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Influence of genotype on contractile protein differentiation in different bovine muscles during foetal life

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Abstract — The purpose of this work was to compare muscle fibre differentiation in two genetic types: "normal Charolais" and double-muscled (DM) "INRA 95" cattles displaying muscle hypertrophy. Six muscles with different contractile and metabolic characteristics in adult animal: *Masseter*, *Diaphragma* (Di), *Biceps femoris* (BF), *Longissimus thoracis*, *Semitendinosus* and *Cutaneus trunci* (CT) were excised from 60 to 260-day-old foetuses of both genotypes. These muscles present different degrees of hypertrophy in DM animals. Fibre types were characterised by immunohistochemistry using monoclonal antibodies raised against different myosin heavy chain isoforms. They were also studied by SDS-PAGE electrophoresis, immunoblotting and ELISA methods. In both genotypes, foetal muscle development involved at least two generations of fibres but their contractile differentiation was more or less delayed in double-muscled animals according to the muscle type. CT muscle, the most hypertrophied in adults DM, showed the most important differences in the appearance of the primary and secondary fibres in DM foetuses. It showed a delay of differentiation which was made up during the last third of foetal life. Other muscles did not differ in the speed of appearance of the two generations of fibres. They were originated from a higher proliferation of secondary fibres. Moreover, all DM muscles presented similar characteristics at 210 days of foetal life. These characteristics seem to be genetically determined. Just before birth, muscles of double-muscled foetuses were composed of a greater proportion of fast type II fibres than normal animals. The most important differences between genotypes were observed in BF and Di muscles.

muscle / foetus / differentiation / cattle / double-muscled

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Résumé — Influence du génotype sur la différenciation contractile de différents muscles de bovin au cours de la vie fœtale. La différenciation contractile a été suivie pendant la vie fœtale chez deux types génétiques de bovins : des culards de la lignée INRA 95 présentant une hypertrophie musculaire et des Charolais à musculature normale. Six muscles différents : *Masseter*, *Diaphragma* (Di), *Biceps femoris* (BF), *Longissimus thoracis*, *Semitendinosus* et *Cutaneus trunci* (CT) ont été prélevés sur les fœtus de 60 à 260 jours de vie fœtale. Ces muscles ont la particularité de présenter des degrés d'hypertrophie musculaire plus ou moins importants chez les bovins culards. L'évolution du type contractile des fibres musculaires a été suivie par immunohistochimie à l'aide de différents anticorps spécifiques des isoformes de chaînes lourdes de myosine lente, rapide, embryonnaire, fœtale et alpha cardiaque. Une analyse complémentaire a consisté à suivre l'évolution du type contractile au niveau tissulaire par électrophorèse SDS-PAGE, immunoblotting et dosage ELISA. Il apparaît que la différenciation contractile se fait selon le même schéma dans les deux types génétiques. Le muscle CT qui est le plus hypertrophié chez le culard adulte, montre le plus de différences dans la mise en place des fibres primaires et secondaires. En revanche, juste avant la naissance, il ne présente pas de différences significatives entre les 2 génotypes. Les autres muscles ne diffèrent pas dans la vitesse de mise en place de la seconde génération en début de vie fœtale. Cependant, il semble que la prolifération de cette génération dure plus longtemps que dans les muscles normaux, induisant un retard de la différenciation contractile des muscles culards. Ce retard est plus marqué dans les muscles présentant une forte hypertrophie musculaire chez le culard adulte. De plus, tous les muscles culards présentent des caractéristiques identiques, en particulier ils contiennent des fibres marquées avec aucun des anticorps à 210 jours de vie fœtale, et ils renferment une plus forte proportion de fibres rapides dès le dernier tiers de vie fœtale. Juste avant la naissance, les différences les plus marquées sont observées dans le BF et le Di.

muscle / foetus / différenciation / bovin / culard

1 . INTRODUCTION

Muscle hypertrophy associated with double-muscled (DM) cattle is due to a specific gene located on chromosome 2 [5] and has recently been identified as the myostatin gene [11, 23]. DM cattle exhibit a greater muscle mass, less bone and less subcutaneous fat than normal (N) cattle [7]. The greater muscle mass arises from increased total number of fibres [3]. Furthermore, adult DM muscles contain a higher proportion of fast-twitch glycolytic fibres [2, 12]. Studies of Swatland and Kieffer [25] and Gerrard and Judge [10] showed that muscle hypertrophy was due to early hyperplasia, since DM cattle developed about twice as many muscle fibres before birth than N cattle. Moreover, studies on myoblast cultures showed that proliferation was higher for cultures arising from DM foetuses [19]. In other species like chicken and quail, muscle hypertrophy is also accompanied by an increase in the total number of muscle fibres [6, 21]. However, a delay in the expression of myosin heavy chain (MHC) is only observed in quail. Similarly to this last species, a recent study on the differentiation of bovine *Semitendinosus* muscle showed that the differentiation was delayed in DM as compared to N foetuses [16]. However, muscle hypertrophy in adult cattle is not uniform throughout the body. When comparisons between N and DM cattle are made at a constant muscle weight, some regions are hypertrophied, isotrophied and even hypotrophied [4, 7]. In particular, muscle hypertrophy is more important in the hindlimbs than in the forelimbs and follows a distoproximal gradient (for review [1]).

To complete the results concerning genetic variability of muscle differentiation in cattle [9, 14], we compared the kinetics of foetal differentiation of several muscles differently affected by hypertrophy such as

Diaphragma (Di) and *Masseter* (Ma), two hypotrophied muscles [13]; *Longissimus thoracis* (LT), an isotrophied muscle [4]; *Biceps femoris* (BF) and *Semitendinosus* (ST), two hypertrophied muscles; and *Cutaneus trunci* (CT), the most hypertrophied muscle in adult DM [13]. Muscle contractile differentiation was studied using several complementary methods: immunohistochemistry, electrophoresis, immunoblotting and ELISA. The same pattern of differentiation was observed in both genotypes, in particular the presence of at least two consecutive generations of fibres, but the timing of muscle differentiation varied according to genotype and muscle types.

2 . MATERIALS AND METHODS

2.1. Muscle samples

Foetuses of each genotype were studied at 60, 110, 150, 180, 210, 230 and 260 days post-conception. Three or four foetuses of each genotype were obtained at each stage. Normal Charolais foetuses were obtained by artificial insemination of Charolais heifers using Charolais sperm of normal type. DM foetuses were obtained by transplantation of frozen embryos of strain INRA 95 (male selected on muscle hypertrophy). This strain was composed of a mixture of breeds (retained embryos contained around 75% of Charolais). After slaughter, foetuses were collected and six muscles (Ma, Di, ST, LT, BF and CT) differing by their contractile and metabolic properties and their degree of hypertrophy in DM adult cattle, were excised. The CT muscle was not sampled at 60 days because it was too small. Muscle samples used for immunohistochemistry were frozen in isopentane cooled by liquid nitrogen. Samples used for electrophoresis and ELISA were directly frozen in liquid nitrogen. All samples were stored at –80 °C until analysis.

