First Meeting of the Interuniversity Institute of Myology

Montegrotto Terme - Padova (Italy), October 16 – 18, 2004
Continental Hotel Terme - Via Neroniane, 8 - 35036 Montegrotto Terme (Padova)

Saturday October 16, 2004

14.30 Welcome Address, G Fanò and U Carraro

STRUCTURAL, FUNCTIONAL AND REGULATORY MUSCLE MOLECULES
Chair: P Bruni and C Reggiani

14.45 IDENTIFICATION OF TWO SITES IN OBSCURIN THAT MEDIATE THE INTERACTION WITH ANKYRIN 1.5
Armani A, Galli S, Sorrentino V

15.00 CROSSBRIDGE PROPERTIES STUDIED BY FAST STRETCHES IN ACTIVATED FROG MUSCLE FIBRES
Colombini B, Bagni MA, Cecchi G

15.15 MYOSIN ORIENTATION IN SKELETAL MUSCLE REVEALED BY X-RAY DIFFRACTION STUDIES DURING SARCOMERE LENGTH OSCILLATIONS
Colombini B, Griffiths PJ, Bagni MA, Amenitsch H, Bernstorff S, Ashley CC, Cecchi G

15.30 THE MOLECULAR MOTOR OF MUSCLE STUDIED BY X-RAY INTERFERENCE
Reconditi M, Linari M, Lucii L, Piazzesi G, Stewart A, Sun Y-B, Narayanan T, Irving T, Irving M, Lombardi V

15.45 MECHANICAL AND KINETIC PROPERTIES OF PURE ISOFORMS OF SKELETAL MYOSIN FRACTION S1 STUDIED BY A SINGLE MOLECULE MECHANICAL APPROACH
Capitanio M, Canepari M, Maffei M, Cicchi R, Pavone FS, Bottinelli R

16.00 ENDOGENOUS TROPONIN COMPLEX REPLACEMENT IN SINGLE SKELETAL AND CARDIAC MYOFIBRILS
Belus A, Piroddi N, Scellini B, Tesi C, Poggesi C

16.15 THE IK-B-HOMOLOGUE CACTUS IS NECESSARY FOR NORMAL NEUROMUSCULAR FUNCTION IN DROSOPHILA MELANOGASTER
Peron S, Beramendi A, Megighian A, Reggiani C, Cantera R

16.30 REGULATION OF GLYCOGEN SYNTHASE BY SARCOPLASMIC RETICULUM-BOUND CAMKII IN RABBIT FAST-TWITCH SKELETAL MUSCLE
Sacchetto R, Bovo E, Damiani E

16.45 TRANSFER OF PLASMID DNA AND OLIGONUCLEOTIDES INTO SKELETAL MUSCLE BY MEANS OF CATIONIC LIPID-BASED VECTORS
Ditadi A, Malerba A, Occhi G, Gamba PG, Scambi I, McLachlan I, Baroni V, Vitello L

17.00 Break

MOTOR CONTROL, AGING AND DENERVATION OF SKELETAL MUSCLE

17.30 AGONIST-ANTAGONIST ACTIVATION DURING ELBOW FLEXION AND EXTENSION AT DIFFERENT ANGULAR VELOCITIES
Bazzucchi I, Marzattinocci G, Felici F

17.45 EFFECTS OF INHIBITORS OF CELL MEMBRANE CALCIUM CHANNELS ON HIGH-FREQUENCY FATIGUE OF FAST AND SLOW SKELETAL MUSCLES
GerminarioE, Esposito A, Midrio M, Palade PT, Betto R, Danieli D

18.00 DIFFERENCES IN FORCE/ENDURANCE RELATIONSHIP BETWEEN YOUNG AND OLDER MEN Bazzucchi I, Marchetti M, Rosponi A , Fattorini L, Castellano V, Sbriccoli P, Felici F

18.15 EFFECTS OF SPHINGOMYELIN DERIVATIVES ON INNERVATED AND DENERVATED RAT SOLEUS MUSCLE
ZaninM, Germinario E, Betto R, Danieli D

18.30 LONG-TERM DENERVATION OF RAT MUSCLE: A TIME COURSE STUDY
Adami N, Biral D, Kern H, Carraro U

18.40 LONG-TERM LOWER-MOTONEURON DENERVATION OF HUMAN MUSCLE: A TIME COURSE STUDY
Caccavale S, Rossini K, Adami N, Kern H, Carraro U

18.50 MYOREGENERATION IN HUMAN DENERVATED DEGENERATED MUSCLE DECREASES AFTER MUSCLE RECOVERY INDUCED BY FES TRAINING
Rossini K, Caccavale S, Adami N, Kern H, Carraro U

19.00 REGENERATION OF LONG-TERM DENERVATED MUSCLE BY FES
Boncompagni S, Kern H, Mayr W, Carraro U, Protasi F

19.15 HELMUT KERN, Guest Speaker
Functional Electrical Stimulation of Skeletal Muscle: Clinical Results of the EU Project RISE

20.00 IIM Social Dinner

Sunday October 17, 2004

REGENERATIVE PATHWAYS AND MUSCLE DISEASES

Chair: A. Musarò and R Bottinelli

9.00 SKELETAL MUSCLE OF MICE OVEREXPRESSING FRG1 SHOWS HYSTOLOGICAL AND FUNCTIONAL SIGNS OF MUSCULAR DYSTROPHY
Brocca L, Pellegrino MA Moggio M, Green M, Tupler R, Bottinelli R

9.15 TRANSGENIC MOUSE MODELS OF MUSCLE WASTING AND REGENERATION
Dobrowolny G, Giacinti C, Pelosi L, Nicoletti C, Barberi L, Molinaro M, Rosenthal N, Musarò A

9.30 HYALURONIC ACID BASED DRESSING AS ANTI-FIBROSIS AGENT IN THE TREATMENT OF MUSCLE INJURY
Vindigni V, Mazzoleni F, Carraro U

9.45 STRESS GENE EXPRESSION IN SKELETAL MUSCLES AFTER MODERATE EXERCISE
Marini M, Lapalombella R, Scordari A, D'Aloia C, Margonato V, Esposito F, Veicsteinas A

10.00 GENE EXPRESSION MODIFICATIONS IN RAT HEART FOLLOWING MODERATE PHYSICAL EXERCISE
Margonato V, Veicsteinas A, Samaja M, Ventura C, Lapalombella R, Scordari A, Carinci P, Marini M

10.15 CONTRACTILE PROPERTIES OF SINGLE MUSCLE FIBERS FROM NORMAL DOGS AND DOGS AFFECTED BY GOLDEN RETRIEVER MUSCULAR DYSTROPY
Rinaldi C, Pansarasa O, Bottinelli R, Blot S, D'Antona G

10.30 Break

MUSCLE DISEASES 2

Chair: A Uncini and V Sorrentino

11.00 IDENTIFICATION AND FUNCTIONAL STUDIES OF MUTATION IN THE RYR1 GENE IN PATIENS WITH MALIGNANT HYPERTHERMIA
Rossi D, De Smedt P, Galli L, Orrico A, Franci D, Petrioli F, Lorenzini S, Tegazzin V, Sorrentino V

11.15 PROTEIN AND MOLECULAR DIAGNOSIS IN LGMD2A
Nascimbeni AC, Fulizio L, Spinazzi M , Fanin M, Angelini C

11.30 LIMB-GIRDLE MUSCULAR DYSTROPHIES TYPE 2A AND 2B: CLINICAL AND RADIOLOGICAL ASPECTS
Borsato C, Padoan R, Stramare R, Fanin M, Angelini C

11.45 AN ENDOCRINOLOGICAL AND NEUROPSYCHOLOGICAL INVESTIGATION IN MYOTONIC DYSTROPHY TYPE 1 (DM1)
Romeo V, Squarzanti F, Mongiat M, Gasparoni P, D'Ascenzo C, Pegoraro E, Angelini C

12.00 FAMILIAL IDIOPATHIC HYPERCKEMIA
Capasso M, De Angelis MV, Pace M, Zuccarini F, Di Muzio A, Uncini A

12.15 FAMILIAL IDIOPATHIC HYPERCKEMIA: A POSSIBLE PREDISPOSITION TO STATIN-INDUCED MYOPATHY
Capasso M, Di Muzio A, De Angelis MV, Uncini A

12.30 MUSCLE INFECTION IN CHRONIC HEPATITIS B
Capasso M, Di Muzio A, Comar M, Campello C, Robuffo I, Gambi A, De Angelis MV, Uncini A

14.00 – 15.00 Meeting of the IIM Council

REGULATORY MECHANISMS OF MYOGENESIS 1

Chair: F. Protasi and G Cecchi

15.00 TNF□ AND AVP EXERT OPPOSITE EFFECTS ON MUSCLE REGENERATION
Moresi V, Adamo S, M Molinaro, Coletti D

15.15 CHARACTERIZATION OF MYOGENIC FACTORS DERIVED FROM A STABLE MACROPHAGE CELL LINES
Malerba A, Scambi I, Segat D, Frigo M, De Coppi P, Gamba P, Boldrin L, Cavallini L, Bellomo R, Fanò G, Vecchiett L, Vitiello L, Baroni D

15.30 3D-CULTURE OF ISOLATED CELLS AND TISSUE EXPLANTS IN RELATIVE MICROGRAVITY: NEW PERSPECTIVES AND POSSIBLE APPLICATIONS
Steimberg N, Rovetta F, Boniotti J, Mazzoleni G

15.45 MUSCLE TISSUE ENGINEERING USING SINGLE FIBRE ISOLATION TECHNIQUE: IN VITRO AND IN VIVO PROSPECTIVES
Boldrin L, Flaibani M, Malerba A, Slanzi E, Pozzobon M, Baroni D, Messina C, Zanesco L, Gamba PG, Vitiello L, Elvassore N, De Coppi P

16.00 THE OXIDATIVE DAMAGE INDUCES MODIFICATIONS OF Ca2+ TRANSPORT SYSTEM IN HUMAN SATELLITE CELLS
Beccafico S, Belia S, Puglielli C, Pietrangelo T, Fulle S

16.15 SPHINGOSINE 1-PHOSPHATE AFFECTS THE ELECTRIC PROPERTIES OF THE PLASMAMEMBRANE IN C2C12 MYOBLASTS
Squecco R, Formigli L, Sassoli C, Chellini F, Quercioli F, Zecchi S, Tiribilli B, Francini F
16.30 ORGANIZATION OF THE SARCOPLASMIC RETICULUM DURING MUSCLE DEVELOPMENT
Cusimano V, Giacomello E, Sorrentino V
16.45 MHC ISOFORM EXPRESSION IN BOVINE SINGLE FIBRES STUDIED AT RNA AND PROTEIN LEVEL
Toniolo L, Maccatrozzo L, Patruno M, Mascarello F, Reggiani C
17.00 MYOSIN HEAVY CHAIN ADULT ISOFORMS EXPRESSION IN DIFFERENT SKELETAL MUSCLES OF CATTLE
Maccatrozzo L, Patruno M, Toniolo L, Reggiani C, Mascarello F
17.15 FIBRE TYPES, MHC ISOFORM EXPRESSION AND SINGLE FIBRE CONTRACTILE PROPERTIES IN FELINE SKELETAL MUSCLES
Pavan E, Toniolo L, Maccatrozzo L, Patruno M, Mascarello F, Reggiani C
17.30 INSIDE MUSCLE –TENDON UNIT BY SURFACE MECHANOMYOGRAM (MMG) AND FORCE SIGNAL
Orizio C, Gobbo M

