Myocardial dystrophin immunolocalization at sarcolemma and transverse tubules

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Abstract. Using monoclonal antibodies against two different regions of the helical rod part of dystrophin[1], we have localized dystrophin on both plasma membrane and transverse tubules in cardiac muscle of man and several animal species. The staining persisted after experimental ischaemia, and was observed in long-standing heart disease. No immunostaining was seen at the intercalated discs. In skeletal muscle the same two antibodies stained only the plasma membrane.
Key words. Dystrophin; transverse tubules; myocardium; ischaemia; muscular dystrophy.

The presence of dystrophin in the transverse tubules of skeletal muscle has been suspected from fractionation studies and immunoelectron microscopy[1-4]. Nevertheless, no such localization has yet been demonstrated by immunofluorescence. The immunostaining most often seen in both skeletal and cardiac muscle[2] is a continuous sharp line on the cytoplasmic side of the peripheral plasma membrane. Additional localizations have recently been reported at post-synaptic junctional folds and at the myotendinous junctions[5].

Dystrophin is as abundant in cardiac as in skeletal muscle[6], so the relatively mild mycardial involvement[7] in patients with Duchenne muscular dystrophy (DMD), who lack the protein, is surprising. However, electrocardiography often reveals sinus tachycardia, and also tachyarrhythmias, especially late potentials[8]. This indicates early changes in the conducting system, perhaps related to the elevated intracellular calcium concentration or to dystrophin loss in cholinergic receptors of the parasympathetic system.

The function of dystrophin is still unknown, but its localization and its structural resemblance to the spectrin and alpha actinin protein family[2] suggest that it has a cytoskeletal role. Indeed, a recent study reports decreased osmotic membrane stability of dystrophin-less myocytes[9].

Myocyte membrane integrity is known to be affected by ischaemia. Experimental work showed that 90 min of anoxia markedly diminished alpha actinin[10].

In this paper, we describe the immunohistochemical localization of dystrophin in normal and ischaemic myocardia.

Materials and methods

The animals used in our study were 3 open-chested minipigs in whom the left anterior descending coronary artery was tied for 2 h (biopsy samples of at risk and control areas were taken) as well as samples of rabbit myocardia left in cold saline for 0, 4, 12 and 20 h. In addition we studied explanted hearts of patients undergoing cardiac transplantation (6 with ischaemic heart disease and 4 with dilated cardiomyopathies) as well as 5 normal myocardia from cases of valve replacement. Human and pig skeletal muscle biopsies were examined as reference controls.

The monoclonal antibodies used here were prepared as previously described[5]. Briefly: a 4.3 Kb dystrophin cDNA fragment, designated cf23, was subcloned in an EcoRI site of pEX2 expression vector. A deletion derivative (pEX2 : cf23b) was used to express recombinant beta galactosidase fusion protein called LacZ-108 kD. This protein was partially purified and injected into Balb/c mice. Hybridomas were then generated and tested for antibody specificity with ELISA and immunohistochemistry. Antibodies to 2 epitopes on the helical rod domain of the dystrophin, MANDYS1 and MANDYS18, were used in the study described here.

The myocardial samples were quick-frozen and sectioned at 3–4 μm in a cryostat. On unfixed sections, the MANDYS1 antibody gave a strong fluorescence at a dilution of 1 : 50 after 1 h of incubation. The MANDYS18 was best in a dilution of 1 : 10; the human and rabbit tissues were weakly positive after 12-h incubation, and became strongly fluorescent after 24 h. In pigs, 2-h incubation was enough for a strong signal. The secondary antibody was a biotinylated sheep antimouse antibody, followed by streptavidin fluorescein (both from Amersham).

In controls, a monoclonal antibody against an autosomal homologue of dystrophin was used on serial sections of the same material. It bound only to microvessels. In additional sections the primary antibody was omitted, and no fluorescence was obtained at all.

Results and discussion

In skeletal muscle, the dystrophin immunofluorescence was peripheral only (with both MANDYS1 and MANDYS18 antibodies), without any intracellular staining (fig. 1).