2.2. Monoclonal antibodies

Five monoclonal antibodies were used. The conditions under which they were produced, purified and characterised have been described [20]. Their specificity has been established previously by Picard et al. [14, 17] in bovine muscle by reference to histochemical techniques and immunoblotting. The antibody F88 8H8, called S, was prepared from adult human auricle. The antibody is specific of slow MHC1 in bovine muscle. Antibody F113 15F4, specific for fast MHC (2a and 2b) in bovine muscle, was generated against rabbit adult myosin of *Tibialis* muscle and was called R. The F158 4C10, called F (foetal MHC), was obtained from a myosin of a 22-week-old cattle foetus [20]. The F88 11H7, called E (embryonic MHC), was prepared from adult human atrium. The last F88 10C2, called A (alpha-cardiac MHC), was prepared from adult human heart. All antibodies were purchased from Biocytex company (Marseille, France). The cross-reactivity of F, E and A antibodies has been analysed by immunohistochemistry, particularly in Ma muscle [18] and by immunoblotting (results not published) indicating that these three antibodies recognised three different MHC in bovine muscle.

2.3. Immunohistochemistry

Myosin heavy chains present at different stages were revealed by immunohistochemical staining using the monoclonal antibodies previously described. Transverse 10 µm thick serial cross-sections, were obtained using a cryostat microtome at –25 °C. Sections were incubated directly with not diluted primary monoclonal antibody, for 30 min at 37 °C. Sections were washed in PBS and incubated with the second antibody (rabbit anti-mouse IgG labelled with dichlorotriazinylaminofluorescein, Interchim) diluted 1/30 in PBS, for 30 min at 37 °C. After washing in PBS, sections

were mounted with mowiol (Calbiochem ref. 475904).

Proportions of the different types of fibres revealed by the 5 antibodies used, were counted only at 110, 180, 210 and 260 days of foetal life.

2.4. Protein preparation

Frozen muscle 200 mg was ground (polytron) for 20 s at speed 6, in 5 ml of buffer solution: 0.5 M NaCl, 20 mM sodium pyrophosphate, 50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol. After 10 min at 4 °C, samples were centrifuged for 10 min at 2500 *g*. The supernatant was mixed with glycerol at 50% (v/v) and stored at –20 °C until analyses.

2.5. Electrophoresis

Protein preparations were diluted 50% (v/v) in a solution containing 1.4% (w/v) SDS, 10% (v/v) 1 M Tris pH 6.8, 17% (v/v) glycerol, 0.6% (w/v) pyronin Y and 7.5% (v/v) mercaptoethanol. After 10 min at room temperature, samples were heated at 90 °C for 10 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on gels of 160 × 160 × 1.5 mm. Separating gel was a 3.5–10% gradient SDS-polyacrylamide gel with a C of 1.3 and a ratio of 1/74.5. Stacking gel was at 3.5%. To improve resolution, separating gel also contained 30–40% (v/v) glycerol gradient according to Suguira and Murakami [24]. Wells were loaded with 5 µg of protein when only electrophoresis was performed, or with 0.5 µg when electrophoresis was followed by immunoblotting. Migration was performed at 110 V overnight at 4 °C. Gels were stained with Coomassie blue R250 or used for immunoblotting.

2.6. Immunoblotting

Proteins were electrotransferred from the SDS-PAGE gel onto polyvinyl (Immobilon P., Millipore) membranes according to the method of Towbin et al. [26]. Membrane were saturated in TBS: 100 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8 with 3% (w/v) powdered skim milk and shaken for 4 hours at room temperature. Membranes were incubated overnight with primary antibodies, either S (1/ 2000), or R or F diluted (1/1000) in TBS containing 3% (w/v) powdered skim milk. The second antibody (rabbit anti-mouse IgG labelled with alkaline-phosphatase, Jackson Interchim) was diluted 5000-fold (v/v) in TBS containing 3% powdered skim milk and applied 1 hour at room temperature. After washings in a buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$ pH 9.5), color development was carried out in a solution of Nitro Blue Tetrazolium (NBT) and 5 Bromo 4 Chloro 3 Indolyl Phosphate (BCIP). Reactions were stopped in a solution of 20 mM Tris-HCl, 10 mM EDTA, pH 8.

2.7. ELISA

This technique was used to quantify either MHC1, MHC2 (2a and 2b) or foetal MHC. The method used was that of Winkelman et al. [27] modified by Picard et al. [15]. Briefly, 2.4 $\mu g \cdot ml^{-1}$ of myofibrillar proteins were loaded on each well of a 96-wells plate (Nunc) and incubated overnight at 4 °C. The following day, aspecific sites were saturated with PBS (138 mM NaCl, 2 mM KCl, 9 mM Na2HPO4, 1 mM KH2PO4, pH 7.4) containing 10% (w/v) powdered skim milk for 30 min at room temperature. Primary monoclonal antibodies were diluted (1/400) S or R and F (1/200) in PBS containing 10% (w/v) powdered skim milk. Second antibodies (rabbit anti mouse IgG labelled with alkaline phosphatase, Jackson Interchim) were diluted in PBS containing 10% (w/v) powdered skim milk either 700-fold (v/v) to determine MHC1 and MHC F percentages or 1400-fold (v/v) for MHC2 percentage. The substrate of the enzyme was paranitrophenylphosphate

(Sigma). Absorbance was measured at 405 nm using a microtiter plate reader.

MHC concentration was proportional to optical density (OD). A standard curve was obtained with a mixture of two muscles whose fibre compositions were opposite. To quantify MHC1 and MHC2 proportions, adult bovine Ma (100% MHC1) and median part of adult bovine CT (100% MHC2) were chosen. To determine foetal MHC percentage, LT of a 180-days old bovine foetus containing 100% foetal MHC, and adult bovine CT (0% foetal MHC) were chosen.

The good relationship between percentage of fibres and ELISA concentrations was verified by Picard et al. [15].

2.8. Statistical analysis

A variance analysis using SAS software [22] was performed to study the effect of genetic type for each muscle at the different stages.

3. RESULTS

Contractile differentiation was analysed by comparing both the position of fibres in the bundles, their size and their response with the different antibodies used.

3.1. Expression of the different types of myosin heavy chain in muscle fibres

3.1.1. 60 days

At this stage, no difference was observed between the two genetic types. Only one category of large myotubes recognised by antibodies S, R, E, F and A, was present in all muscles of N and DM foetuses (data not shown).

3.1.2. 110 days

Two generations of fibres were distinguished in all muscles of both genotypes (Fig. 1). They were present in similar proportion in the two genetic types in all muscles except in CT (Tab. I) where primary fibres were more numerous (51% vs. 21%) and secondary fibres less numerous in DM cattle (49% vs. 79%). Consequently the ratio secondary/primary fibres was smaller in DM than in N CT. It was not different between genotypes in the other muscles.

Two categories of primary fibres were observed in ST, BF, Di and Ma of both genetic types. CT muscle contained only the first category (SREFA) in DM cattle and the two categories in N (Tab. I) showing a delay in the differentiation of these fibres in DM. In LT, only one category (SREFA) was present in the two genotypes (Tab. II). The large myotubes recognised by the five antibodies (SREFA) which displayed the main group of primary fibres (Fig. 1), were less numerous in LT and CT of N than of DM ($P < 0.001$). They were not significantly different in other muscles (Fig. 1). No significant differences were observed in the composition of the secondary fibres in the different muscles of the two genotypes. However, the second generation of fibres was more numerous in CT of N and was not different between other muscles of N and DM foetuses. It was composed of three categories of fibres in all muscles of both genetic types (Tabs. I and II).