18.15 Open Convention of the IIM

Monday October 18, 2004

REGULATORY MECHANISMS OF MYOGENESIS 2
Chair: G Fanò and U Carraro
9.00 SARCOLEMMAL IONIC CONDUCTANCE IN CULTURED MUSCLE FIBRES OF mdx, COL VI KO, dy/dy AND WT MICE
Canato M, Pavan E, Vassanelli S, Megighian A, Reggiani C
9.15 CALCIUM COMPARTMENTS AND GENE EXPRESSION ACTIVATION IN L6 MYOGENIC CELLS
Naro F, De Arcangelis V, Coletti D, Canato M, Molinaro M, Adamo S, Reggiani C
9.30 THE HETEROLOGOUS EXPRESSION OF SARCOGLYCANS
Gastaldello S, Sandonà D, Maddaloni C, D'Angelo S, Martinello T, Chan Yi-umo, Betto R
9.45 DYSTROPHIN-ASSOCIATED-GLYCOPROTEIN AND VINCULIN-TALIN-INTEGRIN-COMPLEXES DURING MYOGENESIS. TIMING APPEARANCE AND LOCALIZATION IN NORMAL HUMAN SKELETAL MUSCLE CULTURE
Di Mauro D, Magaudda L, Mancinelli R, Trimarchi F
10.00 EXPRESSION OF VASOPRESSIN RECEPTORS IN MYOGENESIS
Alvisi M, Ciccone L, Naro F, Adamo S
10.15 EXTRACELLULAR NUCLEOTIDES AFFECT MYOGENESIS
Martinello T, Paganin M, Sandonà D, Betto R
10.30 THE ROLE OF EXTRACELLULAR GTP ON DIFFERENTIATION OF C2C12 MYOBLASTS
Pietrangelo T, Mancinelli R, Fulle S, Fanò G
10.45 ROLE OF cAMP SIGNALLING IN MYOGENIC CELLS
De Arcangelis V, Némoz G, Molinaro M, Adamo S, Naro F
11.00 SPHINGOSINE 1-PHOSPHATE REGULATES MYOGENIC DIFFERENTIATION. A MAJOR ROLE FOR S1P2 RECEPTOR
Donati C, Meacci E, Nuti F, Becciolini L, Farnararo M, Bruni P
11.15 SPHINGOSINE 1-PHOSPHATE INDUCES CYTOSKELETAL REORGANIZATION IN C2C12 MYOBLASTS
Chellini F, Sassoli C, Nosi D, Meacci E, Bruni P, Formigli L, Zecchi-Orlandini S
11.30 INVOLVEMENT OF PHOSPHATIDIC ACID IN ACTIN FIBER FORMATION IN DIFFERENTIATING L6 MYOBLASTS
Komati H, De Arcangelis V, Adamo S, Némoz G, Naro F
11.45 A QUANTITATIVE STUDY ON REGULATORY MOLECULES INVOLVED IN THE POST-NATAL GROWTH OF PIG
Caliaro F, Maccatrozzo L, Toniolo L, Reggiani C, Mascarello F, Patruno M

ABSTRACTS

LONG-TERM DENERVATION OF RAT MUSCLE: A TIME COURSE STUDY

Adami N, Biral D, Kern H(1), Carraro U

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In spite of a large numbers of clinical and experimental studies on effects of denervation on the properties of skeletal muscle, little is known on the characteristics of long-term denervated muscles. We studied in rat (2-9 months after sciatectomy) excitability in vivo and morphologic characteristics of rat EDL and soleus muscles. Chronaxie of the leg muscle increased with denervation time up to values higher than 30 msec (in early denervation cronaxie is 1-2 msec). Cryosection analyses allow to distinguishing three stages during long-term denervation: 1. Up to three-month: myofiber atrophy with distinct ATPase fiber-typing (mean myofiber diameter 20 mm); 2. Up to six-month: Severe myofiber atrophy with loss of ATPase typing (fast-like characteristics prevails also in soleus) and poor, if any contractility; 3. After 7-month: Very severe myofiber atrophy, i.e., loss of any contractile proteins (mean myofiber diameter less than 10 mm) and fat infiltration. We confirm that the time-scale of changes in rats appears to be much shorter than in large mammals.

EXPRESSION OF VASOPRESSIN RECEPTORS IN MYOGENESIS

Alvisi M, Ciccone L, Naro F, Adamo S

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The neurohypophiseal hormone arg8-vasopressina (AVP) is a potent inducer of myogenic cell differentiation (1,2). Signaling studies on the L6 cell line showed that AVP effects are mediated, in these cells, trough a V1a type AVP receptor (3). Gene and protein expression analyses showed that V1aR is down regulated during differentiation. To understand the role of AVP receptors in muscle development we analysed the expression of V1aR and related receptors (V1bR, V2R and OTR) in murine and human primary cultures, isolated fibers and skeletal muscle. The results obtained indicate that either V1aR or OTR or both receptors are expressed in primary cultures, in isolated fibers and in muscle samples. No expression of V1bR or V2 was detected. A developmental modulation of the expression of V1aR and OTR appears to take place. The localisation of these receptors in the developing muscle is under investigation. The demonstration of the expression of such receptors in pre- and post-natal muscle supports the hypothesis of a physiological role of AVP and/or related hormones in muscle development.

1. C. Nervi et al., Cell Growth & Differentiation, 6: 81,1995 2. F. Naro et al., J.Cell.Physiol. 170: 34,1997 3. F. Naro et al., JBC 278: 49308,2003

IDENTIFICATION OF TWO SITES IN OBSCURIN THAT MEDIATE THE INTERACTION WITH ANKYRIN1.5

Armani A, Galli S, Sorrentino V

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Ankyrins are proteins involved in targeting ion channels and other specific proteins to specialised domains of the plasma membrane. More recently, ankyrins have been also found in association with the membrane of some intracellular organells. We are interested in studing a small muscle-specific isoform of the ankyrin1 gene (ank1.5), located on the sarcoplasmic reticulum in striated muscle cells. Ank1.5 is apparently capable to bind obscurin, a protein of the myofibrillar apparatus. We initially identified one ank1.5 binding site in the region of obscurin between residues 6236 -6260. More recent studies have let us to identify a second ank1.5 binding site in obscurin. This novel binding site shows sequence similarity with the first one and is capable of binding ank1.5 in in vitro pull-down experiments. Additional work is being performed to characterize the two binding sites of obscurin for ank1.5 in light of a possible role of these two proteins in mediating an interaction between the sarcoplasmic reticulum and the myofibrillar apparatus

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DIFFERENCES IN FORCE/ENDURANCE RELATIONSHIP BETWEEN YOUNG AND OLDER MEN

Bazzucchi I(1), Marchetti M(2), Rosponi A(2), Luigi Fattorini L(2), Castellano V(3), Sbriccoli P(1), Felici F(1,3)

(1) Department of Human Movement and Sport Sciences, Istituto Universitario di Scienze Motorie, Interuniversity Institute of Myology (2) Department of Human Physiology and Pharmacology, Università "La Sapienza", (3) Fondazione S. Lucia – Scientific Institute for Research, Hospitalization and Health Care – Rome, Italy. E-mail: felici@iusm.it

The aim of the present study was to ascertain if in 6 young (Y; 23-35 yr) and in 6 older (O; 70-72 yr) healthy men matched for comparable absolute and specific maximal force of the dominant elbow flexors, differences in isometric endurance, myoelectrical fatigability and shortening velocity, are still recognisable. To assess the specific force, the muscle cross sectional area (CSA) was determined on MRI scans. The elbow flexors performance was studied by assessing: a) the

isometric endurance times (ET) at different percentages of maximal isometric contraction (MVC); b) the average muscle fibres conduction velocity of action potentials (CV) and the median frequency (MDF) of the surface electromyogram (sEMG) of the biceps brachii. Finally, the torque-velocity curve was assessed by means of maximal isokinetic contractions at six fixed angular velocities. Results showed that: a) ET was longer in O subjects at the highest levels of isometric contraction independently from the absolute force (see table);

	ET [s] 30%MVC		ET [s] 50%MVC		ET [s] 80%MVC	
	Mean	SD	Mean	SD	Mean	SD
Y	193	19.7	62.2	13.6	17.2	6.5
O	202.3	41.3	108	32.1	31.5	13.3
P value	ns		$P<0.01$		$P<0.05$	

b) the modifications of muscle fibre CV during isometric effort progressed less rapidly in O than Y as well as those of MDF; c) at the same angular velocity, O exerted less absolute force than Y. These results suggest an impairment of the neuromuscular system of older men which is less powerful and less fatigable than young men.

AGONIST-ANTAGONIST ACTIVATION DURING ELBOW FLEXION AND EXTENSION AT DIFFERENT ANGULAR VELOCITIES

Bazzucchi I, Marzattinocci G, Felici F

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The aim of the present study was to assess agonist-antagonist activation of the biceps brachii (BB) and triceps brachii (TB) muscles during elbow flexion and elbow extension of the dominant arm by means of surface EMG during isokinetic concentric contractions performed at different angular velocities. Eight subjects (5 males and 3 females: age 23.4±1.1 yr; stature 175.9±9.6 cm; weight 67±13 kg) participated in this study. The EMG signals were recorded from the biceps brachii (BB) and triceps brachii (TB) muscles during 3 maximal voluntary isometric contractions (IMVC) of elbow flexors and extensors and a set of 3 maximal elbow flexions and extensions at 15, 30, 60, 120, 180, 240°s^{-1}. The root mean square (RMS) of sEMG was then calculated as an index of sEMG amplitude. Levels of BB and TB activation were expressed as percentage of average RMS with respect to average RMS of the contraction at 15°s^{-1}. A repeated-measures ANOVA was used to assess statistical differences ($P<0.05$). During the elbow flexion, antagonist TB activation was greater than antagonist BB activation recorded during the elbow extension at all the selected velocities of movement. The normalised RMS values ranged from 48,3% ± 27,1 at 15° s^{-1} and 67,3%± 18,5 at 240° s^{-1} for antagonist TB activation, and from 17.3% ± 5,6 at 15° s^{-1} and 43.1% ±15.1 for antagonist BB activation. Our findings suggest that TB muscle is co-activated to a greater extent with respect to BB when acting as antagonists. The functional specialization of these two groups of muscles, which can be responsible for these different levels of antagonist co-activation, seems to play a pivotal role in the control of movement.

THE OXIDATIVE DAMAGE INDUCES MODIFICATIONS OF CA2+ TRANSPORT SYSTEM IN HUMAN SATELLITE CELLS

Beccafico S, Belia S, Puglielli C, Pietrangelo T, Fulle S

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In humans ageing is characterised by progressive loss of muscle mass and strength (sarcopenia) associated with decline in functional ability. In skeletal muscle there are quiescent mononucleated myogenic cells, called satellite cells (Mauro 1961. J. Biochem. Biophys. Cytol., 9: 493-8), located between the sarcolemma and the basal lamina. Satellite cells contribute to muscle pre and post-natal growth and also to the muscle fibers regeneraton after injury. These cells remain quiescent until external stimuli trigger them to re-entry into the cell cycle. During ageing there is a decrease in anti-oxidative capacity of skeletal muscle that results in an abnormal accumulation of ROS (reactive oxigen species) (Mecocci et al. 1999. Free Rad. Biol. Med., 26: 303-8; Fulle et al. 2004. Exp.Gerontol. 39: 17-24). The aim of this work is to verify if, in aged human muscles, the oxidative damage affects also satellite cell alterating their functional status. The obtained results show that: i) the activity of two main anti-oxidative enzymes, Catalase and Glutatione Transferase, is drastically reduced in satellite cells derived from elderly, compared to that observed in satellite cells from youngs; ii) the cell membrane fluidity is modified with the age; iii) the Ca2+-ATPase activity increases during ageing; iv) ^{3}H-Ryanodine binding does not modify; v) the basal [Ca2+]I levels, measured in resting conditions, in satellite cells increase significantly in an age-dependent manner. We hypothesize that the oxidative damage that occurs during ageing in skeletal muscle may also affect satellite cells probably modifying their functional status by Ca2+ homeostasis alteration.

