Role of Innervation in the Expression and Maintenance of Some Troponin Subunits in Developing and Adult Rat Skeletal Muscles

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Abstract

The role of innervation in the expression of troponin I and troponin T isoforms was investigated by sciatic neurectomy in both newborn and adult rats. Immunoblotting showed that troponin T composition of adult fast skeletal muscles (Extensor digitorum longus, Tibialis anterior, Plantaris and Gastrocnemius) following denervation was affected only slightly. The expression of adult fast troponin T isoforms was maintained but re-expression of developmental isoforms at low level became apparent in all denervated adult skeletal muscles. Unlike the fast muscles, both the number and level of fast troponin T isoforms increased markedly in denervated adult rat soleus. The transition to adult isoforms of fast troponin T in developing muscles was not blocked by neonatal denervation although the level of developmental isoforms was higher during early stages of denervation. The level of slow troponin T was reduced in developing muscles after denervation.

Denervation for up to six weeks did not influence the expression pattern of fast or slow troponin I in most adult fast or mixed skeletal muscles studied. Slow troponin I, however, was undetectable in denervated gastrocnemius and plantaris muscles after 12 weeks. The level of slow troponin I in neonatally denervated fast skeletal muscles gradually decreased except in extensor digitorum longus in which the level of this isoform slightly increased. In soleus, the level of fast troponin I increased at the expense of slow troponin I after denervation of both neonatal and adult muscles.

Key words: muscle, denervation, troponin I, troponin T, developmental, fast, slow.

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Most postnatal skeletal muscles are composed of two main types of cells called slow (type I) and fast (type II) fibres [2, 3]. These fibre types in the adult differ from each other functionally in their speed of shortening, power output and endurance properties. Type I fibres are more oxidative in metabolism and slow in contractile response. Type II fibres have a shorter contraction time and their metabolism is mainly anaerobic. Many myofibrillar proteins in skeletal muscle exist in fast and slow isoforms which are differentially expressed in different muscle cell types and these are in part responsible for the different physiological properties of these cells [23]. It is not known, however, what regulates differentiation into different muscle cell types. The differentiation process may be preprogrammed or affected by extrinsic factors such as innervation, thyroid hormones, workload or stretch. Innervation is regarded as an important factor in the process of muscle cell differentiation, since the muscle cell properties have been shown to change when the nerve supply to a muscle is changed or if the muscle is electrically stimulated [1, 24, 31].

Changes in the composition of muscle proteins can be used to study the role of different factors in muscle cell differentiation. Some muscle proteins change during development and exist in developmental stage specific isoforms. Myosin, for example, exists as embryonic, neonatal and adult isozymes [36]. Troponin T has also been shown to exist in developmental stage-specific isoforms. For example, in addition to small amounts of cardiac and slow troponin T, slow skeletal muscle-like embryonic isoforms (ES1-ES5) of this protein are present in embryonic or early foetal rat skeletal muscles [27]. During late foetal development, these are gradually replaced by two foetal isoforms, FF1 and FF2 followed by neonatal isoforms NF1 and NF2 in the early postnatal period [28]. Muscle specific adult isoforms (AF1-AF5) start appearing during late foe-

tal period and are the exclusive forms present in most limb muscles by weeks 4-10. Such developmental stage specific isoforms of troponin T or myosin provide useful markers to analyse the role of intrinsic and extrinsic factors in the development of mature muscle cell phenotype.

The isoform diversity of myosin heavy chain has been widely used to investigate the role of innervation in muscle cell differentiation. Using myosin as a marker of muscle cell differentiation, the short term paralysis of foetal skeletal muscles does not affect the emergence of slow and fast muscle cell types [8]. Denervation in neonatal rats also does not block the appearance of adult muscle type myosin isozymes [4]. Denervation of adult skeletal muscles, however, has produced conflicting results from different laboratories. While some studies demonstrated the re-expression of embryonic and neonatal isoforms following denervation [7, 32], others detected no such effect on myosin isozymes [6, 21]. Few studies have been undertaken to investigate the role of innervation in the expression of other muscle specific proteins such as troponin components. Troponin components, I, T and C, are involved in the regulation of muscle contraction. Changes in the isoforms of troponin components are functionally important in modulating the physiological response of the muscle [22, 33, 34]. The present study was undertaken to investigate the role of innervation in maintaining the adult pattern of expression of troponin I and troponin T isoforms and its role in completing their transitions during development.