3.1.3. 180 days

The first generation of primary fibres was composed of one category of fibres (S----) which expressed only slow myosin in Di, BF and LT of both genetic types (Tabs. I and II, Fig. 2). CT and Ma of both genotypes were only composed of (S-EFA) fibres which still contained the developmental MHC isoforms. These fibres were more numerous in the DM genotype ($P < 0.01$ in CT). In ST muscle, the primary generation was different between N and DM, in N muscle only slow fibres were present, in DM two types (S----) and (S-EFA) were observed (Tab. II).

The principal difference observed for the second generation was that fibres (SREFA) were present in most N muscles (except BF), while none were observed in DM (Tabs. I and II).

3.1.4. 210 days

The first generation of fibres, corresponding to largest fibres, was only recognised by antibody S in all muscles of both

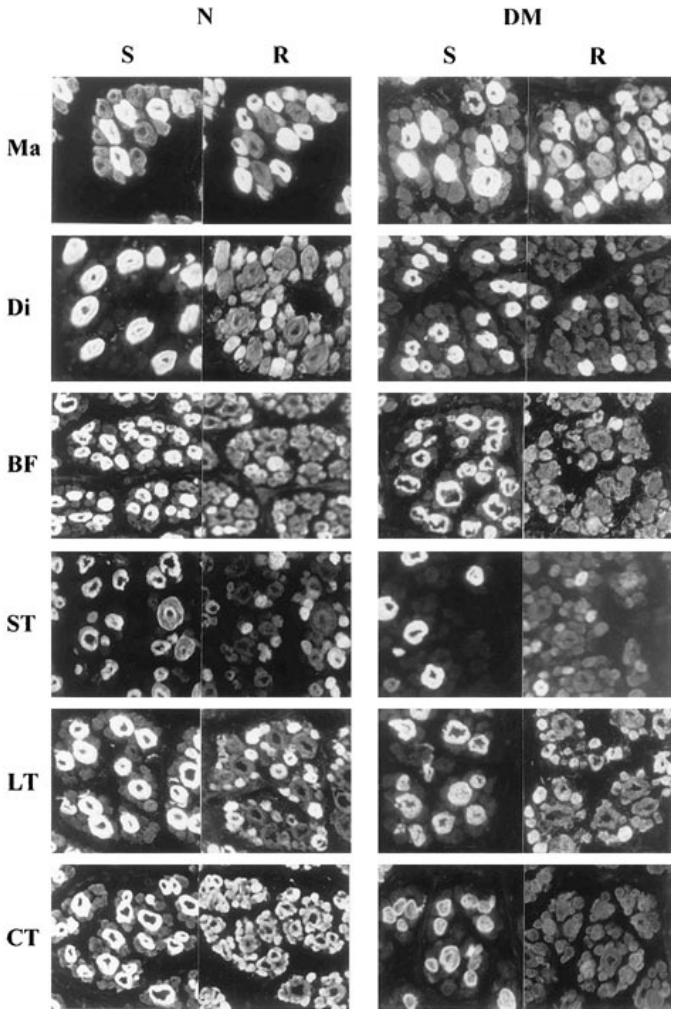

Figure 1. Immunohistochemical analysis of transverse serial sections of the muscles *Masseter* (Ma), *Diaphragma* (Di), *Biceps femoris* (BF), *Semitendinosus* (ST), *Longissimus thoracis* (LT) and *Cutaneus trunci* (CT) excised from normal (N) and double-muscled (DM) 110-day-old foetuses and reacted with monoclonal antibodies raised against slow (S) and fast (R) MHC. The labelling with antibodies raised against embryonic MHC (E), foetal MHC (F) and alpha-cardiac MHC (A) was not shown. Large myotubes were recognised by S, R, E, F, A and others were recognised by S, E, F, A. They corresponded to the first generation of fibres. The second generation of fibres was composed of smaller myotubes recognised by S, R, E, F, A or by R, E, F, A and very small fibres recognised by E, F, A.

Table I. Proportion of the different types of fibres in CT, Di and Ma muscles of the two genetic types at 110, 180, 210 and 260 days of foetal life. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Age (days)	Generation of cells	Labelling with antibodies	Muscle											
			***Cutaneus trunci* (CT) (%)**		Signifi-cance	***Diaphragma* (Di) (%)**		Signifi-cance	***Masseter* (Ma) (%)**		Signifi-cance			
			N	**DM**		**N**	**DM**		**N**	**DM**				
110	**primary**	SREFA	21 | 19	51	***	27 | 7	24 | 7	NS	42.8 | 41	36 | 30	NS		
		S – EFA	21 | 2	–	NS	27 | 20	24 | 17	NS	42.8 | 1.8	36 | 6	NS		
	secondary	SREFA	79 | 33	48.7 | 20	NS	71 | 19	76 | 17	NS	57 | 6	54 | 7	NS		
		– REFA	79 | 14	48.7 | 8.7	NS	71 | 18	76 | 22	NS	57 | 22	54 | 14	NS		
	small cells	– – EFA	79 | 32	48.7 | 20	NS	71 | 34	76 | 37	NS	57 | 29	54 | 43	NS		
180	**primary**	S – – – –	–	–		4.5	4.9	NS	–	–				
		S – EFA	1.4	3	**	–	–		1.7	3	NS			
	secondary	– REFA	19	14	NS	44	45	NS	40	43	NS			
		– rEFA	20	16	NS	24	20	NS	20	23	NS			
		SREFA	0.7	–	NS	3.5	–	NS	0.7	–	NS			
		– – EFA	46	66	*	24	21	*	30	28	NS			
	small cells	– – EFA	13	–	**	–	9	*	7	2.5	NS			
210	**primary**	S – – – –	3.4	1	NS	7.9	3.1	**	5.8	0.9	**			
	secondary	– REFA	58 | 52	25.5 | 25	**	69	60 | 49	*	69	36	NS			
		– R – —	58 | 6	25.5 | 0.5	NS	–	60 | 11	T	–	–			
		SREFA	5.4	12	NS	15	12	NS	25	11	*			
		– – EFA	11	19	NS	5.1	4.5	NS	–	33	NS			
		– – – – –	19	34	*	–	13	*	–	19	**			
	small cells	– – EFA	3.1	3.7	NS	–	1.6	NS	–	–				
		– REFA	–	4.6	NS	–	2.5	**	–	–				
260	**slow fibres:** primary and secondary	S – – – –	–	–		23	18	**	3.9	8	NS			
	secondary	– REFA	89 | 27	93 | 23	NS	35 | 13	60 | 8.2	NS	39 | 18	57 | 22	NS			
		– R – – –	89 | 62	93 | 70	NS	35 | 22	60 | 52	**	39 | 21	57 | 35	NS			
		SREFA	11	5.2	NS	32	20	NS	35	28	NS			
		S – – FA	–	–		9	2.7	***	14	6.1	NS			
		– – EFA	–	–		0.6	–	NS	–	–				
	small cells	– REFA	–	–		–	–		–	–				