ENDOGENOUS TROPONIN COMPLEX REPLACEMENT IN SINGLE SKELETAL AND CARDIAC MYOFIBRILS.

Belus A, Piroddi N, Scellini B, Tesi C, Poggesi C

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The replacement of native troponin complex (Tn) with exogenous Tn in single myofibrils is a powerful tool for the understanding of the regulation mechanism of striated muscle contraction (Piroddi & al., J Physiol. 2003, 552, 917-931). In rabbit psoas myofibrils we have studied the mechanical sequelae of the replacement of native fast skeletal Tn with cardiac

Tn or with chimeric Tn complexes containing different combinations of the C, T, and I subunit isoforms (fast skeletal, cardiac, and slow skeletal). The results show that Tn replacement in myofibrils by Tn of homogeneous origin strongly affects calcium sensitivity and cooperativity of force generation while maximal isometric force and apparent rates of force generation are unchanged. These results suggest that cross bridge kinetics and mechanics are basically set by the myosin isoform and are unaffected by Tn isoforms. We are now extending these replacement studies to cardiac myofibrils. The relative ease of Tn exchange even in human cardiac myofibrils means that replacement can be made in myofibrils using Tn forms associated with cardiac diseases to further investigate the role of regulatory proteins on cross-bridge properties in human cardiomyopathies. Supported by EU grant HPRN-CT-2000-0091.

MUSCLE TISSUE ENGINEERING USING SINGLE FIBRE ISOLATION TECHNIQUE: IN VITRO AND IN VIVO PROSPECTIVES

Boldrin L, Flaibani M(1), Malerba A(2), Slanzi E, Pozzobon M, Baroni D(2), Messina C, Zanesco L, Gamba PG, Vitiello L(2), Elvassore N(1), De Coppi P

Department of Paediatrics, Division of Paediatric Oncohaematology, (1) Department of Chemical Engineering, (2) Department of Biology, University of Padova, Interuniversity Institute of Myology, Italy. E-mail:

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Tissue engineering is a developing strategy to replace or repair large muscle defects and to improve the outcome of muscle dystrophies and others genetic deficiencies. In our work we have combined cell biology and polymer chemistry to create in vitro the most appropriate microenvironment for satellite cells in order to regenerate muscle tissue in vivo. Following this propose, we have cultured satellite cells using single muscle fibre technique. Flexor digitorum brevis (FDB) muscles of adult GFP positive mice were carefully removed. The single muscle fibres, obtained after enzymatic digestion, were plated in Petri dishes coated with matrigel. The satellite cells obtained from the fibres were expanded and seeded on to polymers. The polymers were made in PLGA (poly-glicolic acid), that dissolves progressively when inserted into the muscles, and, according to the chemistry, designed to align the cells and to create a well organized system to vehicle satellite cells in vivo. The polymers, with incorporated GFP positive cells, have been introduced in tibialis anterior muscles of isogenic wild type mice after mechanical damage. At 1, 2, 4, and 8 weeks the animals have been sacrificed and serial sections of treated muscles have been analyzed with immunofluorescence, RT-PCR and Western blot . We observed the in vivo activation of the transplanted satellite cells that were able to form fully differentiated muscle fibres. This approach could be used in the future in degenerative muscle diseases or/and in large muscle defects.

RECOVERY OF LONG-TERM DENERVATED MUSCLE BY FES

Boncompagni S, Kern H(1), Mayr W(2), Carraro U(3), Protasi F

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An innovative rehabilitation procedure, based on modulated and prolonged stimulation (FES) of long-term denervated and degenerated muscles (DDM), has been developed with the aim of reversing muscle atrophy in patients with lower motoneuron lesions. Using electron microscopy, we have studied human muscle fibers from the vastus lateralis in long-term DDM, both before and after FES. The results of our studies are surprising: myofibrils, that in DDM are completely missing, are restored and realigned with one another following FES training. In parallel, the excitation-contraction coupling apparatus is also reassembled and re-associated to the sarcomere I-A junctions after FES treatment. These structural studies are extremely encouraging since they demonstrate, contrary to common belief, that the severe degeneration that follows long-term denervation is reversible in humans.

LIMB-GIRDLE MUSCULAR DYSTROPHIES TYPE 2A AND 2B: CLINICAL AND RADIOLOGICAL ASPECTS

Borsato C, Padoan R, Stramare R(1), Fanin M, Angelini C

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The aims of this study were to investigate the morphologic changes, evaluated by MRI, which involve the muscles of patients affected by calpainopathy (LGMD2A) and dysferlinopathy (LGMD2B), and evaluate their correlation with muscle strength and muscle performance.

18 patients affected by LGMD2B and 10 patients affected by LGMD2A have been evaluated clinically and by MRI imaging. We also tested, in our patients, the muscle strength of 11 upper and lower limbs muscle groups by using MRC scale. Their muscular performance has been evaluated using the GSGCA scale (Gait, Stairs, Gowers, Chair, Arms functional tests). We have studied the skeletal muscles of upper and lower limbs by using MRI imaging (T1, T2, STIR sequences). The fibro-fatty replacement has been evaluated on T1 sequences by using a specific score. Myoedema has been evaluated on STIR sequences by using an edema score. We observed an inverse linear correlation between muscle strength (MRC scale) and functional muscular performance (GSGCA scale) in patients with LGMD2A and 2B. We, also, found a significant inverse linear correlation between the degree of fibro-fatty changes, on MRI, and the strength of the same muscles tested with MRC scale. A direct linear correlation was found between the degree of fibro-

fatty changes, on MRI, and the functional muscular performance, (GSGCA scale). The presence of myoedema was confirmed in upper and lower limbs of patients with LGMD2B and was described for the first time, also, in patients with LGMD2A. These findings have been evaluated on STIR sequences and, in both disorders, a peculiar pattern of myoedema distribution was found: the anterior compartment of lower limb was more involved than the posterior compartment in both dystrophies, the leg more than the thigh for LGMD2B, without significant difference between leg and thigh for LGMD2A. MRI imaging is useful to study LGMD, because the quantification of fibro-fatty replacement and myoedema, associated with the clinical examination resulted of prognostic value in disease progression.

SKELETAL MUSCLE OF MICE OVEREXPRESSING FRG1 SHOWS HYSTOLOGICAL AND FUNCTIONAL SIGNS OF MUSCULAR DYSTROPHY

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Gene over-expression has never been reported as cause of muscular dystrophy (MD). Recently, it has been proposed that expression levels of three genes (FRG1, FRG2 and ANT1) are involved in the pathogenesis of facioscapulohumeral muscular dystrophy (FSHD). We generated transgenic mice over-expressing FRG1, and selected three lines expressing FRG1 at different levels. Mice phenotype and the morphological and functional characteristics of skeletal muscles were studied in female mice from the 12th to the 30th week of age. At all ages, transgenic mice showed a clearly altered phenotype and impaired performance in running tests. Skeletal muscles had clear histological signs of MD. Analysis of specific force of isolated muscle fibers in vitro showed a significant deterioration of force generating capacity. The severity of all signs of MD was related to FRG1 expression levels, being almost absent in the mice with low FRG1 expression.

LONG-TERM LOWER-MOTONEURON DENERVATION OF HUMAN MUSCLE: A TIME COURSE STUDY

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We will show the results of our light microscopy study of long-term denervated human muscle (0.7 to 37 years). Main conclusions are: 1. Human skeletal muscle tissue undergoes three phases during long-term lower-motor neuron denervation: 1.1) Atrophy (up to 18 months post-injury); 1.2) Lipodystrophy (up to 15 year post-injury); 1.3) Fibrosis (after 15-year post-injury); 2. These three phases change over time at a much slower rate than in rodents; 3. Therefore, human skeletal myofibers survive lower motor neuron denervation much longer than generally accepted (years); 4. Cycles of myofiber death/regeneration persistently occur in permanent denervated human muscles and produce up to 37-year post-denervation a population of young throphic myofibers, which contribute to the Functional Electrical Stimulation-induced recovery of paretic human muscles after spinal cord injury.

A QUANTITATIVE STUDY ON REGULATORY MOLECULES INVOLVED IN THE POST-NATAL GROWTH OF PIG

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In vertebrates skeletal muscle originates from paraxial mesoderm which segments into somites on either side of the neural tube and the notochord. Muscle progenitors cells migrate from the somite to develop into *primary myotubes* due to the activation of a number of regulatory factors which are classified as myogenic determination factors, such as *Myf5* and *MyoD*, and myogenic differentiation factors such as *Myogenin* and *MRF4*. The same factors control other waves of myogenesis that take place as development proceeds, originating *secondary myotubes*. The presence of a third generation of myotubes has been described in the sheep, human and bovine during foetal life whereas in pig they appear shortly after birth and massively from the 3rd/4th weeks of post-natal life. Real Time-PCR experiments have shown that MRFs are all expressed post-natally but their levels decrease after the 1st week from birth. *Tertiary myotubes* might derive from satellite cells or more primitive cells, different from satellite cells, called side population cells (SP) since they are “set aside” during embryonic development and sequestered in mature tissue. Studying the positive and negative regulatory molecules involved in the post natal hyperplastic growth of pig have the purpose of discerning satellite cells from other stem cells and so to identify the source of new muscle fibres. Our results indicate the presence of adult stem cells around birth since the high expression of Pax 7 and CD34 genes, established markers of stem cells. The question about if new myoblasts derive from satellite cells or other myogenic stem cells such as SP or Sk-34 cells is still under investigation.

SARCOLEMMAL IONIC CONDUCTANCE IN CULTURED MUSCLE FIBRES OF MDX, COL VI KO, DY/DY AND WT MICE

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Several muscle dystrophies are due to mutations of proteins connecting cellular cytoskeleton and extracellular matrix: among these proteins are dystrophin, laminin and collagen VI. Little is known about the mechanism of development of cellular damage and eventually cell death. Altered ionic conductance of the sarcolemma, mechanical damage and disrupted signalling are among the hypotheses. Here we study membrane electrophysiological properties of muscle fibres of mdx (lacking dystrophin), Col6a-/- (lacking collagen VI), dy/dy (lacking laminin) and C57 (wild type) mouse strains. Adult muscle fibres were enzymatically dissociated and kept in culture for 2-3 weeks. Intracellular recordings with single electrode showed that resting membrane potential, membrane conductance and the parameters of the action potential were significantly altered in the different groups of muscle fibres. To identify which ionic currents are altered, double electrode voltage clamp experiments are in progress and currents running through the adhesion region are under investigation in muscle fibres plated on silicon dioxide microchips.

FAMILIAL IDIOPATHIC HYPERCKEMIA

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Persistent hyperCKemia of unknown cause in subjects with normal neurological examination has been defined Idiopathic HyperCKemia (IH). This condition has been included in the inherited muscular disorders on the basis of just one family reported. Thereafter, as a mutation in the caveolin-3 (CAV3) gene has been reported in another family and two sporadic cases, familial IH has been ascribed to CAV3 gene mutations. However, most cases reported are sporadic and the true frequency and features of familial IH remain undetermined.