Materials and Methods

Denervation Procedure

Twelve week old Wistar rats weighing 350 ± 25g and newborn rats from seven litters with average weight of 6 ± 1g were used for control and surgical denervation. Using Halothane anaesthesia, denervation was performed in the right hindlimb by removal of a sciatic nerve segment (5-6 mm in the newborn and 1 cm in the adult) from the upper thigh region. This led to paralysis of the right hindlimb muscles. Muscles were removed from control and denervated adult rats (1-3) killed at the end of weeks 1, 2, 3, 4, 5, 6, 12 and 16 after checking for the absence of reinnervation . The muscles denervated at birth were removed from 3-6 rats killed at days 3, 5, 7, 10, 14, 21, 28 and 35. The whole lower hindlimb muscles were used for earlier stages up to week 2 but individual muscles were dissected out at the later stages of 21, 28 and 35 days. Because of their small size due to denervation atrophy, individual neonatal muscles for electrophoretic analyses were pooled from 3-6 pups.

Electrophoresis and immunoblotting

After excision, the muscles were weighed and frozen in liquid nitrogen. Compared with the controls, all denervated muscles showed considerable atrophy as shown by their reduced muscle weights (Tables 1 and 2).

The samples for electrophoresis were prepared by homogenising the muscle in 10 volumes of sample buffer (62.5 mM tris pH 6.8, 2% SDS, 5% β mercaptoethanol, 10% glycerol and 0.001% Bromophenol blue. The samples were boiled for 3 minutes and stored at -70°C. The 10 µl samples of clear muscle homogenates were analysed by SDS polyacrylamide (10%) gel electrophoresis according to Laemmli [19]. The resolved proteins from the gels were electrotransferred to nitrocellulose as described by Towbin et al [34]. The Western blots thus prepared were stained with troponin T antibodies, CDC4 [27], F24 [10] or JLT12 (Sigma, Poole, England) using the immunoperoxidase procedure reported earlier [10]. Antibody F24 is specific for the fast isoforms of troponin T and does not recognise the slow skeletal or cardiac isoforms. Antibody JLT12 recognises all the fast isoforms of troponin T but it also reacts with cardiac isoforms in addition. Antibody CDC4 recognises slow skeletal and cardiac isoforms of troponin T. Different isoforms of troponin I were detected with monoclonal antibody 14b which has the same specificity as a previously described antibody 42/25 [26].

Table 1. Muscle weights, as percentage of control, after 1-16 weeks of denervation in adult rats.

Muscle		Time (weeks) after denervation							
		1	2	3	4	5	6	12	16
Extensor digitorum longus		81	59	49	37	31	26	19	15
Tibialis anterior		74	62	51	39	33	26	20	16
Plantaris		79	69	56	42	36	31	24	18
Gastrocnemius	84	68	53	42	35	23	18	15	
Soleus		72	55	46	38	30	27	22	12

Table 2. Muscle weights, as percentage of control, at different times after denervation in newborn rats.

Muscle	Time (days) after denervation							
	3	5	7	10	14	21	28	35
Lower hindlimb	90	87	83	70	53	-	-	-
Extensor digitorum longus	-	-	-	-	-	42	19	11
Tibialis anterior	-	-	-	-	-	42	20	12
Plantaris	-	-	-	-	-	38	23	11
Gastrocnemius	-	-	-	-	32	27	19	
Soleus	-	-	-	-	-	40	10	8

Results

Expression pattern of troponin T and troponin I isoforms in fast and slow skeletal muscles denervated in adult rats

Troponin T: Most adult fast skeletal muscles express adult isoforms of fast troponin T, called AF1-AF5. Using antibody F24 which recognises both adult (AF1-AF5) and developmental isoforms (FF1, FF2, NF1) of fast troponin T, the developmental isoforms are usually not detected in most hindlimb muscles by 6-8 weeks of age [28]. In this study, we also used another monoclonal antibody (JLT12) which by immunoblotting procedure detects both fast skeletal and cardiac isoforms of this protein. Using antibody JLT12, small amounts of FF1 and FF2 isoforms were detectable in extensor digitorum longus (EDL) muscle at both 12 and 24 weeks of age but were undetectable or barely detectable in gastrocnemius, plantaris, tibialis anterior (TA) and soleus muscles investigated in this study (Figure 1). Re-expression of small amounts of developmental isoforms FF1 and FF2 became soon apparent in all denervated fast skeletal muscles including tibialis anterior, gastrocnemius and plantaris muscles in which these isoforms are undetectable in the adult (Figure 1). The level of these developmental isoforms was slightly higher during early stages after denervation. The expression pattern of different adult fast troponin T isoforms was not significantly affected by denervation in most muscles although the level of AF5 isoform appeared to increase in denervated plantaris (Figure 1).