Table II. Proportion of the different types of fibres in ST, LT and BF muscles of the two genetic types at 110, 180, 210 and 260 days of foetal life. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Age (days)	Generation of cells	Labelling with antibodies	Muscle											
			***Semitendinosus* (ST)** (%)			Signifi-cance	***Longissimus thoracis* (LT)** (%)			Signifi-cance	***Biceps femoris* (BF)** (%)			Signifi-cance
			N		**DM**		**N**		**DM**		**N**		**DM**	
110	**primary**	SREFA	29 | 26	31 | 28		NS	34	40		NS	35 | 23	39 | 35		NS
		S – EFA	3	3		NS	–	–			12	4		NS
	secondary	SREFA	28	24		NS	21	22		NS	20	10		NS
		– REFA	71 | 11	69 | 7		NS	66 | 19	60 | 7		NS	65 | 9	68 | 13		NS
	small cells	– – EFA	32	38		NS	26	31			36	45		NS
180	**primary**	S – – – –	3.9	2			5	3.3		NS	6.2	3.7		NS
		S – EFA	–	3.1		NS	–	–			–	–		
	secondary	– REFA	54	31		***	37	47		T	33	36		NS
		– rEFA	29	17		*	17	20		***	11	17		*
		SREFA	1.4	–		T	0.5	–		NS	–	–		
		– – EFA	12	47		NS	32	24		***	28	28		NS
	small cells	– – EFA	–	–		*	7.8	6.9		NS	21	15		NS
210	**primary**	S – – – –	5.4	1.5		***	4.8	3		NS	9	4.7		*
	secondary	– REFA	78 | 58	42 | 30		**	75 | 51	56 | 49		NS	73 | 59	75 | 60		NS
		– R – —	20	12		T	24	17		NS	14	15		NS
		SREFA	11	26		NS	13	13		NS	12	7		NS
		– – EFA	2.3	12		NS	7.2	5.5		NS	–	2.6		NS
		– – – – –	–	17		**	–	9.4		NS	–	8.9		NS
	small cells	– – EFA	–	–			–	1.5		NS	3.6	2.2		NS
		– REFA	2.3	2.3		NS	–	0.9		NS	2.5	–		**
260	**slow fibres:** primary and secondary	S – – – –	7.9	7.1			13	11		NS	12	5.5		**
		– REFA	67 | 20	73 | 19		NS	67 | 17	80 | 31		NS	67 | 18	87 | 23		NS
	secondary	– R – – –	47	54		NS	50	49		NS	49	64		**
		SREFA	14	20		NS	20	8.7		NS	15	5.4		NS
		S – – FA	–	–			–	–			–	1.3		NS
		– – EFA	5.3	–		NS	–	–			–	–		
	small cells	– REFA	5.3	–		T	–	–			5.2	–		*

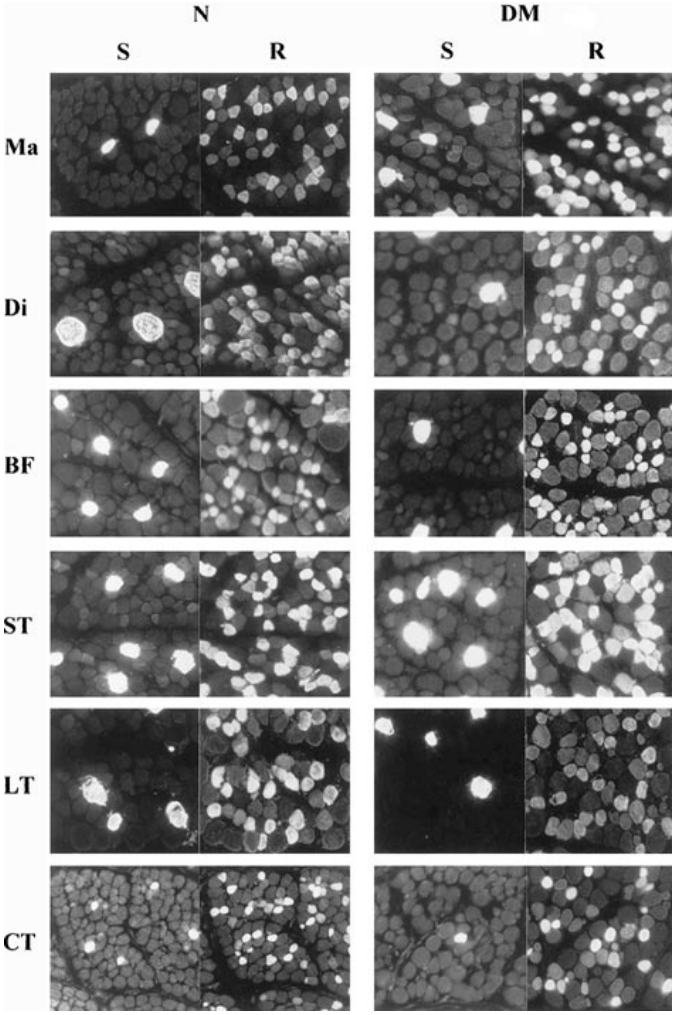

Figure 2. Immunohistochemical analysis of transverse serial sections of the muscles *Masseter* (Ma), *Diaphragma* (Di), *Biceps femoris* (BF), *Semitendinosus* (ST), *Longissimus thoracis* (LT) and *Cutaneus trunci* (CT) excised from normal (N) and double-muscled (DM) 180-day-old foetuses and reacted with monoclonal antibodies raised against slow (S) and fast (R) MHC. The labelling with antibodies raised against embryonic MHC (E), foetal MHC (F) and alpha-cardiac MHC (A) was not shown. The first generation was composed of fibres only recognised by S observed in all muscles of both genotypes and fibres labelled with S, E, F, A only present in Ma. The second generation of fibres was composed of fibres only recognised by S, R, E, F, A corresponding to type IIC fibres; cells recognised by R, E, F, A; fibres recognised by E, F, A and fibres only labelled with R.

genetic types but the proportion was lower in all DM muscles ($P < 0.05$ for BF; $P < 0.01$ for Di and Ma; $P < 0.001$ for ST) (Tabs. I and II). Secondary fibres only recognised by antibody R (-R---) and those which also expressed the developmental MHC (-REFA)

were less numerous in CT, LT and ST of DM than of N. No differences were observed in BF of N and DM (Tabs. I and II). Di muscle did not contain any fibres only expressing fast MHC in N. Ma contained only fibres (-REFA) in both genotypes. Type IIC fibres (SREFA) were present in all muscles of both genotypes. In all DM muscles, many large secondary fibres were recognised by none of the antibodies. They represented 8 to 34% depending on the muscle (Tabs. I and II). Moreover, this fibre type was more numerous in CT than in other DM muscles. It was also observed in CT of N cattle but in a lower proportion than in DM.

3.1.5. 260 days

Just before birth, seven categories of fibres were observed in both N and DM but their proportions varied between genotypes (Tabs. I and II).

First generation type I fibres (S----), expressing only MHC1, were present in all muscles of both genotypes except in CT (less than 1%) (Fig. 3). They were more numerous in BF ($P < 0.01$), LT and Di ($P < 0.01$) of N than DM, while in the same proportions in ST of both genotypes and in lower percentage in Ma of N than DM (Tabs. I and II). Among the secondary generation, fast fibres (-R---) and (-REFA), represented the greatest proportion (from 39 to 93% of secondary fibres). These fibres were present in a higher percentage in all DM muscles (Tabs. I and II). Fibres expressing only MHC2 (-R---) were observed in all muscles and represented the highest proportion in all muscles of both genotypes. However, they were in greater proportion in all DM muscles but the difference was significant only in Di and BF ($P < 0.01$) (Tab. II).