In our Center, extending CK determination to family members of 32 subjects with IH, we identify 15 families for a total of 49 subjects (age: 1-80). About 10% of subjects complained mild myalgias and/or fatigue. Nobody had muscle weakness, including 6 subjects older than sixty. CK were 1.2-14 times the upper limit of normal (ULN) and were twofold the ULN in at least one member from each family. A male to male transmission was found in 9 families, with a significant prevalence of males with hyperCKemia (83%). Muscle biopsy of one member from each family was normal or disclosed heterogeneous patterns of changes in fibre size and distribution at morphometric examination. All biopsies were normal by currently available histochemical, immunohistochemical and biochemical investigations, including immunofluorescence for caveolin-3. In six families molecular genetics also excluded CAV3 gene mutations. Our findings suggest that: 1) IH is familial in half of cases; 2) CAV3 gene mutations accounts for only a minority of cases; 3) familial IH is benign, genetically heterogeneous, and, in at least 60% of cases, autosomal dominant with a higher penetrance in males.

MUSCLE INFECTION IN CHRONIC HEPATITIS B

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Myopathies have been reported in the course of some viral illnesses, including few cases associated with hepatitis B. However, as infection of muscle fibers has never convincingly demonstrated, the muscle is commonly considered resistant to viral infection, and the occurrence of these myopathies has been regarded as fortuitous or due to indirect immunological mechanisms.

We studied two patients with chronic hepatitis B and an asymptomatic or paucisymptomatic myopathy, whose cause had not been found in spite of careful investigations.

Muscle biopsy showed mild necrosis and scanty inflammation in both. Through extractive and in-situ polymerase-chain-reaction (PCR), immunohistochemistry, and immunoelectron microscopy, we found HBV DNA and antigens inside muscle fibers. These fibers were usually structurally intact and expressed major histocompatibility complex class I antigens (MHC-I), which is not expressed normally and make infected fibers a possible target for immune response. Actually, in mice models of DNA vaccination, intramuscular injection of HBsAg-encoding genes leads to muscle expression of HBsAg and immune-mediate necrosis.

We think our findings demonstrate that HBV can infect muscle fibers and was probably responsible for muscle injury in the patients we report. The possible direct involvement of the muscle in viral infections should be reconsidered.

FAMILIAL IDIOPATHIC HYPERCKEMIA: A POSSIBLE PREDISPOSITION TO STATIN-INDUCED MYOPATHY

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The asymptomatic and persistent creatine kinase (CK) elevation, when unexplained in spite of extensive ancillary investigations, is termed Idiopathic HyperCKemia (IH). IH is not rare, seems to not evolve into disabling disorders, and may occur as a familial condition with supposed autosomal dominant inheritance. Statins, the most effective cholesterol-lowering agents, may induce myopathy. We observed a 61-year-old woman, who developed myalgias, muscle weakness and hyperCKemia during treatment with pravastatin. Muscle biopsy revealed a mild necrotic non-inflammatory myopathy. Symptoms regressed after stopping treatment but hyperCKemia persisted . Other possible causes of hyperCKemia, including hypothyroidism, hypoparathyroidism, lipid and glycogen storage myopathies, and subclinical muscular dystrophies, were excluded. Investigation in two asymptomatic adult sons revealed persistent CK elevation, leading to a diagnosis of familial idiopathic hyperCKemia for both mother and sons. Statin-induced myopathy is rare in general population. Then we think that its occurrence in our patient is not coincidental but favoured by the unknown familial defect underlying Idiopathic HyperCKemia. Our findings support the hypothesis that statin-induced myopathy may develop in genetically predisposed individuals and suggest that routine CK investigation before starting therapy could help to identify patient at risk. Moreover, the case we report warns that drugs, and possibly other unknown factors, may induce muscle weakness in the virtually benign Idiopathic HyperCKemia.

MECHANICAL AND KINETIC PROPERTIES OF PURE ISOFORMS OF SKELETAL MYOSIN FRACTION S1 STUDIED BY A SINGLE MOLECULE MECHANICAL APPROACH

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Contractile properties of pure isoforms of skeletal myosin has been widely studied in different experimental conditions (Pellegrino et al. 2003, Weiss at al. 2001). Large variations of unloaded shortening velocity (Vo) were found when pure fibres containing different MHC isoforms were compared. Large variation were also observed when actin sliding velocity (Vf) on the same pure isoforms extracted were studied in in vitro motility assay. Finally kinetic properties in solution have been shown to be closely related to Vo and Vf. At molecular level, differences in Vf among isoforms can be determined by differences in the elementary step displacement (d) and/or in step duration (ton) according to the relation Vf~d/ton (Huxley, 1990). In this study we have developed a technique to directly measure and compare the molecular parameters d and ton among pure myosin skeletal isoforms. A dual optical tweezers assay was used and pure myosin samples were prepared by extraction from a pool of single fibres of identical myosin heavy chain isoform content. In order to exclude any cooperative effects of the two heads, the subfragment S1 from each pure myosin sample was prepared. The experiments were performed at 25°C, 50 mM ionic strength and at [MgATP] from 0.1 to 20 mM. Rat type 1 and mouse type 2B S1 were compared: d was 5.7±2.3 nm and 4.8 ±1.8 and stiffness (Km) was 0.38±0.23 and 0.9±0.35pN/nm respectively. The relation between ton and [MgATP] was best fit by a linear regression (r2=0.99) for the isoform 2B and by a Michaelis-Menten curve fit for the isoform 1.

SPHINGOSINE 1-PHOSPHATE INDUCES CYTOSKELETAL REORGANIZATION IN C2C12 MYOBLASTS

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Sphingosine 1-phosphate (S1P) is a bioactive lipid abundantly present in the serum, which mediates multiple biological responses, including cell adhesion, migration and differentiation. With the aim to extend our knowledge on the role played by S1P in the regulation of cytoskeletal reorganization native C2C12 myoblasts were stimulated with this sphingolipide. S1P treatment induced the appearance of actin stress fibres and focal adhesions. The actin dynamics during these complex morphological changes was also investigated in living C2C12 cells stably transfected with GFP-tagged α and β actins and observed by two-photon excitation fluorescence microscopy. The cytoskeletal response was dependent on the extracellular action of S1P through its specific surface receptors (S1PR), since the intracellular delivery of the sphingolipid by microinjection was unable to modify the actin cytoskeletal assembly. However, inhibition of Gi-protein mediated pathway by pretreatment with PTx did not affect the S1P-induced cytoskeletal reorganization. Experiments aimed to analyze the target molecules involved in the pathway linking S1PR activation to the cytoskeletal effects, showed that Rho/Rho kinase and PLD cascades were both required for stress fibre formation. In fact, defects in the S1P- induced formation of stress fibres were observed after pretreatment with the Rho kinase inhibitor, Y-27632, or in myoblasts in which PLD activity was reduced by the transient overexpression of a catalitically inactive PLD1 mutant (DN-PLD). Moreover, the treatment of with Y27632 of the cells overexpressing DN-PLD completely abrogated the S1P-induced cytoskeletal response.

CROSSBRIDGE PROPERTIES STUDIED BY FAST STRETCHES IN ACTIVATED FROG MUSCLE FIBRES

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During stretching of an activated muscle fibre, tension rises steeply to a point (critical tension, Pc) at which the resistance to elongation greatly diminishes and the tension either remains constant or falls to a lower level. At the same time of Pc, sarcomere elongation reaches a value (critical length, Lc), at which it increased steeply indicating a sudden fall in fibre stiffness caused by the forced crossbridge detachment. Stretching of a skeletal muscle beyond the critical length could provide information about the number and the properties of the attached crossbridges (Flitney and Hirst 1978a,b, J. Physiol. 276). This technique was extended here to dynamic conditions by using very fast stretches on activated single fibres from the tibialis anterior muscle of the frog (Rana esculenta). Stretches were applied at various tension levels during the tetanus rise and relaxation. Force was measured with a fast transducer and sarcomere length was measured using a striation follower device. During the tetanus rise Pc was directly proportional to the tension developed, suggesting a direct proportionality between the number of attached crossbridges and tension. Lc was instead constant throughout the rise. At high tensions during the isometric phase of relaxation Pc was proportional to tension but at lower levels, Pc was relatively smaller suggesting the presence of a smaller number of crossbridges developing a greater individual force compared to the rise. Interestingly during this phase, Lc decreased indicating a different crossbridge configuration. These preliminary data show that: 1) the number of crossbridges attached at any time can be estimate by measuring Pc, 2) Lc provides a measure of crossbridge extension.

MYOSIN ORIENTATION IN SKELETAL MUSCLE REVEALED BY X-RAY DIFFRACTION STUDIES DURING SARCOMERE LENGTH OSCILLATIONS

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Tilting lever arm hypothesis of force generation in skeletal muscle was tested here by using X-ray diffraction technique to measure the changes in intensity of the meridional X-ray reflection at 14.5 nm (IM3), resulting from elastic and active (power stroke) changes in S1 lever arm orientation. IM3 was investigated in tetanized fibre bundles from a toe muscle of Rana temporaria, mounted between a stretcher and a force transducer, and exposed simultaneously to X-rays (Elettra Synchrotron, Trieste, Italy) and sinusoidal length oscillations of various amplitudes at a frequency (2.8kHz) at which the myosin motor's power stroke is almost abolished. At low oscillation amplitude IM3 signal was roughly sinusoidal, but at high amplitudes it showed a characteristic double peak distortion similar to that previously observed under experimental conditions (Griffiths et al., 2002, PNAS 99) in which active S1 tilting was substantial. This means that the increased elastic tilting (due to the increased oscillation amplitude) can compensate the active tilting lost at high frequency and shows that effects of the two mechanisms on the lever tilting are additive. Simulation of the IM3 data with a model including the contribution from detached S1 in each actin-bound dimer and the dispersion of the attached heads, showed that the isometric orientation of myosin lever arm was similar to that previously determined at low frequency or high temperature where S1 tilting is strongly influenced by the power stroke movement.

ORGANIZATION OF THE SARCOPLASMIC RETICULUM DURING MUSCLE DIFFERENTIATION

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Differentiation of muscle cells is accompanied by synthesis and assembly of myofibrillar proteins and by the development and organization of the sarcoplasmic reticulum. We are interested to study the subcellular localization of ank1.5 a small muscle specific ankyrin localized on the sarcoplasmic reticulum. Since ank1.5 is capable of interacting with obscurin, a myofibrillar protein, these two proteins may mediate an interaction between the sarcoplasmic reticulum and the sarcomere. We have studied the expression and localization of ank1.5 and obscurin during development of skeletal muscle cells in comparison with that of a-actinin and of markers of the endo/sarcoplasmic reticulum. Our results show that obscurin starts to be detected with a striated pattern early in myofibrillogenesis, while ank1.5 displays a striated pattern only after myofibrils are completely organized. The relationship between ank1.5 staining and that of the endo/sarcoplasmic reticulum markers will be presented.