Compared with the fast hindlimb muscles, the troponin T composition of slow twitch soleus, was affected markedly by denervation. Normal adult rat soleus contains mostly slow troponin T with barely detectable levels of AF2 and AF3 fast isoforms (Figure 1). The level of both these fast isoforms in this muscle increased considerably after denervation (Figure 1). In addition to increased levels of AF2 and AF3 that are detectable in trace amounts in normal soleus, the expression of another adult isoform, called AF4, was also observed soon after denervation. The level of all these fast isoforms remained higher throughout the denervation period of 16 weeks. Low level expression of developmental isoforms NF1, FF1 and FF2 was also apparent during both earlier stages and at 16 weeks post-denervation.

Cardiac tropooin T was not detected by the immunoblotting procedure in any of the adult normal or denervated hindlimb muscles investigated (Figure 1).

Troponin I: Until week 6, the composition of troponin I isoforms did not change significantly in any adult fast skeletal muscles following denervation (Figure 2). The slow troponin I, however, became undetectable in gastrocnemius and plantaris muscles at 12 week stage of denervation, whereas it was detectable in the age matched normal controls and during earlier stages of denervation. As was the case for troponin T, the troponin I isoform composition in denervated soleus changed considerably. The level of fast troponin I in this muscle gradually increased while the relative level of slow troponin I decreased after denervation (Figure 2).

Expression pattern of troponin T and troponin I isoforms in fast and slow skeletal muscles denervated at birth:

Troponin T: Newborn rat lower hindlimb muscles express developmental isoforms FF1, FF2, NF1, and a small amount or trace amount of adult fast isoforms AF1 and AF2 (Figure 3). After reaching maximum level of expression at 2-3 weeks, the level of FF1 and FF2 isoforms decreases, becoming very low or undetectable in most muscles by week 4-5 although a low level persists in some muscles such as EDL (Figure 1). The maximum level of neonatal isoform NF1 is observed during weeks 3-5. All adult isoforms, AF1-AF5, are detectable by week 3, AF1 and AF2 being the first to appear after birth with AF4 and AF5 appearing by about week 1 and AF3 by week 2.

No significant effect of denervation was observed on the expression or transitions of fast troponin T isoforms in developing muscles except for a slightly raised level of developmental isoforms during early stages (Figure 3). The denervation in the neonate did not interfere with the appearance of adult isoforms of fast troponin T either although the muscle mass (Table 2) and the total protein content in denervated muscles was considerably reduced.