Finally, fibres (S--FA) were only observed in Di and Ma. They were in greater proportions in slow N muscles than in DM ones ($P < 0.001$ in Di) (Tab. I).

3.2. Expression of different myosin heavy chains by electrophoresis and immunoblotting – quantification by ELISA method

3.2.1. MHC1

MHC1 was found throughout the gestation in all muscles except in CT of both genotypes. Measured by ELISA, its percentage was low and consistent during the first two-thirds of foetal life and increased subsequently in both genotypes (Figs. 4–6). MHC1 increased ($P < 0.05$) after 150 days in Di (Fig. 4), 210 days ($P < 0.01$) in BF (Fig. 6) and 230 days ($P < 0.001$) in LT (Fig. 5) in both N and DM. MHC1 concentration also increased significantly from 210 days in ST (Fig. 5) and Ma (Fig. 6) of DM foetuses and only from 230 days in those of N ones ($P < 0.001$). In contrast, the MHC1 proportion decreased significantly after 110 days in CT of both genetic types (Fig. 4). Just before birth, MHC1 percentage was higher in BF, Ma, Di, and ST of N than in DM animals (Figs. 4–6). It was not different in LT between N and DM.

3.2.2. MHCF

Foetal MHC isoform was found throughout foetal life in all muscles of both genotypes. Its proportion increased from 60 to 180 days and decreased thereafter ($P < 0.001$) in all muscles except in CT in both N and DM (Figs. 4–6). In this last muscle, foetal MHC proportion was lower than in other muscles at most stages of development. It reached a maximum at 110 days in CT of N and at 150 days in that of DM (Fig. 7). Thus, the MHCF percentage decreased ($P < 0.001$) earlier in CT of N than of DM (Fig. 4). In the same way, MHCF proportion decreased ($P < 0.001$) earlier in ST of N than of DM (Fig. 5) but this decrease only began during the last third of foetal life (180 days vs. 210 days, respectively). The decrease in MHCF occurred at the same stage (after 180 days) in BF, Di and Ma of

both genetic types (Figs. 4 and 6). In contrast, it decreased earlier in LT of DM (after 180 days) than in N (after 210 days). Foetal MHC proportion was higher in ST and CT of DM than of N from 110 days but differences were only significant at 210 days in ST ($P < 0.05$) and at 150 and 180 days in CT ($P < 0.001$) (Figs. 4 and 5). In contrast,

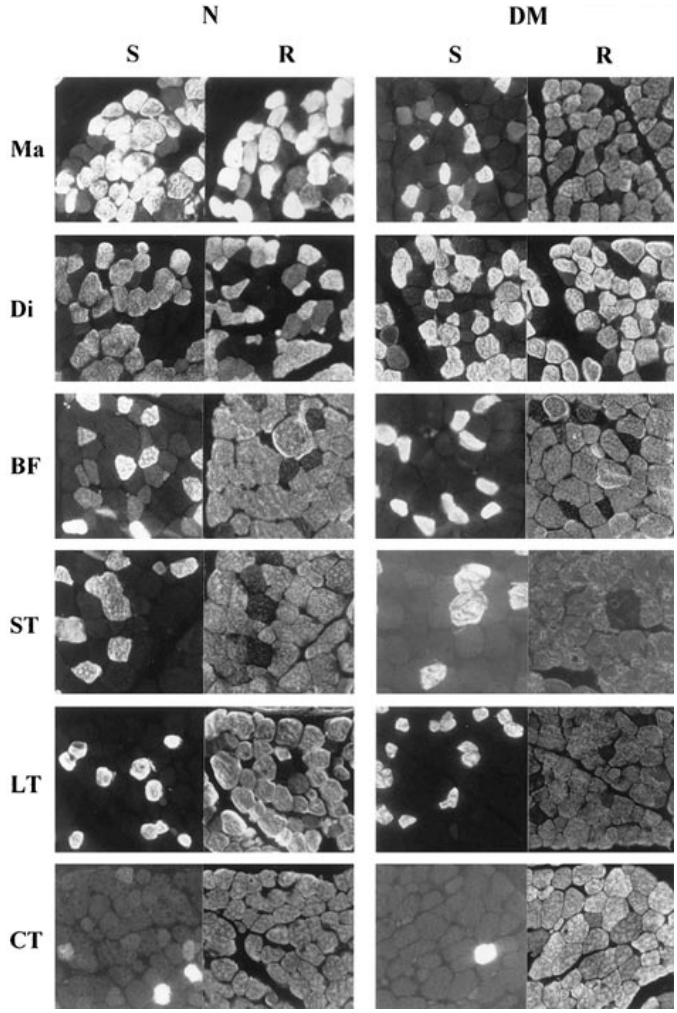

Figure 3. Immunohistochemical analysis of transverse serial sections of the muscles *Masseter* (Ma), *Diaphragma* (Di), *Biceps femoris* (BF), *Semitendinosus* (ST), *Longissimus thoracis* (LT) and *Cutaneus trunci* (CT) excised from normal (N) and double-muscled (DM) 260-day-old foetuses and reacted with monoclonal antibodies raised against slow MHC (S) and fast (R) MHC. The labelling with antibodies raised against embryonic MHC (E), foetal MHC (F) and alpha-cardiac MHC (A) was not shown. Fibres of the first generation were only recognised by S corresponding to adult type I fibres. The second generation of fibres was composed of fibres only recognised by S, R, E, F, A corresponding to type IIC fibres; fibres recognised by R, E, F, A; fibres recognised by E, F, A; fibres only labelled with R corresponding to adult type IIA and IIB fibres; fibres recognised by S, E, F, A only observed in Di and Ma.

LT, BF and Ma did not differ significantly between N and DM cattle (Figs. 5 and 6). Di of DM exhibited a lower foetal MHC percentage than that of N only at 110 days ($P < 0.01$) (Figs. 4 and 6).

3.2.3. MHC2 (2a and 2b)

Electrophoretic data showed that two fast MHC isoforms, MHC2a and 2b, are observed from 230 days in BF, CT, LT and

