Actin Isoform and Intermediate Filament Protein Expression in Human Developing Skeletal Muscle

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Abstract

The expression of intermediate filament proteins and actin isoforms has been sequentially studied in the human embryo from the 8th week to the 36th week of gestation by means of immunohistochemistry and immunoblotting using specific antibodies for vimentin, desmin, α-smooth muscle (SM) and α-striated (SR) actins. Our immunohistochemical results show that desmin is present in myotubes throughout the observation periods and assumes a striated pattern around the 20th week; vimentin is present diffusely in myoblasts and myotubes at the 8th week and disappears at the 20th week. From the 8th to the 12th week, there is coexistence of α-SM and α-SR actins ; both isoforms are initially distributed diffusely in the cytoplasm. The first isoform practically disappears at the 12th week whereas α-SR actin increases in quantity and assumes a striated pattern already at the 16th week. These results were confirmed by immunoblotting.

Our results describe for the first time the coordinate distribution of intermediate filament proteins and actin isoforms in developing muscle of the human embryo and may be useful for the interpretation of the expression of these proteins in soft tissue tumors.

Key words: desmin, vimentin, microfilaments, α-smooth muscle actin, rhabdomyosarcoma.

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The concept that tumor cells generally express cytoskeletal features typical of their tissues of origin [9] is widely accepted in tumor pathology and utilized for diagnostic purposes (for review see 23). Conversely, if a given cytoskeletal protein, absent in normal tissues, is observed in tumor cells, a conceivable explanation is that this protein is expressed temporarily during development. This furnishes a working hypothesis for embryological studies. Thus, the observation that α-smooth muscle (SM) actin, one of the six actin isoforms present in higher vertebrates [25], can be localized in rhabdomyosarcoma cells of humans [22] and rats [1] has suggested the possibility that this isoform is temporarily expressed during striated muscle development. This has been shown to be true for rat and mouse skeletal (and cardiac) muscle [2, 18, 26], as well as for chicken cardiac muscle [17].

The general scheme of muscle development in man is established [14]. However, the current knowledge of the normal sequence of human myogenesis results from the integration of investigations on human muscle cells growing in vitro in the absence of innervation, and on myogenesis of animals such as mice and rats, in which the duration of myogenesis as well as the locomotory pattern differ importantly from those of humans.

In this study, we have followed systematically by means of immunohistochemistry (and, for α-SM and α-striated (SR) actins, immunoblot analysis) with specific antibodies, the expression of α-SM actin, α-SR actin, desmin and vimentin in human embryos, from the 8th to the 36th week of gestation, and show that expression of α-SM actin takes place in myotubes between the 10th and the 12th week of gestation with a characteristic pattern, different from that previously observed in rats and mice.

Materials and Methods

Specimens

We have examined samples of femoral quadriceps and brachial biceps muscles of 70 human fetuses obtained from

legal abortions at the Clinic Villa Gina in Rome (Italy). We examined 8 fetuses at 8 weeks of gestation, 10 at 9 weeks, 9 at 10 weeks, 21 at 11 weeks, 14 at 12 weeks, 2 at 14 weeks, 2 at 16 weeks, 2 at 20 weeks and 2 at 36 weeks. The age of the fetuses was calculated on the basis of the last maternal menstruation. In no case, there was an history of familial neuromuscular disease and at the histological examination, we have never seen changes indicative of a pathological situation. The specimens have been constantly collected in the middle of the thigh and the arm in order to obtain the largest diameter of the muscle in the histological sections. They were fixed as follows: 1) in 10% neutral formaline, then embedded in paraffin and stained with hematoxylin and eosin and Verhoeff-Van Gieson staining; 2) in a solution of 60% methanol, 30% chloroform and 10% glacial acetic acid for 12 hours, then embedded in paraffin and used for immunohistochemical staining (see below); 3) when possible, part of the specimens were frozen in isopentane immersed in liquid nitrogen and utilized for electrophoretic and immunoblot studies.