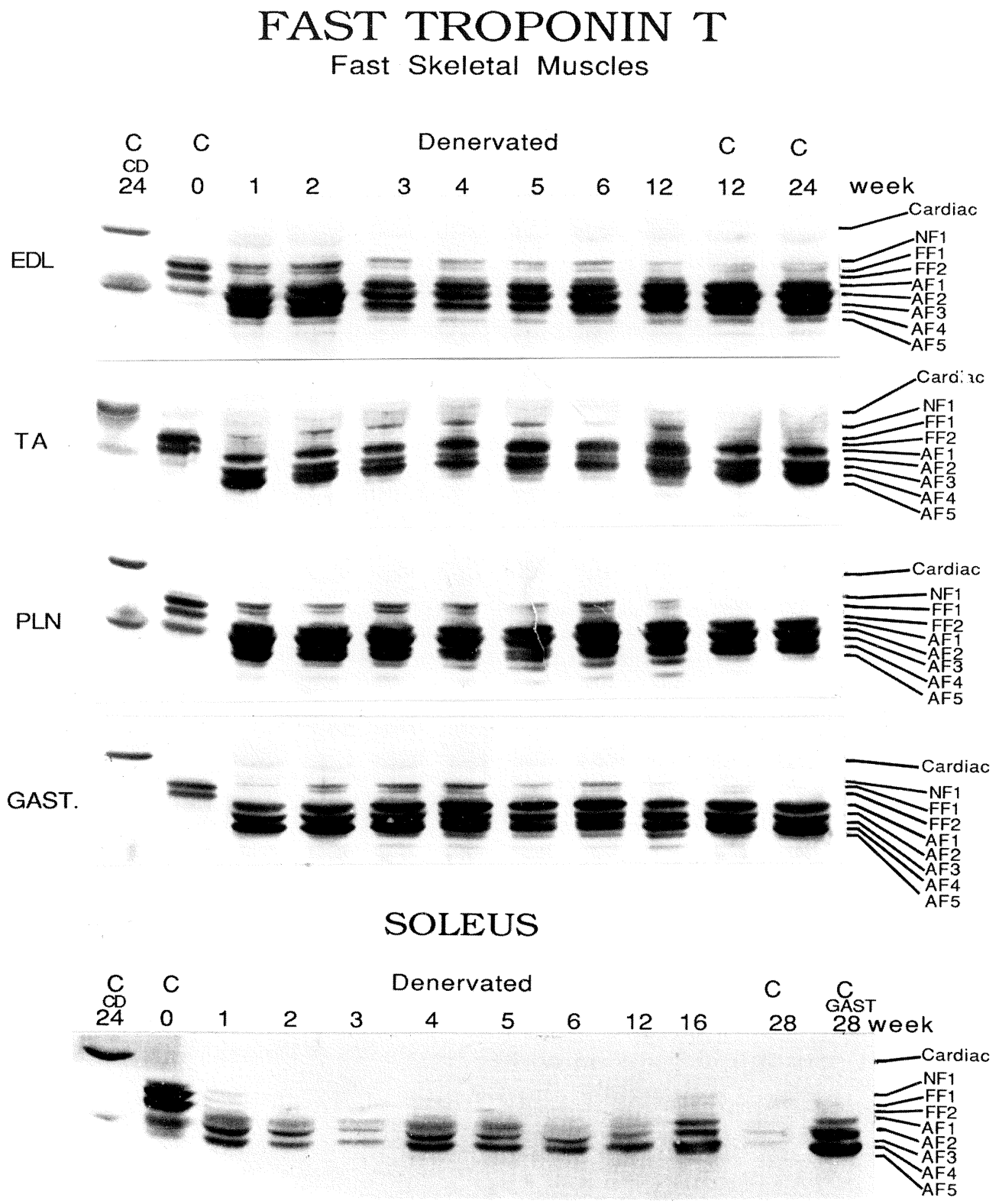

Figure 1. Expression pattern of different troponin T isoforms in normal and denervated rat skeletal muscles after different time periods of surgery, determined by immunoperoxidase procedure using antibody JLT12. EDL = Extensor digitorum longus; TA = Tibialis anterior, PLN = Plantaris; Gast. = Gastrocnemius; CD = cardiac; C,0 = Control muscle at birth; C,12 = Control muscle from 12 week old normal rat (at the time of surgery); C,24 = Control muscle from a 24 week old normal rat. Cardiac muscle sample also shows the presence of a troponin T isoform present in the skeletal muscle size range [18].