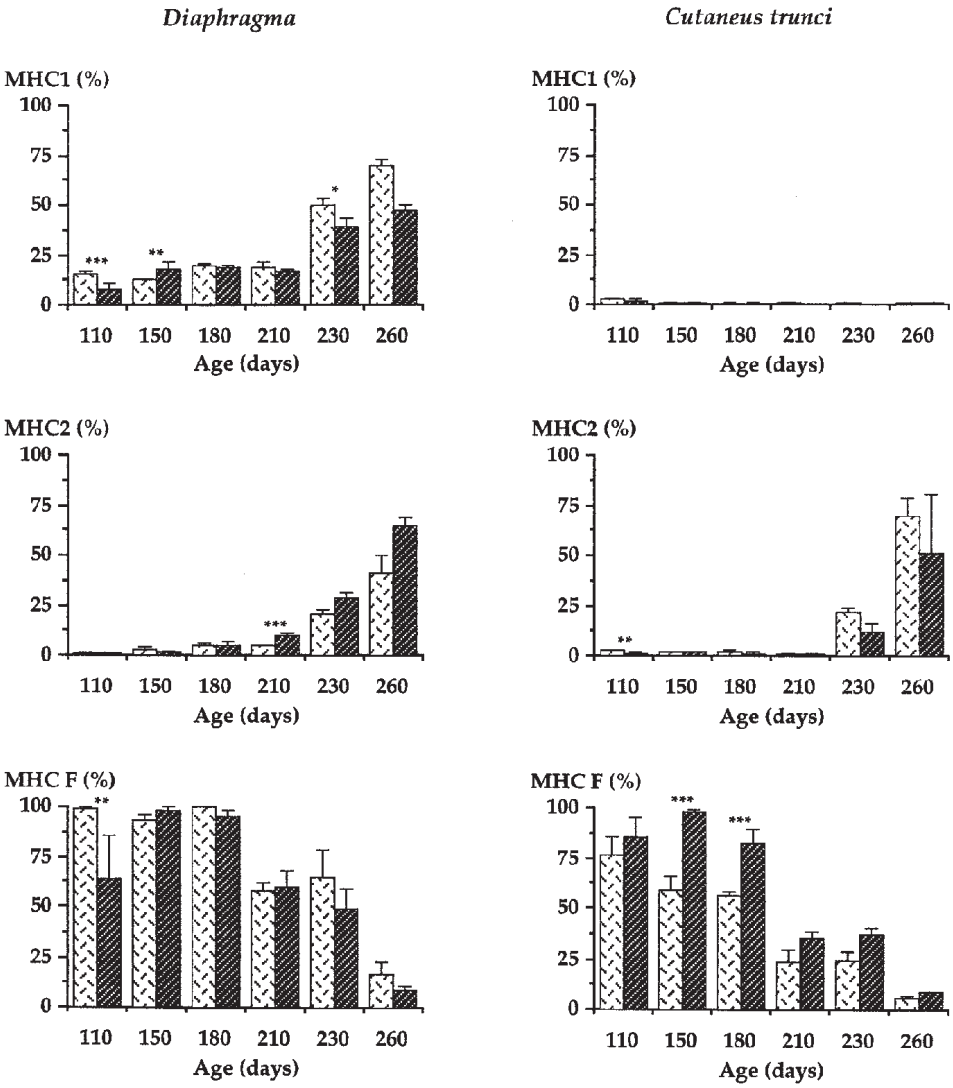

Figure 4. Evolution of MHC1, MHC2 and foetal MHC percentages in *Diaphragma* and *Cutaneus trunci* muscles of normal and double-muscled foetuses at different stages of development.

ST of DM and N foetuses (Fig. 7). Only MHC2a was present in Ma of both foetus types from 230 days. Di of DM was composed of MHC2a and 2b from 230 days, whereas Di of N only expressed MHC2a (data not shown). MHC2 quantity determined by ELISA increased ($P < 0.001$) during the last third of foetal life in all muscles of both genetic types (Figs. 4–6). This increase was significant after 180 days in Di of DM but only after 210 days in that of N (Fig. 4). In contrast, the increase of MHC2 percentage significantly increased later in LT (Fig. 5) and CT (Figs. 4 and 7) of DM

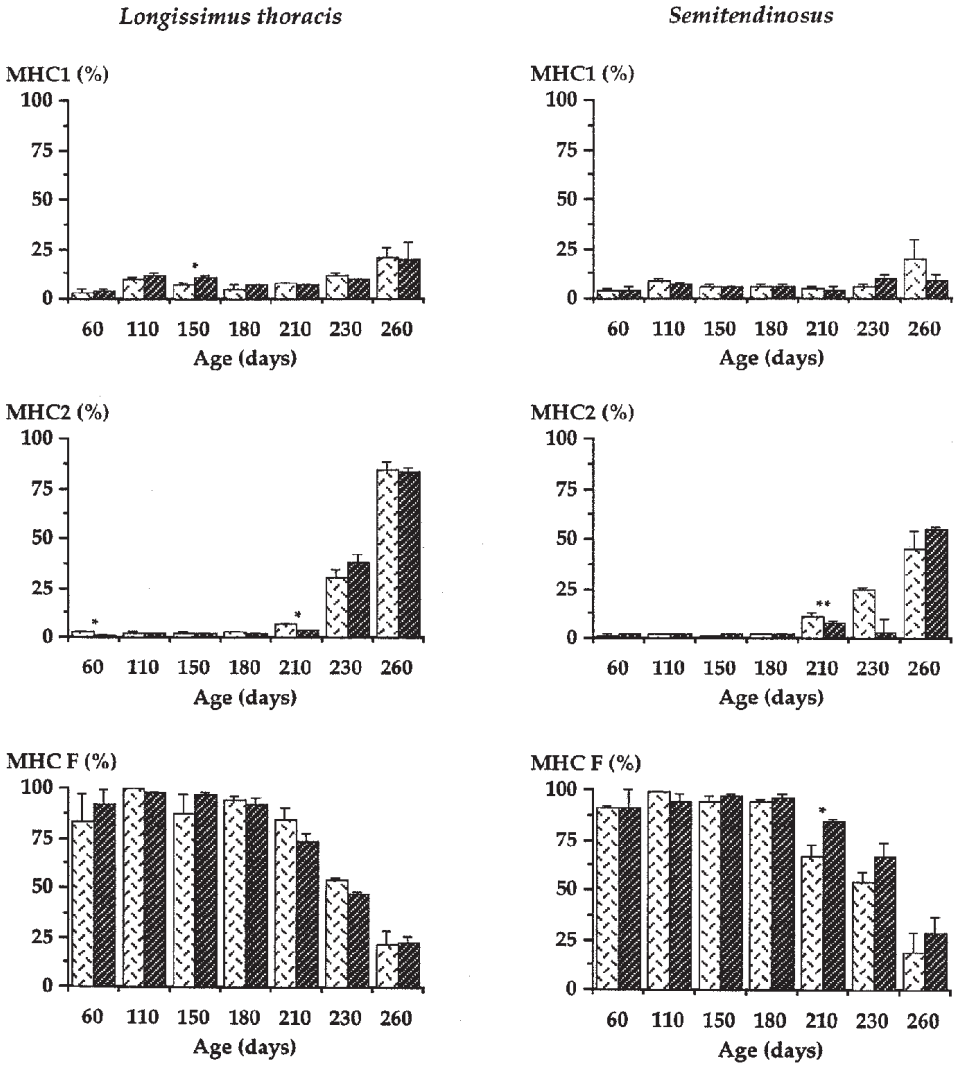

Figure 5. Evolution of MHC1, MHC2 and foetal MHC percentages in *Longissimus thoracis* and *Semitendinosus* muscles of normal ▨ and double-muscled ▨ foetuses at different stages of development.

than of N (210 days vs. 180 days and 230 days vs. 210 days, respectively). For ST (Fig. 5), BF and Ma (Fig. 4) MHC2 concentration increased significantly after the same stage in both genetic types (180 days in ST, 210 days in BF and Ma).