Immunohistochemical Staining

Immunoperoxidase staining was performed on 8 µm-thick sections from paraffin-embedded blocks using the avidin-biotin-peroxidase complex (ABC-P) method as described previously [1]. Two mouse IgG monoclonal antibodies specific for α-SM actin (anti-αSM-1 [21]) and vimentin (Amersham Corp., Arlington, Heights, IL, USA), a mouse IgM monoclonal antibody specific for α-SR actin (anti-αSR-1 [22]) and a rabbit polyclonal antibody specific for desmin [10] were used. Sections were deparaffinized and endogenous peroxidase was blocked with methanol/hydrogen peroxide. Sections were then incubated with one of the cytoskeletal antibodies followed by biotinylated second antibody using the Vectastain kits anti-mouse IgG or IgM or anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). After incubation with ABC-P, peroxidase activity was revealed using 3,3' diaminobenzidine-HCl. After counter-staining with hematoxylin, sections were mounted with permount.

SDS-PAGE and Immunoblotting

SDS-PAGE [11] and immunoblotting [24] were performed on human embryonic muscle at 8, 11 and 12 week of gestation and on human adult muscle. Tissues were mixed with sample buffer containing 2% sodium dodecyl sulfate (SDS, Bio-Rad Laboratory AG, Glattbrug, Switzerland), 1% dithiothreitol (DTT, Fluka Chemical AG), 1 mM phenylmethylsulfonyl fluoride (PMSF, Merck CO., Darmstadt, FRG), 1 mM Nα-p-Tosyl-L-arginine methyl ester (TAME, Sigma Chemical Co., St Louis, MO, USA), 50% glycerol in 0.4 M Tris HCl pH 6.8 [11], sonicated and boiled for 5 minutes.

After protein determination [4], proteins were separated on a 5%-20% gradient gel and stained with Coomassie blue. The different samples contained variable amounts of albumin (see fig. 2). The total actin content of each sample was determined by scanning the gel with a laser beam scanner (Genofit SA, Geneva, Switzerland) connected by means of an analogue to digital converter to an IBM AT compatible microcomputer running on MS/DOS operating support. Proteins were then loaded on a 5%-20% gradient gel using for each sample, amounts of total proteins containing the same quantity of total actin, separated and stained with Coomassie blue or transferred to nitrocellulose paper which was incubated with anti-αSM-1 or anti-αSR-1 followed by a goat anti-mouse Ig conjugated with alkaline phosphatase diluted 1:7500 (Promega Corp., Madison, WI, USA). The proportion of α-SM and α-SR actins per total actin was determined by scanning of immunoblottings as described above.

Results

Light Microscopy

At the 8th week of gestation, rare nests of 4 to 10 first generation myotubes, with a scarce eosinophilic cytoplasm and a diameter varying from 6 to 12 µm, were present and showed large spherical central nuclei aligned in a chain. These cellular nests were separated by large spaces of connective tissue containing fusiform cells, i.e. fibroblasts, and several rounded cells with a large nucleus and a slightly basophilic cytoplasm, referred to as myoblasts. Finally, some small vessels were seen. At the 9th week, the myoblasts were uniformly distributed. In addition, the islets of first generation myotubes were more numerous than at the 9th week. At the 10th week, the largest proportion of the tissue was still constituted of myoblasts. First generation myotubes were more numerous than at the previous weeks, their cytoplasm was more abundant and the islets were constituted of 8 to 15 units. Several myotubes showed fusiform nuclei at the periphery of the cytoplasm with a size slightly inferior compared to the

Figure 1. Immunoperoxidase staining of muscle from human embryo at 8 (a, b, c, d), 10 (e, f, g), 11 (h), 16 (i, k, l), 20 (j, m) and 36 weeks (n, o, p) of gestation with anti-vimentin (a, m, n), anti-desmin (b, e, i, j, o), anti-αSM-1 (c, g, h, l) and anti-αSR-1 (d, f, k, p). At the 8th week, myotubes express vimentin (a), desmin (b), and α-SR actin (d) but are negative for α-SM actin (c); vimentin is also expressed in myoblasts. At the 10th and 11th week, myotubes are positive for desmin (e), α-SR actin (f) and α-SM actin (g, h); α-SM actin staining is distributed in the periphery of the cells (arrowheads). Myotubes show desmin staining in a diffuse pattern at the 16th week (i) and in a striated pattern at the 20th week (j). At the 16th week, striations are positive for α-SR actin (k) and myotubes are negative for α-SM actin (l). Vimentin is still present at the 20th week (m). At 36th week, myotubes are negative for vimentin (n) and striations are positive for desmin (o) and α-SR actin (p).