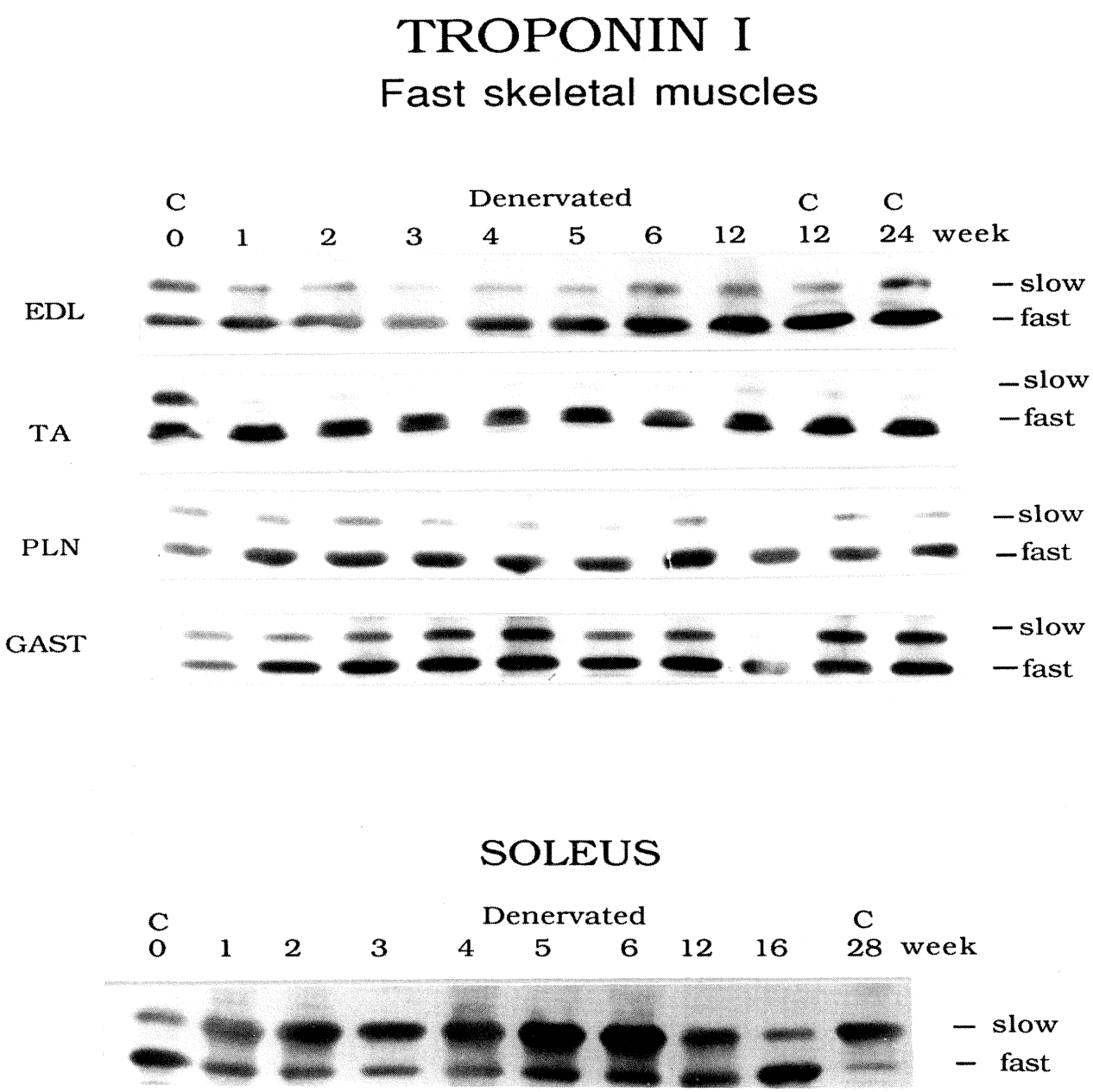

Figure 2. Expression pattern of fast and slow troponin I isoforms in normal and denervated rat skeletal muscles after different time periods of surgery, determined by immunoperoxidase procedure using antibody 14b. EDL = Extensor digitorum longus; TA = Tibialis anterior, PLN = Plantaris; Gast. = Gastrocnemius; C,0 = Control muscle at birth; C,12 = Control muscle from 12 week old normal rat (at the time of surgery); C,24 = Control muscle from a 24 week old normal rat.

AF3, AF4 and AF5 isoforms which are not present in a newborn rat at the time of denervation but appear around weeks 1-3 in normally developing muscles, appeared about the same time in denervated muscles. The level of fast troponin T isoforms, however, was higher in denervated developing soleus muscle (Figure 3).

The expression of slow troponin T detected by antibody CDC4 was gradually reduced in all denervated developing muscles becoming barely detectable after 14 days (not shown). Unlike the adult muscles, trace amounts of cardiac troponin T were detected in some denervated developing skeletal muscles (Figure 3).

Troponin I: Both fast and slow troponin I isoforms were present in normally developing pooled hindlimb muscles (Figure 4). Compared with the adult, the level of slow troponin I was higher in newborn muscles. In normally