4. DISCUSSION

Our results showed that myosin heavy chain isoforms in DM muscles involve the same process than that described for muscles of "normal" foetuses [9, 14]. However, some

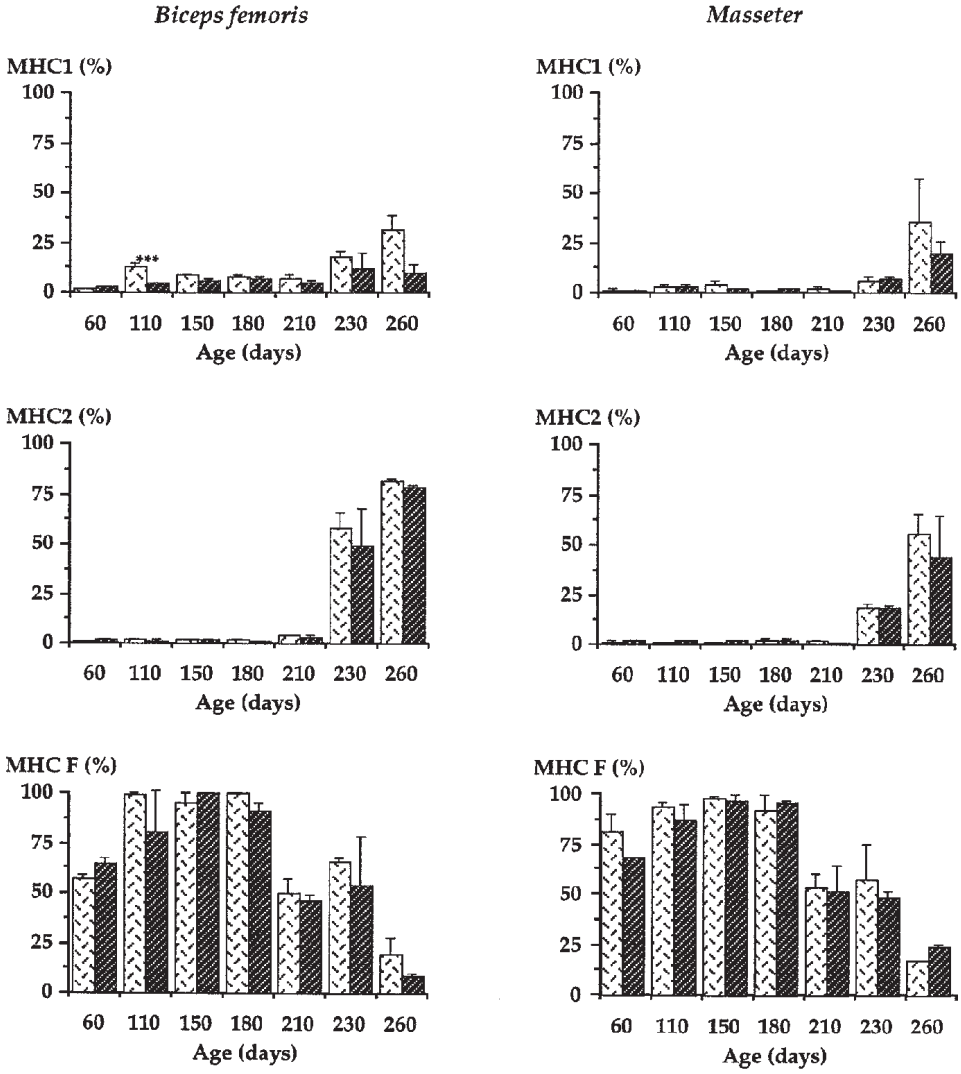

Figure 6. Evolution of MHC1, MHC2 and foetal MHC percentages in *Biceps femoris* and *Masseter* muscles of normal ▨ and double-muscled ▩ foetuses at different stages of development.

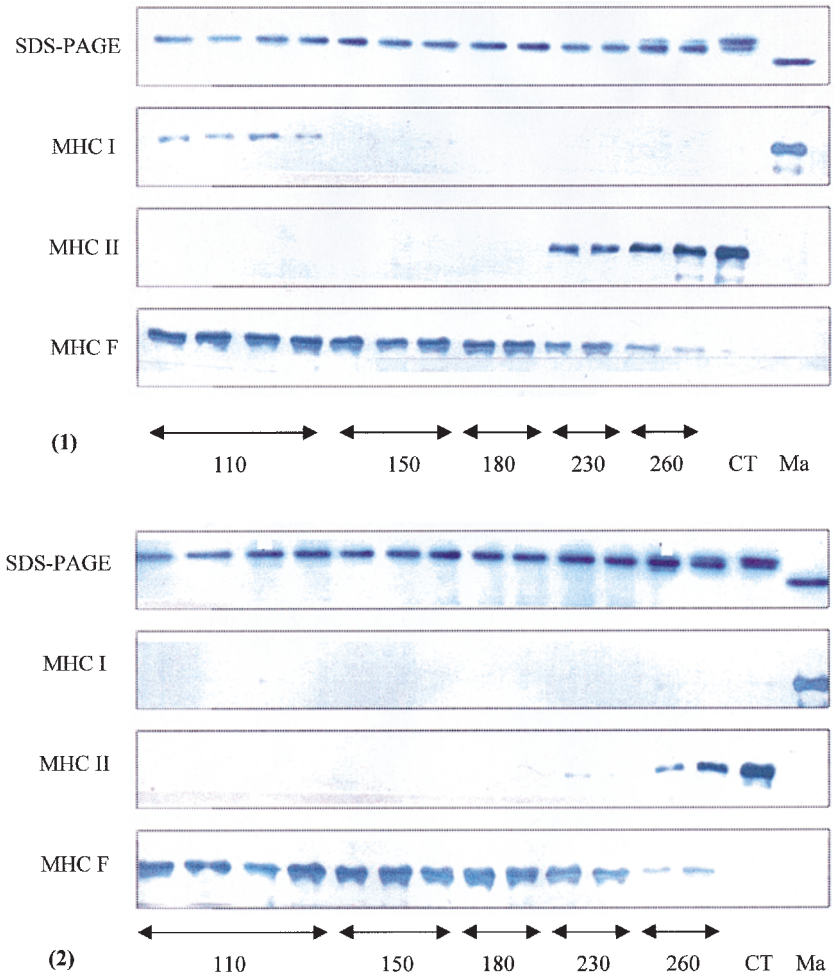

Figure 7. Different myosin heavy chains revealed by immunoblotting in N and DM *Cutaneus trunci* (CT) muscle from 110 to 260 days of foetal life. (1) Normal; (2) Double-muscled.
CT: adult CT muscle; Ma: adult *Masseter* muscle.
MHC I: slow MHC; MHC II: fast MHC 2a and 2b; MHC F: foetal MHC.

differences were observed in a time and genotype specific manner.

4.1. Differentiation of the primary fibres

As already described for normal muscles [14], the first generation of fibres contained initially all MHC isoforms, then stopped to contain fast MHC, and finally lost developmental MHCs (embryonic, foetal and alpha cardiac) from 180 days onwards in all muscles. According to the different MHC contained into fibres, the differences of precocity (Di > CT > BF > ST > LT > MA) described for N muscles [9], was also observed for DM muscles. On 110 days, the differentiation of the primary fibres presented a marked delay in CT of DM,

because no fibres have stopped to express R MHC. However, differences between the two genotypes were not evident in the other muscles suggesting that proliferation of primary fibres was identical in N and DM muscles except in CT. In this muscle proliferation phase of primary fibres seems to be longer in DM genotype. At 180 days, ST of DM also showed a marked delay which has been already described by Picard et al. [16], all primary fibres contained only slow MHC in N but not in DM muscle. From 210 days onward, primary fibres were in lower proportion in DM muscles. This suggests a longer proliferation phase of secondary fibres in DM muscles. Moreover, primary fibres were completely differentiated as they contained only MHC1 in all muscles of both genotypes. Therefore, the regulation of the differentiation of the first generation may be similar in N and DM muscles.