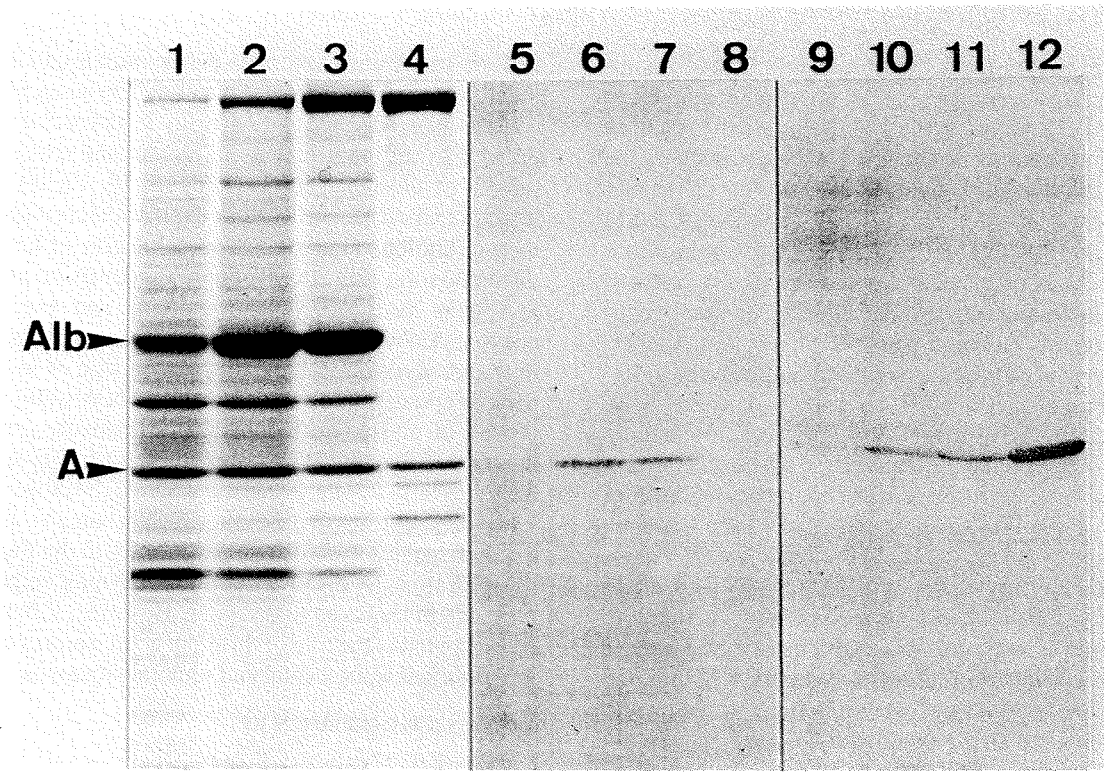

Figure 2. Immunoblotting with anti-αSM-1 (5, 6, 7, 8) and anti-αSR-1 (9, 10, 11, 12) after SDS-PAGE of human muscle protein extracts (1, 2, 3, 4) after 8 (1, 5, 9), 11 (2, 6, 10), and 12 weeks (3, 7, 11) of gestation and in adult (4, 8, 12). The Coomassie blue stained actin band is constant in each sample. Immunoblotting shows that α-SM actin appears at the 11th week, then decreases and has desappeared in adult. α-SR actin constantly increases during development of muscle. (Alb, albumin; A, actin).

round central nuclei. One could also observe many degenerating cells. At the 11th week, both myoblasts and myotubes were still increased in number compared to the previous week. A beginning of muscular organization could be seen and several myogenic cells were still undergoing necrosis. At the 12th week, single muscle cells appeared well delineated, myotubes contained both central and peripheral nuclei and many myoblasts were still present. The number of necrotic cells was decreased compared to the previous weeks. At the 14th, 16th, 20th and 36th week of gestation, the organization of single myofibers became more and more typical.

Immunohistochemistry

The results of immunohistochemical stainings (Fig. 1) are summarized in Table 1. At the 8th week, myoblasts and myotubes were distinguishable, the first being positive for vimentin and negative for desmin and α-SR actin, and the second positive for the three proteins. In all cases, the staining distribution was diffuse throughout the cytoplasm. α-SM actin was absent in these cells, but present in small vessels, probably in both smooth muscle cells and pericytes. Additional work (not reported here) showed that at this time a general anti-prekeratin antibody did not stain either myotubes or myoblasts. At the 9th week, myotubes

Table 1: Immunohistochemical Staining with Cytoskeletal Antibodies of Human Embryonic Muscle at Different Gestation Times.