In the cardiac myocytes, in addition to the peripheral staining, intracellular structures running mainly transversally were clearly visible (fig. 2). That these represent the transverse tubules was confirmed by immunostaining of laminin (unpublished data), which gave a

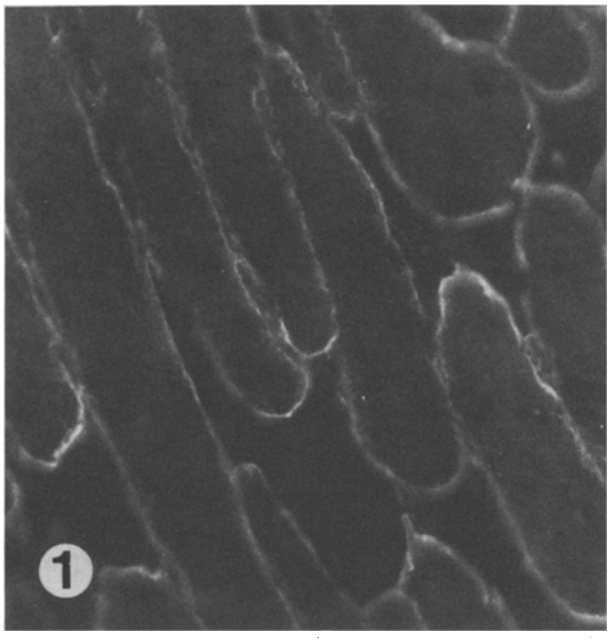

Figure 1. Skeletal muscle of pig cut tangentially, showing dystrophin immunofluorescence of the peripheral sarcolemma only. (MANDYS 18, × 320).

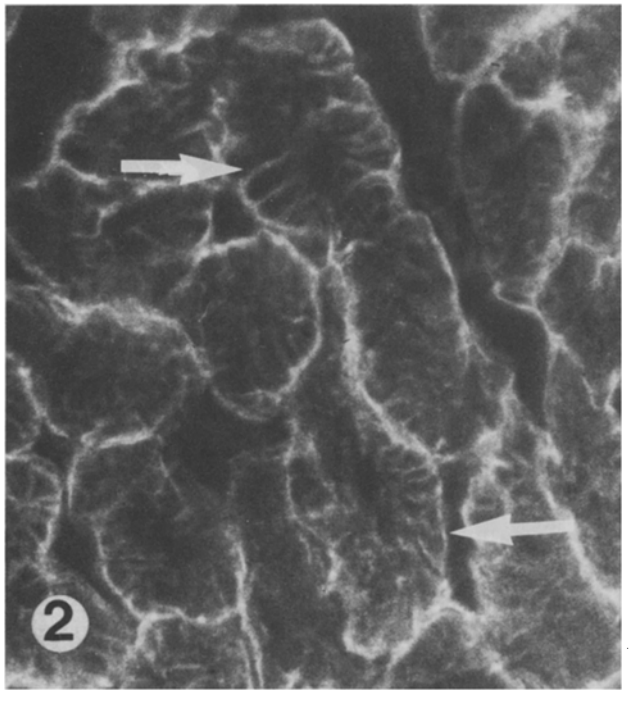

Figure 2. Myocardium of pig cut tangentially, showing transverse tubule staining (arrows) in addition to the peripheral staining, while the intercalated discs are not stained. (MANDYS 18, × 400).

similar picture. Anti-laminin antibodies stain the basement membrane on the outside of the transverse tubular plasma membrane, but the slight difference in the localization cannot be resolved by light microscopy. It is noteworthy that parts of the sarcolemma involved in the

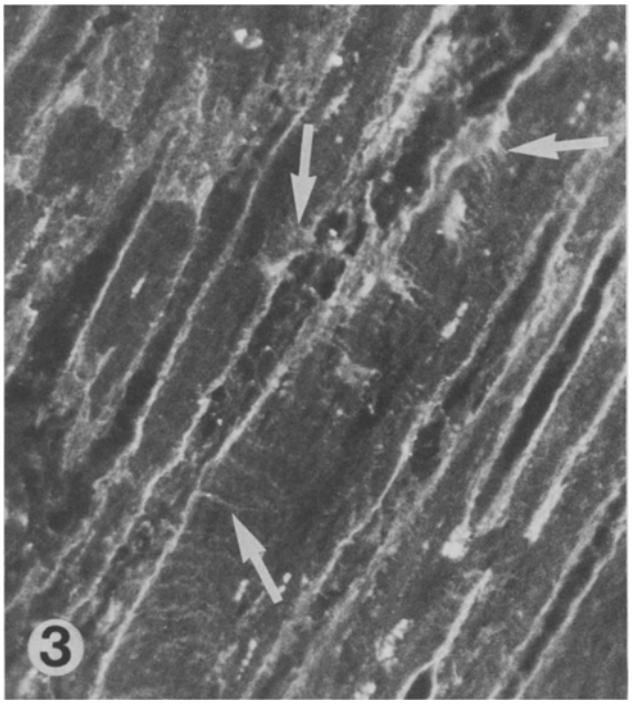

Figure 3. Explanted heart of patient with long-standing ischaemic heart disease. Myofibres cut longitudinally showing the same features as in fig. 2 (MANDYS 18, × 500).

formation of the intercalated discs were completely devoid of stain (anti-alpha actinin antibodies do stain these structures).