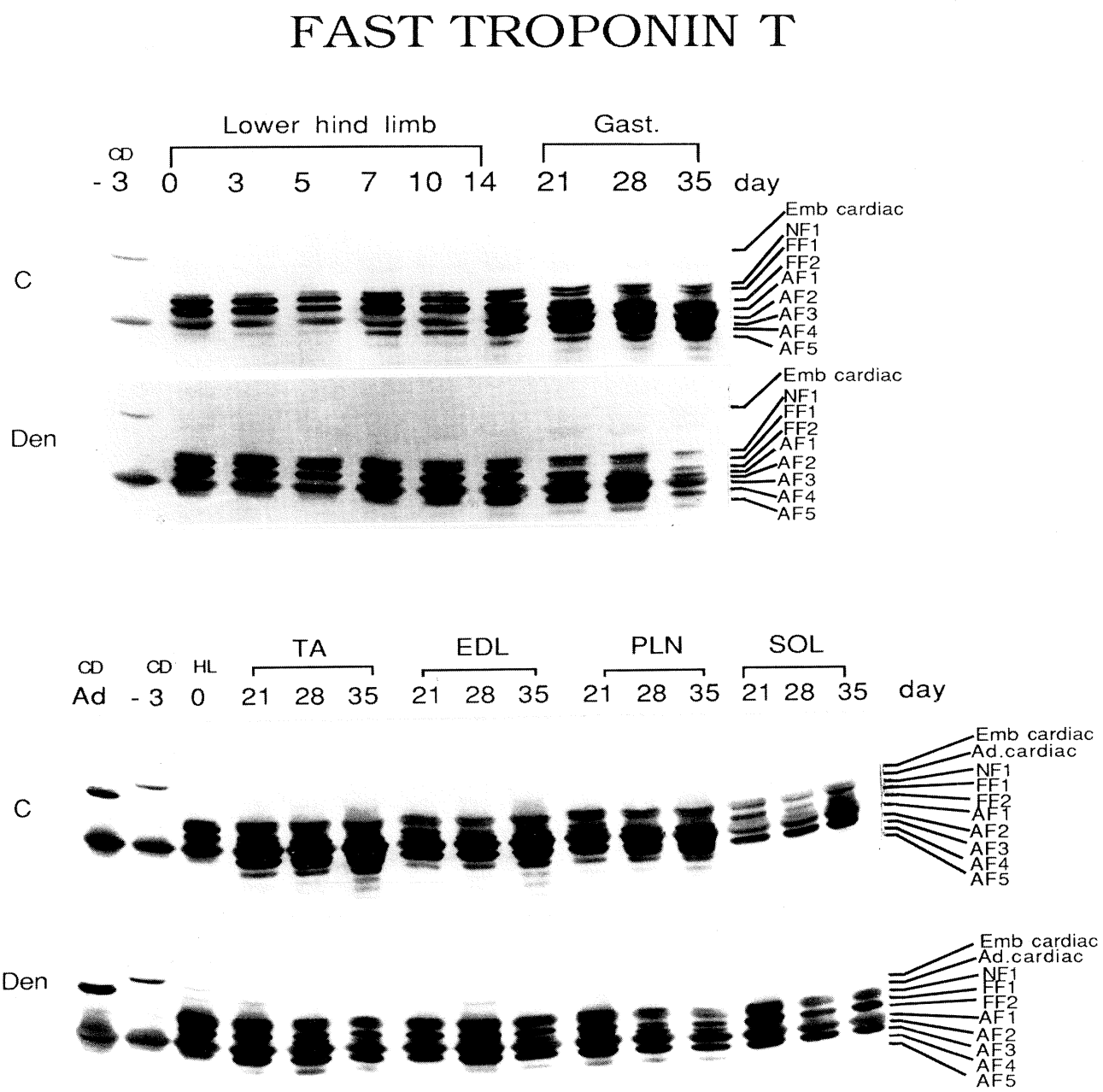

Figure 3. Expression pattern of different troponin T isoforms in some fast and slow skeletal muscles during normal development and at different time intervals after denervation determined by immunoperoxidase procedure using antibody JLT12. EDL = Extensor digitorum longus; TA = Tibialis anterior; PLN = Plantaris; Gast. = Gastrocnemius; C = Control; Den. = Denervated; HL = Hindlimb; Ad = Adult; CD,-3 = cardiac muscle from foetal rat 3 days before birth.

developing muscles, the proportion of slow troponin I gradually decreased with growth. At 35 days, the level of slow troponin I in normal gastrocnemius muscle was judged as approximately 20% but quite low in EDL and TA with moderate level of expression in plantaris and high level in soleus. In response to denervation, no difference in the pattern of expression of fast and slow troponin I was observed until day 14. With the exception of EDL, the level of slow troponin I was reduced at and after 14 days in all denervated muscles. Slow troponin I was undetectable after 4-5 weeks of denervation in all muscles except in EDL in which the level of this isoform increased slightly (Figure 4). The level of fast troponin I increased markedly in denervated soleus. Slow troponin I in this muscle was undetectable after 3 weeks of denervation.