ROLE OF cAMP SIGNALLING IN MYOGENIC CELLS

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Differentiation of several myogenic cell lines is potently stimulated by vasopressin (AVP) via activation of PLC and PLD (J. Cell. Physiol. 171: 34, 1997). We reported that AVP promotes myogenesis in L6 myoblasts cultured in synthetic

medium through long lasting modifications of cAMP intracellular concentration (Mol. Biol. Cell 10: 4355, 1999). AVP decreases cAMP level and PKA activity by inducing a sustained increase in the activity of cAMP-specific phosphodiesterase (PDE4). Transfection experiments showed that overexpression of the PDE4D3 isoform both potentiated AVP-induced differentiation and unmasked the ability of IGF-1 to promote L6 differentiation in serum-free medium (Mol. Biol. Cell. 14: 1392, 2003). A complex role of the cAMP pathway in this model was suggested by further experiments showing that AVP induces a transient cAMP generation in L6 cells (J Biol Chem. 278:49308, 2003). We got evidence that the cAMP increase is due to activation of PLA2 with subsequent prostaglandins PGD2 and PGE2 generation. AVP-dependent early cAMP increase is effective in activating PKA and increasing phosphorylation of the transcription factor CREB. Inhibition of this pathway by prior incubation of the cells with the protein kinase A inhibitor H89 led to a significant reduction (about 65%) of AVP-stimulated differentiation. These results show that cAMP plays a key role in the control of myogenic differentiation, and that both an early activation and a later down-regulation of the cAMP pathway are required for AVP induction of myogenesis. Experiments performed to define the molecular mechanism involved in cAMP regulation of myogenesis show the existence of a 24 kDa protein specifically associated with myogenin in the cytoplasm of myogenic cells.

DYSTROPHIN-ASSOCIATED-GLYCOPROTEIN AND VINCULIN-TALIN-INTEGRIN-COMPLEXES DURING MYOGENESIS. TIMING APPEARANCE AND LOCALIZATION IN NORMAL HUMAN SKELETAL MUSCLE CULTURE

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The dystrophin-glycoprotein complex (DGC) and the vinculin-talin-integrin (VTI) anchorage system play an important role in sarcolemmal function and in the cell signalling. The integrity of the complexes is essential for the viability of muscle cells because their mutations has been reported to cause various forms of muscular dystrophies1-2. The relationship between DGC and VTI elements and time-course of their formation are still not known in detail. In order to test the timing appearence and the localization of some proteins of DGC and VTI complexes during the proliferation and the differentiation, we used a human muscle cells culture. As regard the VTI and DGC complex, in proliferative cells labelling for all proteins considered appeared as granular cluster mainly localized in the perinuclear region. In young myotubes (4 days) all proteins showed a very intence and granular labelling also seen in the whole cytoplasm. At day 7 the labelling of all proteins was strongest and was still seen in the cytoplasm. At 15 days the proteins distribution began to be placed on the membrane. At this time some myotubes displayed a significant degree of precostameric banding pattern. As fusion proceeded at 21 day, the cytodistribution progressively changed and appeared along fibrillar longitudinal structures and myotubes showed a clear periodic distribution (costameres) . In conclusion, in normal human muscle cultures DGC and VTI proteins are first localized to the perinuclear region, then diffuse in the cytoplasm and finally locate at the plasma membrane.

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TRANSFER OF PLASMID DNA AND OLIGONUCLEOTIDES INTO SKELETAL MUSCLE BY MEANS OF CATIONIC LIPID-BASED VECTORS

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The aim of our project is to develop a lipid-based gene delivery system capable of introducing therapeutic DNA or antisense morpholino oligonucleotides into skeletal muscle after systemic delivery. We have used both DODAC-based lipopoliplexes and SPLP (stabilized plasmid-lipid particles). We had already shown that intra-arterial delivery yielded good results in skeletal muscle and we have optimized our surgical technique to inject the lipid-DNA complexes in the femoral artery. Experiments carried out on adult rats in which muscle regeneration was chemically induced in the Tibialis Anterior muscles of the lower limb showed that SPLP containing the luc reporter gene yielded expression levels higher than 1 ng luciferase per mg of muscle extract, while a GFP-coding plasmid yielded approximately 10% of transfected fibers. Importantly, we also showed that such high levels are maintained for at least three weeks after a single administration without affecting myoblasts' viability and differentiation, both in vitro and in vivo. In our latest experiments we have transferred this protocol to a murine model, using C57BL6 animals. Somewhat surprisingly, SPLP yielded disappointing results in mice, with transfection levels 10 to 100-fold lower than in rat. Following a series of recent reports about exon-skipping therapeutic approaches, we have then applied our surgical protocol to the delivery of oligonucleotide-loaded lipoplexes to murine skeletal. Our initial results are quite encouraging, in that we have been able to restore the correct reading frame of the dystrophin mRNA in mdx mice as old as 10 months.

TRANSGENIC MOUSE MODELS OF MUSCLE WASTING AND REGENERATION

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In the last decade transgenic animals have been become a powerful and exciting research models to study the molecular mechanisms underlying the cellular and physiological processes, such as cell growth, differentiation, apoptosis. In the context of skeletal muscle, transgenic mice and gene-targeting approaches have led to the definition of specific roles for Muscle Regulatory Factors (MRFs) during embryogenesis, although less is known about the molecular mechanism underlying different muscle diseases and the regenerative pathways. Recent studies using specific models of transgenic mice have added new insights into the muscle wasting process, providing a baseline for designing appropriate strategies to attenuate or to reverse the cumulative effects of muscle diseases and to improve muscle regeneration. We generated transgenic mice in which an alternate isoform of IGF-1 (mIGF-1) exhibits sustained muscle hypertrophy, producing pronounced increases in muscle mass and strength with no undesirable side effects (Musarò et al., 2001). Expression of the mIGF-1 transgene safely enhanced and preserved muscle fiber integrity and improve muscle regeneration even at advanced ages (Musarò et al, 2001) and in dystrophic mdx mouse model (Barton et al, 2002). More recently, we demonstrated that the regenerative effect of mIGF-1 enhances the recruitment of stem cells into the site of muscle injury, improving muscle repair (Musarò et al, 2004). In addition, preliminary results suggest that mIGF-1 expression is able to counteract muscle decline and to extend the survival of motor neuron in a mouse model of ALS disease. Here we discuss the different model of transgenic mice that are useful to characterize the molecular markers of muscle wasting and regeneration. In particular, the use and generation of appropriate experimental models is an important determinant to develop therapeutic strategies to attenuate muscle wasting and improve muscle regeneration.

SPHINGOSINE 1-PHOSPHATE REGULATES MYOGENIC DIFFERENTIATION. A MAJOR ROLE FOR S1P2 RECEPTOR

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The adaptation of skeletal muscle involves muscle regeneration, a function mediated by the satellite cells which are mononucleated myoblasts.

Sphingosine 1-phosphate (S1P) is a sphingolipid metabolite that regulates a wide spectrum of biological processes. Initially regarded as intracellular messenger, S1P has been demonstrated to act as ligand of G-protein coupled receptors, called S1PR. We recently reported that S1P1, S1P2, S1P3 are expressed in C2C12 myoblasts and that S1P can trigger various key signalling pathways. Moreover, in myoblasts S1PRs appear to be subjected to changes in their expression during myogenic differentiation. In this study a novel biological activity of S1P, acting in myoblasts as an anti-proliferating and pro-differentiating agent, is reported.

In C2C12 cells S1P (1μM) was found to reduce serum-induced cell proliferation, to promote cell cycle exit and enhance the expression of myogenic differentiation markers such as myogenin, myosin heavy chain and caveolin-3. Manipulation of S1PR expression highlighted a key role of S1P2 in the biological action of the bioactive lipid. Notably, overexpression of S1P2 increased per se the content of myogenic markers and anticipated the onset of the differentiated phenotype. On the contrary, S1P1 appeared not to be involved in the myogenic program while S1P3 exerted a minor anti-differentiating action. Among the various signalling pathway, the activation of ERK1/ERK2 and p38 MAPK, both identified as downstream effector of S1P2, was found to be required for the inhibition of cell proliferation and the stimulation of myogenic differentiation respectively.

THE HETEROLOGOUS EXPRESSION OF SARCOGLYCANS

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Mutations in sarcoglycans cause autosomal-recessive limb-girdle muscular dystrophy. Sarcoglycans exist as a complex of four transmembrane proteins (a, b, g and d) within the major complex formed by dystrophin at cell membrane of striated muscles. We have recently shown that a-sarcoglycan is an ecto-ATPase, i.e. an enzyme able to hydrolyze extracellular ATP. The ecto-ATPase activity and assembly of the sarcoglycan complex was examined in heterologous expression systems. Individual or all four sarcoglycans were expressed in HEK 293 and COS-1 cells by using different expression vectors. Our results show that in COS-1 cells localisation to cell membrane of the sarcoglycans requires the contemporary expression of all four isoforms. In contrast, a-sarcoglycan can be expressed alone in HEK cells, while the other isoforms remain in the cytoplasm. Funded by TELETHON ITALY and NIH.

EFFECTS OF INHIBITORS OF CELL MEMBRANE CALCIUM CHANNELS ON HIGH-FREQUENCY FATIGUE OF FAST AND SLOW SKELETAL MUSCLES

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This work investigated the role of extracellular Ca2+ influx through cell membrane Ca2+ channels during high-frequency fatigue (HFF) in slow and fast skeletal muscles of mice. The study was performed in both innervated and in 14-day denervated soleus and EDL muscles of CD1 mice (3-month old). Stimulation in nominally Ca2+-free conditions caused a dramatic increase of fatigue in the slow-twitch soleus muscle, while in the presence of high Ca2+ levels (5 mM) fatigue was reduced. In the fast-twitch EDL muscle, HFF was not affected by external calcium levels either way. These results indicate that HFF of soleus muscle is sensitive to the entry of Ca2+. The possible involvement of store-operated Ca2+ channels (SOCs), mechano-sensitive or stretch-activated cation channels (SACs), L-type voltage-gated Ca2+ channels, and of P2X receptors in HFF development of soleus muscle was investigated by using specific inhibitors. Calciseptine, a specific antagonist of the a1C isoform of the DHPR, did not influence the development of HFF. Gadolinium, a blocker of both SACs and SOCs, and a mixture of P2X receptor inhibitors significantly reduced fatigability of soleus muscle. Interestingly, EDL muscle, which is insensitive to extracellular Ca2+ became sensitive after 14 days of denervation.

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FUNCTIONAL ELECTRICAL STIMULATION OF SKELETAL MUSCLE: CLINICAL RESULTS OF THE EU PROJECT RISE

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Prior clinical work showed that electrical stimulation therapy with exponential current is able to slow down atrophy and maintaining the muscle during non permanent flaccid paralysis. But exponential currents are not sufficient for long-term therapy of denervated degenerated muscles (DDM). We initiated a European research project investigating the rehabilitation strategies in human but also studying the underlying basic scientific knowledge of muscle regeneration from satellite cells or myoblast activity in animal experiments. In our prior study we were able to show that high intensity stimulation of denervated degenerated muscles is possible. At beginning of training only single muscle twitches can be elicited by biphasic pulses with durations of 120 – 150ms. Later tetanic contraction of the muscle with special stimulation parameters (pulse duration of 30 – 50ms, stimulation frequency of 16 – 25Hz, pulse amplitudes of up to 250mA) can improve the structural and metabolic state of the DDM. Since there are no nerve endings for conduction of stimuli large size anatomically shaped electrodes are used. This ensures an evenly contraction of the whole muscle. Contrary to the current clinical knowledge we were able to stimulate and train denervated muscle 15-20 years after denervation. The estimated amount of muscle fibers that have to be restored is about 2 – 4 million fibers in each m. quadriceps. To rebuild such a large number of muscle fibers it takes up to 3 – 4 years. Despite constant stimulation parameters and training protocols there is a high variation in the developed contraction force and fatigue resistance of the muscle during the first years of FES. Over the next years, the goals of the EU supported RISE project will be to identify, using experiments in animals and clinical research, safer stimulation protocols needed to induce early effects with, hopefully, a reduced stimulation burden for the patients. If all myofibers in the thigh could consistently attain endurance, the increased muscle mass and fatigue resistance will “rise” patients and their quality of life.