In all the species, whether in normal control states or in disease and ischaemia, the dystrophin showed a similar localization without obvious differences in the intensity of staining (fig. 3).

This resistance to ischaemic and perhaps other forms of injury distinguishes dystrophin from some other cytoskeletal proteins like alpha actinin, vinculin or tubulins which are lost or relocated under similar conditions [10]. Skeletal muscle destruction in DMD is thought to be a result of intracellular calcium elevation caused by calcium leakage through various channels [11, 12]. The excellent myocardial calcium homeostasis [13], and the difference in the localization of dystrophin in the transverse tubules, may help to explain the relatively limited myocardial damage in DMD. The voltage-gated calcium channels (1,4-dihydropyridine receptors), which are situated mainly in the transverse tubules, differ in cardiac and skeletal muscle [14]. In excitation-contraction coupling, cardiac muscle needs external calcium while skeletal muscle does not [14]. The very low external calcium entry in skeletal muscle is apparently controlled by the alpha-1 subunit of the receptor, which is specific for different muscle types. Dystrophin as well as the alpha subunit bind to a membrane glycoprotein [15]. If these three proteins interact to maintain low external calcium entry in skeletal but not cardiac muscle, it might explain the different effects of dystrophin absence in DMD on the two tissues.

The difference in the transverse tubular staining by anti-dystrophin antibodies in cardiac and skeletal muscle might be caused by a variation in antibody accessibility as a result of protein-protein interactions or a difference in dystrophin concentration. It has recently been reported that chimaeric myocytes with either cardiac or skeletal muscle calcium channel alpha-1 subunits are available [16]. It would be interesting to study the interactions of dystrophin with the two subunit proteins in order to further our understanding of the paucity of cardiac pathology in DMD.

The significance of the absent intercalated disc staining remains unclear.

To conclude, our study demonstrates the presence of dystrophin in cardiac myocyte sarcolemma and transverse tubules, but not the intercalated discs, and the resistance of this dystrophin localization to ischaemic injury.

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Insecticidal effects of essential oils. A study of the effects of essential oils extracted from eleven Greek aromatic plants on *Drosophila auraria*

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Abstract. Effects of the essential oils (EOs) extracted from eleven aromatic plants belonging to the Lamiaceae family (common in the Greek flora) were examined upon three different developmental stages of *Drosophila auraria*. All of the EOs examined exhibited insecticidal effects, either by preventing egg hatching, or by causing the death of larvae and adult flies. In several cases, malformation and/or prohibition of puparium formation was also observed.
Key words. Aromatic plants; essential oils; *Drosophila*; insecticides.

During the last decade, a growing body of evidence concerning the biological activity of plant-derived compounds has emerged [1, 2]. Among these compounds, essential oils (EOs) possess biological activity against prokaryotic and eukaryotic organisms (e.g., antibacterial, antifungal, insecticidal) [3, 4]. The necessity of finding safer insecticides has led to the exploitation of the mechanisms of chemical defense that plants naturally possess [5]. Recently it was shown that juvocymenes, the active ingredients of the EO of *Ocimmum basilicum*, and farnesol, a component of many EOs, exhibit insecticidal activity [5]. These compounds have a limited use on a commercial scale, but have been used as prototypes for the chemical synthesis of other, commercially available, insecticides [6, 7].

In the present study, eleven EOs, extracted from Greek aromatic plants have been tested for insecticidal activities. *Drosophila auraria* (eggs, larvae and adults) was used as an insect model system, in a controlled environment.

Materials and methods

Plant material and essential oil extraction. Using the Clevenger apparatus (constructed according to the specifications of the American Spice Trade Association) essential oils of eleven aromatic plants were extracted (table). The distillation yield (ml of EO per 100 g of dried plant material) as well as the major constituents of each EO are given in the table.

Animals. Drosophila auraria, an oriental member of the *montium* subgroup of the *melanogaster* species group [15],