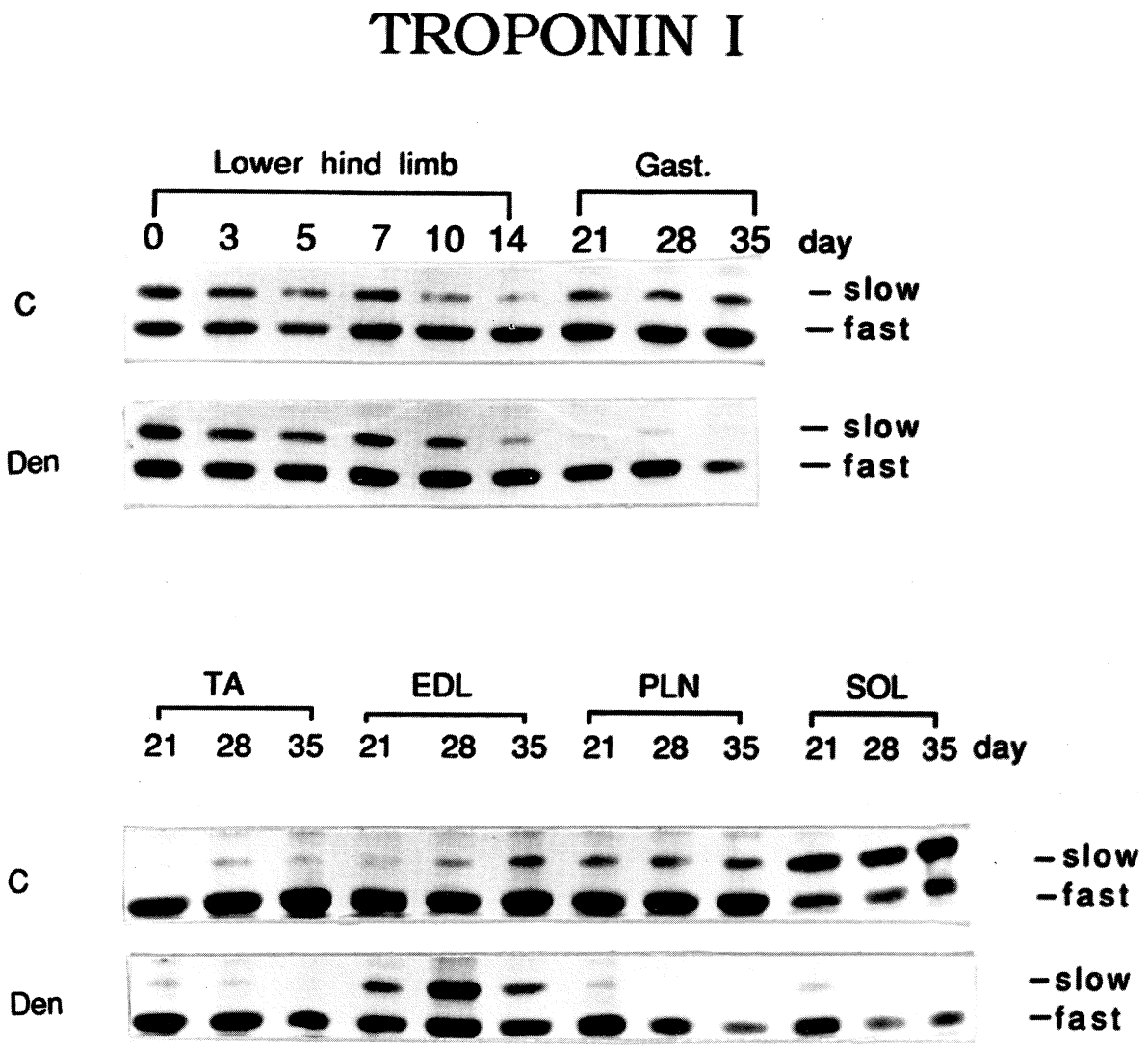

Figure 4. Expression pattern of fast and slow troponin I isoforms in some skeletal muscles during normal development and at different time intervals after denervation determined by immunoperoxidase procedure using antibody 14b. EDL = Extensor digitorum longus; TA = Tibialis anterior; PLN = Plantaris; Gast. = Gastrocnemius; C = Control; Den. = Denervated; HL = Hindlimb; Ad = Adult; CD,-3 = cardiac muscle from foetal rat 3 days before birth.