INVOLVEMENT OF PHOSPHATIDIC ACID IN ACTIN FIBER FORMATION IN DIFFERENTIATING L6 MYOBLASTS

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We had previously shown that phospholipase D (PLD) is involved in myogenic differentiation of cultured L6 myoblasts (Naro et al J Cell Physiol 171:34-42, 1997) and that stimulation by myogenic agents such as arginin-vasopressin or TPA is accompanied by the PLD-dependent formation of actin stress fiber-like structures (SFLS), which suggested that cytoskeletal rearrangements are involved in PLD myogenic effects. We thus set out to define more precisely the mechanism by which PLD modulates myogenesis. Immunocytofluorescence studies showed that myogenic stimulation induced the translocation of PLD1 from the Golgi apparatus to SFLS. We then used a fluorescent probe recognizing selectively the product of PLD, phosphatidic acid (PA), to monitor the spatiotemporal changes in PA concentration. This probe constituted by the PA-specific binding domain of Raf-1 fused to GFP allowed to observe the accumulation of PA at the level of nascent SFLS. These data strongly support the hypothesis of a direct involvement of PLD1 and PA in actin remodelling. Furthermore, we observed that myogenic stimulation induced a PLD-dependent neosynthesis of PIP2, and we showed by immunofluorescence that this compound was accumulated along SFLS. It seems thus likely that PLD and PA participate in actin rearrangements by

triggering a localized production of PIP2, the role of which as an effector of cytoskeleton is well documented. The pivotal role played by cytoskeleton remodelling in myogenesis is well recognized. Our results allow to propose the hypothesis that PLD1 is involved in this process through PA- and PIP2-dependent actin fiber formation.

MYOSIN HEAVY CHAIN ADULT ISOFORMS EXPRESSION IN DIFFERENT SKELETAL MUSCLES OF CATTLE

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Myosin heavy chain (MHC) isoforms of adult skeletal muscles are codified by four genes named: slow, or type 1, and fast types 2A, 2X and 2B. The slow, 2A and 2X isoforms have been found expressed in all mammalian species studied so far whereas there is a large inter-species variability in the expression of MHC-2B. Histochemistry experiments carried out with the use of specific monoclonal antibodies did not clarify if the histochemically "conventional" 2B fibres express 2B or 2X MHC; therefore, the use of RT-PCR was necessary to analyse the expression of MHC adult isoforms in cow muscles. RT-PCR experiments showed that three MHC isoforms (1, 2A, 2X) were expressed in trunk and limb muscles (*pectoralis*, *extensor carpi radialis*, *longissimus dorsi*, *retractor bulbi* and *rectus lateralis*) two (1 and 2A) were expressed in *diaphragm* whereas only type 1 was expressed in *masseter*. Slow or type 1 expression was confirmed using a specific antibody (BA-F8) whereas the detection of fast MHC isoforms were validate by means of BF-35 antibody although not by the SC-71 antibody. MHC-2B was present only in specialized eye muscles (*rectus lateralis* and *retractor bulbi*) whereas was absent in limb and trunk muscles as consistently showed by RT-PCR and reactivity with a specific antibody (BF-F3).

CHARACTERIZATION OF MYOGENIC FACTORS DERIVED FROM A STABLE MACROPHAGE CELL LINE

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The interplay between macrophages and muscle precursors, critical for myogenesis, is still poorly known. We have already reported that the murine macrophage cell line J774 can produce, in the absence of serum, a Macrophage-Conditioned Medium (MCM) that contains muscle specific growth factors. Here we show that MCM can strongly stimulate (up to 2 fold) the proliferation rate of primary rat myoblasts and pure rat satellite cells. Interestingly, in both cases cell division and cytoplasmic growth were partially uncoupled by MCM, with a loss of 30-35% of the mean cell mass respect to control. Primary rat myoblasts, fully inhibited to form myotubes when co-cultured with other cell types, could instead produce a very extensive network of contractile myotubes if exposed to MCM.

Some of the in vitro properties of MCM were also compared to those of purified myogenic factors. The presence of known myogenic factors in active MCM preparations is also under investigation. The potential usefulness of MCM for expanding myoblast populations will be discussed.

GENE EXPRESSION MODIFICATIONS IN RAT HEART FOLLOWING MODERATE PHYSICAL EXERCISE

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Clinical and epidemiological studies have demonstated that moderate physical exercise is beneficial for the prevention of cardiovascular diseases and other degenerative pathologies. However, the cellular and molecular mechanisms underlying such effects are still largely unknown. Variations in cardiac gene expression were studied in rats; moderate aerobic exercise of gradually increasing intensity was the model of choice in order to simulate what may be advisable for sedentary adults starting a maintenance training program. It was our purpose to detect, at the gene expression level, the early cardiac-related events following physical exercise, that might provide a molecular basis for the eventual onset of preconditioning. Sprague-Dawley rats aged 2 months run on a treadmill 3 times a week, one hour/day. Speed and distance were weekly increased in order to reach in 11 weeks a work load close to 60% VO2max, which was then maintained for further 3 wks. Matched siblings were sham exercised. Within 48 hrs from the last training session animals were sacrificed, hearts removed and RNA extracted. Gene expression analysis was carried out by means of Superarray® membranes. The immobilized 96 gene sequences belonged to the pathways of heating, oxidative, metabolic stress, proliferation/carcinogenesis, growth arrest/senescence, inflammation, DNA damage and repair, and apoptosis. Positive and negative controls were included. Results were validated by Real Time PCR. Plasma malonyldialdehyde concentration, an oxidative stress index, was determined as well. Results show that physical exercise, though mild and gradual, is nevertheless source of oxidative stress and stimulates inflammatory responses. At the same time, an increase in expression of a number of cardioprotective genes, such as heme oxygenase, shows that cardioprotective responses are stimulated as well. This double-edged mechanism may constitute the molecular basis for the eventual onset of exercise-induced cardioprotection.

STRESS GENE EXPRESSION IN SKELETAL MUSCLES AFTER MODERATE EXERCISE

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Excessive exercise may damage skeletal muscle cells, while regular physical training of moderate intensity may provide protection against cellular damage by inducing the synthesis of heat shock and antioxidant proteins. Evidence from the literature shows experimental systems whose reference models were highly endurance trained athetes rather than adults involved in a maintenance aerobic training. Thus, aim of the present study was to analyse the effect of moderate exercise on expression of genes that may be representative of stress-induced pathways. Sprague-Dawley rats 2-month old ran on a treadmill 3 times a week (1h/day). Speed and distance were weekly increased in order to reach a work load close to 60% VO_{2max}. Matched siblings were sham exercised. From the sacrificed animals, 3 skeletal muscles - plantaris, soleus and EDL from the hind legs - were analyzed, along with tongue, as a reference muscle not involved in training. The analysis was carried out by side-by-side hybridization on Superarray® membranes, where 96 stress and 6 negative or positive control gene sequences were immobilized. Genes belonged to the following pathways: proliferation/carcinogenesis, growth arrest/senescence, inflammation, oxidative and metabolic stress, heating stress, DNA damage and repair, and apoptosis signaling. As expected, no significative difference in gene expression in control vs. trained animals' tongue was found, whereas differences were found for all leg muscles. However, changes in gene expression followed a different pattern in the 3 muscles. In particular, soleus displayed signs of oxidative stress, whereas in EDL 2 inflammation genes were upregulated in trained rats. Plantaris displayed a mixed response, with less signs of damage. We conclude that exercise - though moderate - may deeply affect stress gene expression in the muscles and induce adaptive – and possibly protective – mechanisms.

EXTRACELLULAR NUCLEOTIDES AFFECT MYOGENESIS

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Extracellular nucleotides exert important biological actions in several cell systems. Signalling of extracellular nucleotides occurs through the activation of specific cell surface receptors. Two families of nucleotide receptors, P2X and P2Y, have been described which are associated to diverse signal transduction pathways. When activated, ionotropic P2X receptors (P2X1-7) form non selective channels permeable to Ca2+ and Na+, while metabotropic P2Y receptors are functionally coupled either to phospholipase C (P2Y1, 2, 4, 6, and 11) or to adenylate cyclase (P2Y12-14). The overall signalling action of nucleotides is under the control of ectonucleotidases (NTPDases), a family of enzymes that by degrading nucleotides terminate the signalling.

Growing evidence shows the occurrence of extracellular nucleotides signalling in skeletal muscle. In the present work, this signalling was characterized in differentiating C2C12 cells. Mechanical or electrical stimulation of C2C12 myotubes produced significant release of ATP, while it was without effects in myoblasts. The ecto-ATPase activity of cells progressively increased in parallel with differentiation. Consistently, the expression level of NTPDases (1, 2 and 3 and of a-sarcoglycan) also increased. Responsiveness to nucleotides changed dramatically during differentiation, consistent with substantial changes in the expression of P2X and P2Y receptors. Nucleotide appears to stimulate proliferation of myoblasts, while the prolonged exposure to and excessive levels of ATP seems to compromise the survival of myotubes. Funded by TELETHON ITALY.

TNF-α AND AVP EXERT OPPOSITE EFFECTS ON MUSCLE REGENERATION

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TNF-α inhibits skeletal muscle differentiation in vitro via NFkB-dependent down-regulation of the MRFs and via PW1-dependent caspase activation. PW1 is early expressed during myogenesis and induced in p53/c-myc-mediated apoptosis. Vasopressin (AVP) induces myogenesis in cell lines and primary cultures upregulating Myf-5 and myogenin.

With the aim to clarify in vivo the regulatory mechanisms of muscle regeneration we studied injured murine Tibialis anterior treated 2 and 4 days later with AVP, TNF-α and their combination.One week after injury, AVP, TNF-α or their combination induced an increase of inflammatory and myogenic cells, and smaller centronucleated fibers; however, TNF-α determined a robust reduction in the number of regenerating fibers. Two weeks after injury, the regeneration proceeded in all but the TNF-α -treated samples, where the regenerating fibers were smaller and less numerous; in contrast, muscle injected with either AVP or TNF-α + AVP showed abundant regenerating fibers. PW1 is expressed in both infiltrated cells and most of the nuclei of the regenerating myofibers. Since in vitro PW1 mediates TNF-α signaling by activating Bax-dependent caspases leading to inhibition of differentiation, we studied the effects of caspase inhibitors on muscle regeneration. A pan-caspase inhibitor injected alone or in combination with TNF-α positively modulated muscle regeneration totally abolishing TNF-α effects.Our data suggest that TNF-α negatively affects muscle regeneration by caspase activation. On

the other hand, AVP not only enhances muscle regeneration but also contrasts TNF-α effects. As already known in vitro, PW1 may mediate TNF-α -induced caspase activation in the regenerating muscle and AVP could act downstream of such activation.