4.2. Differentiation of the secondary fibres

Like primary fibres, the most important difference at 110 days between N and DM secondary fibres was observed in CT. At this stage the proportion of secondary fibres was smaller in DM muscle illustrating a delay in the appearance of these fibres. At 180 days, IIC fibres began to appear in most N muscles, but they were not present in DM ones. These intermediate fibres have been described in N muscles by Picard et al. [14] and Gagnière et al. [9]. They appeared from 180 days and then were transformed into fast or slow fibres according to the muscle. Our results showed that the differentiation of these fibres was delayed in all DM muscles. So, we speculate that the mechanisms of regulation implicated in the differentiation of these fibres are different between the two genotypes.

At 210 days, all DM muscles contained fibres unlabeled with all 5 antibodies. These fibres have been already described in four DM muscles by Picard et al. [17]. Present results confirm that they seem to be present in all DM muscles. They were also observed in CT of N bovine [17]. The fact that CT is a fast muscle containing a large proportion of fast fibres, particularly IIB fibres [15], and that DM muscles are known to contain more IIB fibres than N muscles, let us to suppose that these fibres contained a particular fast MHC. However, the present results showed that these fibres were not observed at 260 days. So, we can speculate that these "unknown" fibres contained a developmental MHC which disappear after this stage, or a fast MHC which was coexpressed with another known fast isoform after 210 days. This fast MHC could be 2× MHC which has not been yet identified in bovine muscle by immunological techniques. Further investigations are necessary to confirm this hypothesis.

Just before birth (at 260 days), all DM muscles (except CT and Ma) contained a higher proportion of fast fibres (-REFA + -R----) and a lower proportion of IIC fibres. So, the contractile characteristics specific of adult DM muscles [3] are already present just before birth. They took place during foetal life. However, the fast antibody used in this study did not allow the distinction between MHC 2a and MHC 2b, so we can not tell if the fast fibres more numerous in DM muscles contain MHC 2b. However, other results concerning the metabolic differentiation [8] have shown that DM muscles were less oxidative than N ones from the last third of foetal life. So, we can hypothesise that DM muscles contained a greater proportion of IIB fibres at birth. Further analyses are in progress to confirm this hypothesis.

4.3. MHC isoforms transition

The five MHC isoforms described in N muscles [9, 14] were also present in all DM muscles. MHC1 and MHC2 were observed in low proportions during the first two-thirds of foetal life and then increased when foetal MHC decreased. These data show that adult

MHC1 and MHC2 appear when the foetal MHC isoform disappears as has already been observed in several N muscles [9, 14] and in ST of DM [16]. Nevertheless, most DM muscles were composed of higher proportions of foetal MHC just before birth. Therefore, the disappearance of foetal MHC appears to be delayed in DM than in N which confirms the delay of differentiation in DM as previously observed at cellular level [19].

4.4. Muscles variability

Our results showed that muscles with different degree of muscle hypertrophy in adult DM differ in their timing of MHC production.

CT muscle described as the most hypertrophied muscle in DM bovine [13] was the most different between N and DM in the present study, particularly from 110 to 210 days. It showed an important delay of differentiation of the first and the second generations of fibres from 110 days. At this stage, the ratio of secondary/ primary fibres was very small in DM. This indicates a delay in the appearance of the secondary generation of fibres which could be the consequence of a longer proliferation phase of primary fibres. Indeed, Picard et al. [19] have shown in vitro that 110 days DM myoblasts of ST muscle proliferated more than N ones. Because CT is highly hypertrohied in DM, it is logical to think that proliferation of myoblasts in this muscle was more intense than in the other DM muscles to generate a larger number of fibres. This will be verified in primary cultures of myoblasts from N and DM CT. The fact that MHC F proportion was higher in DM CT confirms a delay of differentiation. Moreover, this muscle was the only N muscle which contained unknown fibres (----) at 210 days. The proportion of these fibres was higher in DM CT. However, the differences between the two genotypes disappeared just before birth.

Among other hypertrophied muscles in DM bovine, ST and BF were studied. Picard et al. [16] have already described a delay of differentiation in ST which was confirmed in the present study. However, the differences were less important than in CT, which could be explained by a less important hypertrophy in this muscle. In BF of DM only weak differences were observed during foetal life, but just before birth, it showed the most important differences in the proportions of fast and slow fibres between the two genotypes. Our results in N muscles [9] have demonstrated that the differentiation of this muscle was delayed comparatively to ST. This explains why this muscle shows differences between N and DM only at the end of gestation.

LT muscle, isotrophied in DM [4], did not present any delay of differentiation. This result argues in favour of a direct relationship between a more intense proliferation in hypertrophied muscles and a delay of differentiation. In N and DM LT muscles, the proliferation could be similar. However, LT presented the characteristics of DM muscles such as unknown fibres at 210 days and more type II fibres just before birth. This led us to believe that proliferation (at the origin of hypertrophy in adult DM) and differentiation (at the origin of the particular characteristics of adult DM muscles) are controlled differently.

The differentiation of Di, hypotrophied in DM [13], was consistent with the above hypothesis. During the first stages of foetal life (from 60 to 180 days), we did not observed large differences between N and DM. However, from 210 days onwards we observed the same particularity in Di than in other DM muscles. Moreover, this muscle presented another important difference between N and DM. Electrophoretic data showed that MHC 2b was not present in N, whereas it was observed in DM Di. This result is coherent with the fact that DM muscles contain more type IIB fibres, and could explain that DM bovine present some respiratory problems.

The Ma, also hypotrophied in adult DM [13], presented weak differences from 60 to 180 days between N and DM. However at 210 days, it showed the same differences than in other muscles. Just before birth, Ma was composed of a greater proportion of type IIC fibres than other muscles in DM and many type II fibres. Moreover, small fibres (S–FA) only present in slow muscles were observed from 230 days of gestation in Ma of N and 260 days in that of DM which illustrates a delay of differentiation of DM MA comparatively to N one, whereas they were present at 210 days in Di of both genotypes.

All these results show that the delay of contractile differentiation already described in vivo and in vitro for DM ST muscle [16, 19] was more or less marked according to the muscle. It seems that a direct relation exists between the hypertrophy of muscle in the adult stage and the delay of differentiation during foetal life. We speculate that the most hypertrophied muscles exhibit the most important proliferation of myoblasts which induce a delay in the differentiation phase. In CT muscle the proliferation of both primary and secondary fibres seems to be higher in DM animals. In all other muscles studied, speed of appearance of fibres is the same in the two genotypes, only the proliferation of the secondary generation of fibres appears to be longer in DM. Further studies are in progress to verify this hypothesis. However, all DM muscles present similar characteristics, particularly at 210 days, which seems to be independent of their degree of hypertrophy.

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