Discussion

Denervation of adult fast skeletal muscles over 12 week time period showed that the expression of adult fast isoforms of troponin T can be maintained in the absence of innervation. Expression of troponin T was not generally affected in fast muscles except for the re-expression of a small amount of fast developmental isoforms. This is similar to myosin heavy chain, embryonic and neonatal isoforms of which at low level occur in some denervated adult skeletal muscles [7, 32]. Such developmental isoforms of myosin, however, have not been observed in all studies [6, 21]. The level of re-expressed developmental isoforms of fast troponin T is similarly low after denervation but can be detected by the immunoperoxidase procedure.

The expression of slow troponin T was reduced in developing muscles after denervation but the transition from foetal to adult isoforms of fast troponin T continued in the absence of nerve activity. The appearance of adult fast isoforms of troponin T in developing muscles was also not prevented by denervation. Myosin isoform transitions have also been reported to continue after sciatic neurectomy of 1 week old rats [4]. Developmental transitions in both myosin heavy chain and troponin T may be influenced by factors other than innervation. For example, the fetal to adult isoform transition of myosin isozymes is blocked by hypothyroidism [5, 15]. Removal of thyroid hormones during development, however, does not block the foetal to adult fast troponin T transition in hypothyroid rat muscles completely although the rate of this transition is consider-

ably reduced and the developmental isoforms persist for much longer periods after birth [29].

In this study, the expression of cardiac troponin T was not observed in denervated adult skeletal muscles. Saggin et al [30] using an immunocytochemical procedure have described the re-expression of cardiac troponin T in some denervated soleus muscle fibres. It is possible that such low level expression of cardiac troponin T would not be detected by the immunoblotting procedure used in this study. In the present study, the level of cardiac troponin T in neonatally denervated skeletal muscles was apparent but was also quite low.

Denervation did not appear to affect the pattern of troponin I isoforms in most adult fast skeletal muscles for at least six weeks. The slow troponin I, however, was barely detectable in plantaris and gastrocnemius muscles after 12 weeks of denervation. The proportions of fast and slow troponin I isoforms in developing muscles were also not affected in the short term but the level of slow troponin I in most muscles decreased becoming undetectable during later stages of denervation. This may result from atrophy as well as the failure to suppress fast troponin I in developing neonatal fibres positive for slow troponin 1 [12]. Unlike all the other fast skeletal muscles, the level of slow troponin I, however, increased in denervated neonatal EDL muscle. This probably results from selective hypertrophy of a proportion of type I fibres in this muscle [14].

The proportion of fast troponin I increased considerably in both adult and neonatally denervated soleus muscle. Compared with the fast skeletal muscles, the changes in proportions of both troponin I and troponin T isoforms after denervation were much more pronounced in soleus. Unlike most fast skeletal muscles, the proportions of fast and slow isoforms of a number of proteins in soleus continue to change throughout postnatal development [13, 20]. Continuing changes of myofibrillar proteins in postnatal developing soleus are believed to be driven by increasing workload of this muscle with growth [17]. Denervation of soleus muscle clearly impairs such postnatal development as the normal changes in both troponin I and troponin T are affected after such a procedure.

It appears that innervation is more important in maintaining the muscle cell phenotype than the developmental transitions from foetal to adult isoforms. The expression of both slow troponin I and slow troponin T is reduced in most denervated developing muscles. Similarly, the number of slow myotubes is reduced in both mammalian and avian paralysed embryos [8, 9, 25]. This may explain why cross-sinnervation has been shown to change the muscle cell phenotype in terms of both myosin heavy chain [16] and troponin components [11] while denervation does not block the transition to adult myosin isozymes [4] or fast troponin T isoforms (this study). The role of the nerve in maintaining the fast and slow muscle cell phenotype is also supported by the observations that soleus regenerating in the absence of the soleus nerve fails to develop slow myosin although the foetal to adult fast myosin isozyme transition in regenerating muscles takes place in the absence of the nerve [37].

In conclusion, innervation influences the ratios of fast and slow muscle cell type proteins particularly when changes in their expression are still taking place during development but not the developmental stage-specific isoforms of either myosin or troponin T.

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