CALCIUM COMPARTMENTS AND GENE EXPRESSION ACTIVATION IN L6 MYOGENIC CELLS

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Modulation of intracellular calcium concentration ($[Ca^{2+}]_i$) is an important regulative mechanism for the establishment of the muscular phenotype and for myofiber function. The variety of the biological responses regulated by Ca2+ suggest that the cell is capable to discriminate different Ca^{2+}signals. To investigate this issue we took advantage of a new experimental approach based on exposing L6 myogenic cells to high extracellular Ca^{2+} ($[Ca^{2+}]_o$) concentrations. Exposure of L6 transiently increases $[Ca^{2+}]_i$ through a selective increase of the conductance of membrane channels (DHPR) and ion exchangers (Na+/(Ca^{2+}) present in skeletal muscle cells (Naro et al., AJP, 2003). This method allows to study the effects of the increase of Ca^{2+} concentration in specific subcellular compartments. The obtained results (De Arcangelis et al., JCP, 2004) show that an increase of $[Ca^{2+}]_i$ due to the plasma membrane influx is capable to stimulate the hypertrophic growth of the myotubes. The $[Ca^{2+}]_i$–dependent hypetrophy was blocked by cyclosporin A, FK506, overexpression of a calcineurin-dominant negative protein, suggesting the involvement of the Ca^{2+} responsive phosphatase calcineurin. Furthermore, transient exposure of L6 cells to high $[Ca^{2+}]_o$ increased the expression of luciferase reporter driven by myoglobin and b-MHC promoters but not IIB-MHC and MCK promoters. Luciferase transcription driven by MCK promoter was, instead, enhanced by mobilizing Ca^{2+} from the intracellular stores. These data indicate that a transient increase of ($[Ca^{2+}]_i$ due to plasma-membrane influx is sufficient to induce a hypertrophic phenotype and an increased expression of slow-fiber genes but not fast-fiber genes.

PROTEIN AND MOLECULAR DIAGNOSIS IN LGMD2A

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Limb girdle muscular dystrophy type 2A (LGMD2A) is caused by mutations in the CAPN3 gene encoding for calpain-3, a muscle specific protease. The diagnosis of LGMD2A has recently shifted from molecular genetics towards biochemical assay of defective protein. However, an estimate of sensitivity and specificity of protein analysis remains to be established. Thus, we first correlated protein and molecular data in our large LGMD2A patient population. By a preliminary immunoblot screening of calpain-3 protein of 548 unclassified patients with various phenotypes (LGMD, myopathy, hyperCKemia), we selected 270 cases for CAPN3 gene mutation analysis: 83 had protein deficiency and 187 had normal expression. Mutation search was conducted using SSCP, DHPLC, ARMS-PCR and direct sequencing methods. We identified 70 LGMD2A mutant patients: 53 (76%) had variable degree of protein deficiency and 17 (24%) had normal calpain-3 amount. We calculated that the probability of having LGMD2A is very high when patients show a complete calpain-3 deficiency (84.4%) and progressively decreases with the amount of protein; this new data offers an important tool for genetic counseling when only protein data are available. A total of 47 different CAPN-3 gene mutations were detected, 22 of which are new. In our population, 86% of mutant alleles were concentrated in 7 exons (1,4,5,8,10,11,21). The prevalence of LGMD2A in North-Eastern Italy is 9.5 per million. In the Venetia district and in Friuli region it appears particularly frequent due to the recurrence of single mutations (a founder effect is likely). This study reports the largest collection of LGMD2A patients so far where both protein and gene mutation were obtained to draw genotype/protein/phenotype correlations and provide insights into critical protein domain.

INSIDE MUSCLE –TENDON UNIT BY SURFACE MECHANOMYOGRAM (MMG) AND FORCE SIGNAL

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The surface mechanomyogram (MMG) (detectable at the muscle surface by an accelerometer) is the summation of the single motor units (MUs) activity. Each MU contribution is related to the pressure waves generated by the active muscle fibres. During an isometric force ramp from 0 to 90% of the maximal voluntary contraction (MVC) of biceps brachii the analysis of MMG may provide informations on the MUs activation strategy. MMG amplitude Vs %MVC and MMG frequency content Vs %MVC relationships present, at near-maximal contraction levels, a clear decrement and a steeper increment, respectively. These behaviours may mirror the high global firing rate of the MUs at the end of recruitment with a fusion-like situation determining low pressure waves of the active MUs. In fatigued muscle and during 90%-0%MVC ramp the peak of MMG amplitude Vs %MVC relationship is not present or shifted according to the expected changes in the MUs activation strategy. During stimulation of the muscle at the motor nerve the force and MMG compared analysis provide data about the muscle tendon unit mechanics. When the stimulation frequency is linearly varied, in 10 s time, from 2

Hz to fusion frequency and back to 2 Hz the force/frequency and the MMG/frequency relationships present a counter-clockwise and a clockwise hysteresis, respectively. These results suggest that the force hysteresis could be due to the fact that muscle is kept shortened for longer time (see lower MMG because of the better fusion of the mechanical events) between one motor command and the following. When the amplitude of a 30 Hz stimulation train is sinusoidally varied, in the 0.4-6 Hz range, the amplitude and the phase of the force output at the tendon and of the MMG, detected by a laser distance sensor, behave as the response of a second order system with coincident poles. The different position of the poles may suggest that the different components of the muscle mechanical model may specifically affect the force or the MMG.

FIBRE TYPES, MHC ISOFORM EXPRESSION AND SINGLE FIBRE CONTRACTILE PROPERTIES IN FELINE SKELETAL MUSCLES

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Little information are available on contractile properties of feline muscle fibres. In this study we performed a comparative analysis of the mechanical parameters of maximally activated skinned single muscle fibres from two species of felids: the tiger and the cat, Fibres were dissected from temporalis, masseter, diaphragma, longissimus dorsi and soleus. In both species four MHC isoforms, expressed in skeletal muscles, were separated by gel electrophoresis and identified as slow or 1, 2A, 2X and 2M; this last was restricted to masticatory muscles. Soleus was composed of slow fibres in both species, diaphragma by slow and fast 2A , whereas both fast 2A and 2X fibres were present in longissimus dorsi. Fibres were grouped on the basis of their MHC isoform composition: maximum shortening velocity (Vo) and isometric tension (Po) were determined in each group of fibres. In both species Vo was lower in slow than in fast fibres and among fast fibres it was lower in fibres containing 2A myosin than in fibres containing 2X myosin. Vo of fibres containing 2M myosin was similar to that of 2X fibres.

THE I-KB-HOMOLOGUE CACTUS IS NECESSARY FOR NORMAL NEUROMUSCULAR FUNCTION IN DROSOPHILA MELANOGASTER

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The Drosophila I-□B homologue Cactus acts as an inhibitor of the Rel-transcription factor Dorsal. In blastoderm cells and immune competent cells, Cactus inhibits Dorsal by preventing its nuclear localization. Cactus and Dorsal are also expressed in somatic muscles, where they are enriched at the neuromuscular junction, and mutations in dorsal cause neuromuscular defects and miss-localization of Cactus. Here, we investigated whether mutations in Cactus affect the neuromuscular system and subcellular localization of Dorsal. Using locomotion assays, as well as physiological and immunochemical methods, we found that wild type Cactus is necessary for the normal function of the larval neuromuscular system. The phenotype caused by Cactus mutations includes i) altered bouton numbers and impaired neurotransmitter release in the neuromuscular junctions in the abdominal segments, ii) muscular weakness and iii) poor locomotion performance, probably reflecting a general neuromuscular impairment. Interestingly, the subcellular localization of Dorsal in muscle is not affected in cactus mutants, suggesting that in larval muscles the function of Cactus might be other than the cytoplasmic retention of Dorsal.

THE ROLE OF EXTRACELLULAR GTP ON DIFFERENTIATION OF C2C12 MYOBLASTS

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In our previous study we characterized the effect of extracellular guanosine 5' triphosphate (GTP) on Ca2+ homeostasis of mouse skeletal muscle cell line C2C12 via specific binding sites for GTP on plasma-membrane (Pietrangelo et al., JMRCM 23:107, 2002). GTP, 500μM, is able to increase [Ca2+]i and hyperpolarize the myoblats via intermediate Ca2+-activated K+ channels IK (Pietrangelo et al FENS, 2002). The differentiation of C2C12 cells is characterised by proliferation before the establishment of the postmitotic state (myogenin and p21 dependent, respectively) followed by the expression of myosin heavy chain (MHC) proteins and cell fusion (Andres V. and Walsh K., JCB 132:657, 1996). In this study we investigated the role of extracellular 500μM GTP during C2C12 differentiation. Our data indicate that the presence of GTP in the differentiation medium (DM) was able to accelerate mitoses and then increase the number of cells expressing MHC proteins. The incubation with both 200 nM

ChTX, a known blocker of IK channels, and 500μM GTP counteracts the myogenic GTP action. The cells differentiated in presence of 500 μM GTP for 4 and 24 hours reveal different up-regulated genes, as Pax7 and MyoD, and many others genes according to C2C12 gene expression profile during differentiation (Moran et al, JPhysGen 10:103, 2002). Moreover, the cells differentiated in presence of GTP were able to anticipate the physiological responsiveness to pharmacological agonists like nicotine or caffeine. Considering all together the data presented here, we suggest that the specific GTP-binding sites on C2C12 cell membranes is capable to accelerate the myogenesis via Ca2+-correlated transcriptional factors.

THE MOLECULAR MOTOR OF MUSCLE STUDIED BY X-RAY INTERFERENCE

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The regular repeat of myosin motors in each half of a bipolar filament produces an X-ray interference effect that allows their axial motions to be followed in an intact muscle fibre with a precision of about 1Å. We used this effect to measure the unitary working stroke of myosin motors *in situ*, as they pull the actin filaments towards the centre of the myosin filament during muscle shortening (Reconditi *et al*., *Nature*, 428, 578, 2004). The action of the motors was synchronised by superimposing force steps on the isometric contraction. At low load (0.25 times the isometric force) the average working stroke was 12 nm, consistent with crystallographic studies. The working stroke was smaller and slower at higher load.

CONTRACTILE PROPERTIES OF SINGLE MUSCLE FIBERS FROM NORMAL DOGS AND DOGS AFFECTED BY GOLDEN RETRIEVER MUSCULAR DYSTROPY

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Golden retriever muscular dystrophy (GRMD) is an inherited degenerative disease due to the absence of dystrophin and genetically homologous to human Duchenne muscular dystrophy (DMD). Studies on the function-structures relationship in DMD have been mostly carried out on the murine model of DMD, namely the mdx mice, and have provided contradictory results. However, dogs affected by GRMD display more pronounced clinical and pathological signs of MD and more evident similarities with the human DMD than mdx mice. It is therefore of interest to clarify whether in such a model the function of skeletal muscle fibers is significantly altered. Here (i)we identify and characterize the different types of muscle fibers of normal (WT) dogs, and (ii)we compare the contractile properties of single muscle fibers from WT and dogs affected by GRMD (GRMD). We have studied cross section area (CSA), specific force (Po/CSA) and unloaded shortening velocity (Vo) of individual muscle fibers. Fibers were chemically skinned, maximally activated at 12 °C and classified on the basis of myosin heavy chain (MHC) isoform composition. We have also extracted pure myosin isoforms from single muscle fibers and studied myosin function in a reconstituted contractile system in vitro, i.e. in vitro motility assay (IVMA). Analyses of fibers from WT dogs indicate that 3 types of pure muscle fibers can be found (type 1, 2A and 2X) and that they have distinctive functional properties. The preliminary analysis of single muscle fibres from GRMD suggests that functional parameters (Vo, Po/CSA and CSA) were not significantly different in GRMD and CTRL.