	Week of Gestation								
	VIII	IX	X	XI	XII	XIV	XVI	XX	XXXVI
Antibody									
α-SM actin	0	0	+	+	+/-	0	0	0	0
α-SR actin	+	+	+	+	+	+	+(S)	+ (S)	+ (S)
Desmin	+	+	+	+	+	+	+	+ (S)	+ (S)
Vimentin	+	+	+	+	+	+	+	+/-	0

Scale: 0 = negative; +/- = weakly positive; + = positive; + (S) = positive in striations.

appeared more numerous, but the distribution of immunohistochemical stainings was not changed compared to the 8th week. However, at the 10th week, when myotubes were more numerous and appeared larger than in the previous weeks, a high proportion of them showed a positive staining for α-SM actin. The distribution of α-SM actin staining was diffuse throughout the cytoplasm but clearly more abundant at the periphery of the cells, as compared to desmin and α-SR actin which are more regularly distributed. Vimentin was still present in myotubes. At this time, several mesenchymal cells appeared to undergo degeneration and death. This pattern of α-SM actin positivity continued up to the 12th week, when the staining was weak. At this time, the distribution of the other cytoskeletal markers was still diffuse in the cytoplasm of myotubes. At the 14th week, no more α-SM actin staining was seen in myotubes, whereas the other three proteins remained present, and at the 16th week, α-SR actin staining appeared localized in a striated pattern. At the 20th week, desmin staining was organized in a striated pattern and a diffuse vimentin staining was barely visible in the cytoplasm; at the 36th week, the staining for vimentin was negative, and myotubes contained α-SR actin and desmin distributed in the typical striated pattern. This staining pattern was maintained at the end of gestation when myofibrils were clearly visible in the different muscles examined.

Immunoblotting

Immunoblotting revealed with anti-αSM-1 and anti-αSR-1 confirmed the results obtained with immunoperoxidase staining (Fig. 2). α-SM actin was not detectable at the 8th week, whereas α-SR actin was present at a very low level. At the 11th week, α-SM actin was detectable and α-SR actin was expressed at a high level compared to the 8th week. At the 12th week, α-SM actin content decreased of about 50% while α-SR actin content slightly increased compared to the previous week. In adult muscle, α-SM actin had disappeared and the proportion of α-SR actin increased about 9 times compared to the 12th week.

Discussion

Our results show that α-SM actin is expressed temporarily in developing human striated muscle, similarly to what has been previously shown in rat, mouse and chicken [2, 17, 18, 26]. However in humans, α-SM actin is expressed for a short period, compared to rat and mouse, and its localization is essentially at the periphery of the cytoplasm of myotubes, whereas in rats and mice, α-SM actin is incorporated in the I-bands. It is noteworthy that in humans α-SM actin disappears before a sarcomeric organization becomes visible. The sequential expression of actin isoforms is a typical feature of striated muscle development [5]. Only recently, α-SM has been recognized to participate in this phenomenon [18, 26]. Although much speculation has been made on the possible role of actin isoform sequential expression during muscle cell development, no definite role for this phenomenon has been found.

Actin isoforms and α-SM actin in particular are expressed to different extents in human and experimental tumors of muscular origin [1, 6, 12, 16, 19, 20, 22]. In leiomyosarcomas, α-SM actin expression corresponds to the morphological and biochemical degree of tumor cell differentiation [20]. In rhabdomyosarcomas however, α-SM actin has been found irregularly and generally is coexpressed with α-SR actin. The presence of α-SM actin in rhabdomyosarcoma cells is rather a sign of tumor cell dedifferentiation [22]. The expression of desmin, another marker of muscle differentiation, is relatively parallel to that of α-SR actin in rhabdomyosarcomas [6-8, 13, 15 22,], similarly to what is observed during development [3].

In conclusion our findings show that in humans, similarly to other species, α-SM actin participates in the process of striated muscle differentiation. This may explain its presence in rhabdomyosarcomas. However, it remains to be seen what is the role of different actin isoform expression during developmental and tumoral situations.

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