the thin nerves which form simple buttons and rings on human extraocular muscle fibers. It has been suggested in earlier morphologic studies that these thin nerves with simple endings might be parasympathetic in nature. However, they could perhaps also be the gamma efferents of human eye muscle, since true muscle spindles with gamma intrafusal nerves have not been demonstrated in man to our own satisfaction. 17

3. The more complicated networklike endings of the other thin (sympathetic?) nerve fiber type of human eye muscle exhibit no cholinesterase deposits.

4. The sensory nerve endings also show no relation to cholinesterase. The fact that the thin nerves which end with simple endings in addition to motor end plates on striated eye muscle fibers have cholinesterase at their ends remains as the most important finding of this study. This presence of cholinesterase confirms that these thin nerves are efferent elements, but the question whether they are autonomic or gamma efferent in nature has to be left unanswered.
Cholinesterase deposits in normal human eye muscles

REFERENCES


Discussion

Irving H. Leopold, Philadelphia, Pa. In the present studies, Dr. J. Reimer Wolter has continued his pursuit of basic information concerning histology of innervation of ocular structures, this time searching for cholinesterase. There are two methods of value for cytotologic localization of cholinesterase. Each of these has modifications. These are microscopic histochemistry and ultramicro analysis. For his studies, Dr. Wolter utilized the microscopic histochemistry technique. This has the advantage of allowing detailed definition of enzymatic activity in terms of cellular structure. Ultramicro analysis techniques provide a superior degree of quantitation. For Wolter's purpose, the histological technique would appear to be adequate. A third method of measuring cholinesterase consists of fractional centrifugation of tissue homogenates. This would not appear to be applicable to Dr. Wolter's study.

The histochemical technique allows preservation of structure, accuracy of localization, specificity, sensitivity, and avoidance of artifacts. However, to fulfill maximally any one of these demands requirements of the histochemical technique that may sacrifice several of the others. For example, as pointed out by Koelle, preliminary fixation is necessary in order to maintain optimal structural integrity, but this invariably results in a decrease in enzyme activity. At sites of low initial activity, the enzyme may be inhibited completely.

The frozen section technique provides maximum enzyme activity at the cost of structural detail. Embedding in paraffin or celloidin does the reverse. It is necessary, therefore, to employ more than one modification of a technique before arriving at any conclusion. It must be realized also that microscopic histochemistry may permit detection of low concentrations of the enzyme cholinesterase, but this should be recognized as only a very general approximation of relative activities.

It is known that the electron microscope may allow histochemical techniques to be utilized. However, there are many factors which must be controlled before this can be applied to the present study. With the available methods, one must pay strict attention to details especially to control of pH and time of incubation for each tissue studied. Optimal conditions must be found. Dr. Wolter's present investigations demonstrate the importance of pH and the duration of exposure to substrate, and indicate how erroneous conclusions can be drawn from using only one method and not determining the optimum pH and time of exposure to substrate.

Couteaux, in 1958, recommended that tissues studied should be stained with and without fixation in order to overlook sites of low activity and that optimal conditions of fixation must be determined for each tissue.

With respect to localization of acetylcholinesterase, the neuromuscular junction has been studied more extensively than any other region. The most precise histochemical results with light microscopy on the motor end plate are those of Couteaux, summarized in 1958. Robertson in 1956 and Anderson and Cedergran in 1959 confirmed Cou-
teaux's findings using electron microscopy on the motor end plate. Dr. Wolter's studies have confirmed Kupfer's report of 1960 in regard to localization of cholinesterase on the motor end plate of extraocular muscles.

Several years ago in the early fifties, Dr. Wolter studied a series of papers on the nonmedullated fibers in extraocular muscles. The function of these nonmedullated fibers is disputed. Some end in grapelike clusters and have been thought to be sensory. Most are agreed that the nonmedullated nerve fibers which are present within the walls of the blood vessels are of an autonomic nature related particularly to the sympathetic system. In a series of papers in 1953, 1954, and 1955, Wolter considered those fibers derived from the perivascular plexuses and ending in a delicate network to be sympathetic, those accompanying the cranial nerves and ending in loops to be parasympathetic in nature. The absence of cholinesterase staining in any of the former type of fiber would tend to confirm Dr. Wolter's initial impression. However, this should be checked by other techniques.

It might also be interesting to determine the type of enzyme in the various areas of the muscle fibers. Do all of the stained areas represent true cholinesterase or is some of the staining due to pseudocholinesterase? Use of cholinesterase inhibitors could be helpful here. Also, histochemical stains for adrenergic enzymes might be of value. One must be impressed with the persistency and the meticulous efforts with which Dr. Wolter approaches the histologic nature of the ocular tissues. He has been exceptionally productive.