AN ENDOCRINOLOGICAL AND NEUROPSYCHOLOGICAL INVESTIGATION IN MYOTONIC DYSTROPHY TYPE 1 (DM1)

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We searched a correlation between neuropsychological tests results and GH-RH plus arginine stimulation response in a group of patients affected with Myotonic Dystrophy type 1 (DM1), a disorder due to multisystemic mRNA toxic mechanism. We studied a group of DM1 patients aged from 15 to 64 years. They underwent the following neuropsychological tests: Raven's Coloured Progressive Matrices 47; Wechsler Memory Scale; Rey's Complex Figure; Corsi's Blocks; Verbal and Phonemic Fluency Test; Stroop Test. We obtained neuroimaging data by SPECT, to find out any possible hypoperfused cerebral areas. Our patients underwent GH-RH plus arginine stimulation test and blood dosage of human growth hormone (H-GH) was evaluated at the following times after stimulation: -15, 0, 30, 60, 90 minutes. The results of the above tests were matched using statistical analysis. SPECT neuroimaging revealed hypoperfusion in several cortical areas, especially in left hemisphere. Neuropsychological testing revealed low performances in Rey's Complex Figure test, in Fluency tests and in Stroop test, with significantly prolonged time of execution. GH-RH plus arginine stimulation test revealed a partial H-GH secretion defect in about 30 % of patients. Statistical analysis did not show significant correlation between H-GH blood levels and Rey's Complex Figure, Fluency test, Corsi's blocks, Stroop-time test, Wechsler Memory Scale, PM47. Our endocrinological study detected low blood levels of H-GH to GH-RH plus arginine response in about 30 % of patients affected

with DM1. This study suggests to extend the analysis to a larger sample in order to establish for DM1 patients a possible replacement therapy by H-GH.

IDENTIFICATION AND FUNCTIONAL STUDIES OF MUTATION IN THE RYR1 GENE IN PATIENS WITH MALIGNANT HYPERTHERMIA

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MYOREGENERATION IN HUMAN DENERVATED DEGENERATED MUSCLE DECREASES AFTER MUSCLE RECOVERY INDUCED BY FES TRAINING

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We assess the extent of myofiber regeneration in denervated and degenerated muscle (DDM) during long-term lower motor neuron denervation, and their contribution to FES-induced muscle recovery. SCI subjects suffering complete conus cauda syndrome (CCCS) were biopsied from 0.7 to 37 years after lesion. CCCS subjects were submitted to FES at least after 3- to 10-year post-SCI, and biopsied after 1.3- to 9.4-year FES-training. Muscle biopsies from CCCS subjects show the characteristics of long-term denervation, that is, they present a few severely atrophic myofibers dispersed among adipocytes and loose or fibrous connective tissue. On the other hand, monoclonal antibody for embryonic myosin (anti-MHCemb) shows that many regenerative events continue to spontaneously occur in human long-term denervated muscle. Up to 19-year post SCI, about 4 percent of fibers are of recent regeneration. In the extreme case they represent 50% of the myofibers recognized by light microscopy. In the FES-trained muscle the cryosections consist of large round myofibers and a few regenerated fibers. Anti-MHCemb positive fibers were present in all the biopsies we studied, but10 myofibers per CCCS biopsy were stained by anti-MHCemb, while only 5 myofibers were recently regenerated in the muscle biopsy of FES-trained subjects. The structural studies confirm that the protocol used is effective in reverting long-term atrophy/lipodystrophy of CCCS muscles and in maintaining the trophic state of regenerated myofibers in the FES-trained muscles of DDM subjects.

REGULATION OF GLYCOGEN SYNTHASE BY SARCOPLASMIC RETICULUM-BOUND CaMKII IN RABBIT FAST-TWITCH SKELETAL MUSCLE

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Multifunctional Ca^{2+}-calmodulin (CaM) dependent protein kinase II (CaMKII) is a Ser/Thr protein kinase uniformly distributed within sarcoplasmic reticulum (SR). In fast-twitch skeletal muscle, triadin is the main junctional SR-specific substrate. On the other hand, the function of CaMKII bound to longitudinal SR (LSR) remains unknown. Previous electron microscopy data showed that glycogen particles associate with LSR. Therefore, we hypothesised that SR-bound CaMKII might participate in regulation of glycogen metabolism, during physiological conditions involving raising of intracellular Ca^{2+}. Here, we report that glycogen synthase (GS), and protein phosphatase 1 catalytic subunit (PP1c) bind to SR membranes isolated from fast-twitch muscles of adult rabbits, only when animals were injected intravenously with propranolol. GS and PP1c were substantially, but not completely, released from membranes, following either β-adrenergic-mediated glycogen breakdown *in vivo,* or treatment by amiloglucosidase *in vitro*, indicating the existence of a glycogen-independent, GS-targeting mechanism to SR, likely involving glycogen- and PP1c targeting subunit G_M. Microsomes were subfractionated by isopycnic sucrose-density centrifugation. Immunoblot analysis showed that GS, PP1c and G_M protein specifically associated to LSR. Phosphorylation experiments with [γ-^{32}P]ATP showed that GS was the main substrate of LSR-bound CaMKII, and that it underwent a phosphorylation-dephosphorylation cycle *in vitro*. By using a low G6P/high G6P assay for measuring GS activity, we found that pre-phosphorylation of GS by CaMKII significantly reduced fractional velocity. We suggest that activation of LSR-bound CaMKII regulates GS activity in response to the Ca^{2+} signals.

SPHINGOSINE 1-PHOSPHATE AFFECTS THE ELECTRIC PROP-ERTIES OF THE PLASMAMEMBRANE IN C2C12 MYOBLASTS

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The lipid mediator sphingosine 1-phosphate (S1P) is present in the se-rum in physiological concentration. It may act as an external agonist on specific S1P receptors of the plasmamembrane. One of its effect on myoblasts is to induce stress fibres formation. In accord, the aim of this work was to investigate

whether the stress fibres formation produced some mechanical perturbation of the plasmamembrane and in turn the Ca2+ influx through putative stretch-activated (SA) cation channels.

The mechanical stimulation by an atomic force microscope was able to increase the cytoplasmic Ca2+ concentration in almost all the C2C12 stretched cells that had been previously loaded with Fluo-3. To investi-gate the electric properties of the presumably expressed SA channels we used the patch clamp technique in whole-cell configuration. The transmembrane currents (Im) were recorded in voltage-clamp from C2C12 cells without or with S1P pretreatment. S1P treatment significantly in-creased Im amplitude whereas treatment with the SA channels blocker GdCl3, dramatically decreased the effect of S1P. Moreover, the treat-ment with dihydrocytochalasin B, that induces actin filaments depolymerization, strongly reduced the Im increase caused by S1P suggesting the involvement of S1P-mediated stress fibres formation in the modula-tion of SA channels. Thus, we conclude that S1P induces actin polymerisation and stress fibres formation, it increases the plasmamem-brane tension and finally favours the opening of SA channels. This in turn can enhance Ca2+ influx that may represent an alternative pathway by which actin remodelling may positively influence skeletal muscle development.

3D-CULTURE OF ISOLATED CELLS AND TISSUE EXPLANTS IN RELATIVE MICROGRAVITY: NEW PERSPECTIVES AND POSSIBLE APPLICATIONS

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The need of reliable *in vitro* models of human tissues alternative to the use of intact organisms is becoming a research priority in the actual scientific and industrial context. Traditional monolayer cultures of isolated cells present several limitations, mainly due to the poor cell viability and/or to the rapid loss of tissue-specific differentiated functions. It is well known how cyto-architecture and heterotypic cell interactions greatly influence the differentiation state and the metabolic behaviour of cell cultures. To overcome the limits of cell monolayers, several *in vitro* systems, such as 2D-co-cultures and 3D-models (i.e. cell aggregates, spheroids or tissue slices), were developed; nevertheless, none of them has actually reached optimal characteristics for allowing long-term preservation of tissue-like organisation and differentiated cell phenotypes. Since it has been recently proved that the conservation of original cell-cell and cell-extracellular matrix interactions is of pivotal importance for the maintenance of cell integrity and functions, we developed a new 3D-culture system based on the use of the Rotating Wall Vessel device (RWV, Syntecon). Operating at a very low shear regimen, this innovative technology allows the establishment of a particular microenvironment where hydrodynamic forces are strictly controlled (relative microgravity), thus providing a powerful tool for reproducing specific conditions for 3D-tissue morphogenesis. Examples of different human-derived 3D-culture models based on RWV technology (3D organotypic cell aggregates and long-term culture of tissue explants), their characterisation and testing, as well as their exploitation potential, will be illustrated.

MHC ISOFORM EXPRESSION IN BOVINE SINGLE FIBRES STUDIED AT RNA AND PROTEIN LEVEL

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Identification of MHC isoforms is generally based on separation on gel electrophoresis and on specific antibodies, which can be used in immunohistochemistry or in Western blot. Unfortunately the specificity of the antibodies is species-dependent and the migration of the MHC bands is also different from one species to another. A possible alternative to identify MHC isoforms is to combine electrophoretic separation and mRNA amplification in the same fibre, under the assumption that there is a correspondence between the protein and the messenger. In this study we examined MHC expression in bovine muscle fibres identifying in the same preparations mRNAs using RT-PCR and proteins using SDS-polyacrylamide gel electrophoresis. Single fibres dissected from diaphragm and small bundles dissected from eye muscles were analysed. The comparison lead to the reliable identification of the electrophoretic bands corresponding to MHC isoforms and showed that in most cases there was a complete correspondence between mRNA and protein composition.

HYALURONIC ACID BASED DRESSING AS ANTI-FIBROSIS AGENT IN THE TREATMENT OF MUSCLE INJURY

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Defects caused by traumatic or post-surgical loss of muscle mass may result in severe impairments of skeletal muscle functions. Muscle tissue loss often is substituted by scar tissue: the reparative process wins the competition with the regenerative events. Prompt treatment can minimize the degree of muscle fibrosis. We tested a hyaluronic acid based dressing (Hyalofill™) to avoid muscle fibrosis after trauma, and favouring muscle regeneration in an *in vivo* rat model. Hyalofill™ was used to fill the muscular defect following surgical muscle ablation of 2/3 of the tibialis anterior muscle in the right leg of 10 rats (group A). In group B (10 rats), we performed a crush injury of 2/3 of tibialis anterior muscle, observing the natural events of healing. After 21 days, the entire muscle was harvested, and we performed histological

muscle was harvested, and we performed histological analysis (haematoxylin-eosin stain) on serial cross-sections. Muscle dressed with Hyalofill™ showed a good recover in terms of tissue healing: we observed only few fibrosis, the muscle healed resembling features of a linear scar, and showing partial tissue regeneration. This strategy reduces considerably the amount of connective tissue adhesion permitting to myogenic cells to win the competition with fibroblasts during the wound healing process, and favouring the regenerative events as shown by histological analysis. This type of interactive biological dressing should also represent the first step to obtain complete muscle regeneration. The space filled with the biomaterial at the proper time, after its integration and before its complete degradation, could be an optimal site where to inject myoblasts to promote regeneration.

EFFECTS OF SPHINGOMYELIN DERIVATIVES ON INNERVATED AND DENERVATED RAT SOLEUS MUSCLE

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Present work is aimed at studying the effects of natural bioactive sphingomyelin derivatives, on normal and denervated slow-twitch skeletal muscle. The effects on fibre cross sectional area and myosin heavy chains composition of sphingosine (SPH), sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) were studied. Denervation was bilaterally performed cutting the sciatic nerve at the level of trochanter in adult rats. A group of animals was used as controls. Sphingolipids were continuously released by a mini-osmotic pumps implanted subcutaneously in the scapular region and connected by a catheter to the left (control or denervated) soleus muscles. Supplementation of SPC to adult control soleus muscle, produced significant atrophy of fibres and small changes to fibre type composition. No significant effects of SPH and S1P were found. In contrast, SPH and, to a smaller extent, S1P reduced the atrophy and the slow-to-fast transformation produced by 7-14 days of denervation. Preliminary results indicate that these sphingolipids may exert their action by reducing the overall muscle apoptosis and by activating satellite cells.

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