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Expression of the dystrophin gene in cultured fibroblasts

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Summary: The dystrophin whose defect is responsible for Duchenne and Becker muscular dystrophies is present in muscle, brain and cerebellum. We describe here the detection of dystrophin in human cultured skin fibroblasts, L809 cells and murine 3T6 cell line. Dystrophin transcripts initiated at the muscle specific first exon can also be amplified by cDNA-PCR from various fibroblastic cells. The expression of the dystrophin gene in fibroblasts could account for some abnormalities observed in patient's fibroblast cultures.

Introduction: The gene which is altered in Duchenne and Becker muscular dystrophies (1,2), codes for several products. Three different promoters control the expression of a 14 kb mRNA (3) in muscle, brain and cerebellum, respectively. The muscular mRNA encodes a sarcolemmal 427 kDa protein (4). In non-muscle tissues, a smaller messenger was recently characterized (5,6,7) which contains a new alternative exon and exon 63 to 79 of the dystrophin messenger. This distal transcript encodes a 70-75 kDa protein of an unknown function (5,7,8).

Muscle necrosis is the major phenotypic effect of the dystrophin deficiency in Duchenne patients. However, other various abnormalities have been reported (9), including abnormal properties of cultured fibroblasts and lymphocyte capping deficiency. The biochemical defect underlying these observations is yet unknown.

In this brief report, we describe the detection of dystrophin gene products in human and murine fibroblast cultures.

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Materials and Methods

Fibroblast culture: Human skin fibroblast cultures were obtained as described in reference 10. Cells were cultured in Dulbecco's-modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum, 50 IU/ml penicillin, 50 mg/ml streptomycin and 200 mM L-glutamine. Cells were harvested 3 days after confluency in 40 mM Tris-HCl buffer pH 7.4, 1 mM EDTA, 150 mM NaCl.

cDNA-PCR: Reverse-transcription and PCR-amplification were performed on 500 ng total RNA as described in detail in reference 11. L-pyruvate kinase mRNA from rat liver was used as an internal standard.

Protein detection: Electrophoresis and western blotting were performed according to Nicholson *et al* (12) with some modifications. 1×10^7 fibroblasts were directly homogenized in 500 µl of buffer containing 75 mM Tris-HCl pH 6.8, 15% (wt/vol) SDS, 20% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol and 0.001% (wt/vol) bromophenol blue. Rat brain was homogenized with an Ultra-Turrax for 2x15 s in 10 volumes of the same buffer. Samples were placed in boiling water for 3 min, centrifuged 10 min at 10,000 x g and supernatants were loaded on 1.5 mm thick SDS-polyacrylamide gels using a 4%-10% (wt/vol) gradient resolving gels and 4.0% (wt/vol) stacking gel. After electrophoresis, proteins were transferred to a nitrocellulose filter. The membrane was then incubated for 1h with TBSA (5% (wt/vol) dried milk in a buffer TBST containing 10mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% (vol/vol) Tween 20). After 1h incubation with the primary monoclonal anti-dystrophin antibody, NCL-DYS1 (Novocastra) diluted at 1:600 in TBSA, blots were washed 4x15 min in TBST, incubated 45 min with the secondary antibody (peroxidase-labelled anti-mouse Ig diluted 1:2000 with TBSA) and washed 4x15 min in TBST. Chemiluminescent development was accomplished using ECL Western Blotting analysis system (Amersham).

Results

To investigate the presence of the dystrophin and distal mRNAs in various tissues, we amplified by cDNA-PCR a fragment of the dystrophin messenger corresponding to exon 62 and 63 (13) and a portion of the distal transcript corresponding to the specific exon (5) and exon 63. As expected, expression of the distal transcript was detected in brain, cultured skin fibroblasts, lymphoblastoid cells and was at the limit of detectability in skeletal muscle (Fig. 1). In contrast, dystrophin mRNA was present in brain and muscle but not in lymphoblastoid cells. Surprisingly, significant amounts of dystrophin transcript were detected in fibroblasts where it was previously reported to be only present in very low amounts corresponding to illegitimate transcription (14). Amplifications with pairs of primers amplifying fragments spanning

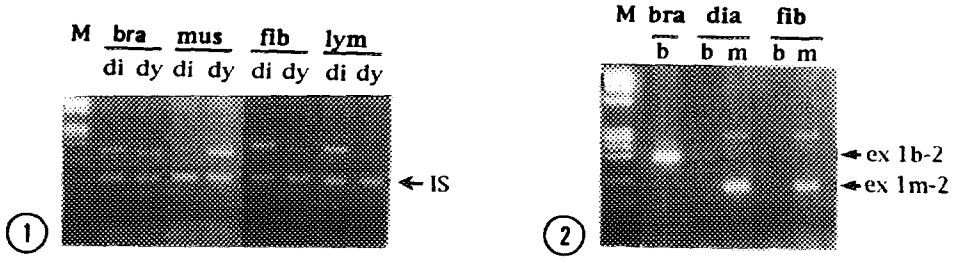

Figure 1. Detection of dystrophin and distal transcript. Ethidium bromide visualization of DNA fragments amplified by 30 PCR cycles and fractionated in an 2 % (wt/vol) agarose gel. The reactions were performed on human total RNAs extracted from brain (**bra**), skeletal muscle (**mus**), cultured skin fibroblasts (**fib**) and lymphoblastoid cells (**lym**). Part of the distal transcript (**di**) was amplified with a primer identical to a sequence of its specific exon (5'-GGCTCTGGGAAGCTCACTC-3') (5) and a complementary primer spanning nucleotides 9460-9480 of the dystrophin cDNA (14). This oligonucleotide was also used with an identical primer spanning nucleotides 9361-9381 of the dystrophin cDNA in order to amplify part of dystrophin mRNA (**dy**). IS: internal standard.

Figure 2. Initiation of the dystrophin transcript. DNA fragments obtained by 35 cycles of PCR amplification were fractionated on a 2% (wt/vol) agarose gel then stained with ethidium bromide. The reactions were performed on human total RNAs extracted from brain (**bra**), diaphragm (**dia**) and cultured skin fibroblasts (**fib**). Primer identical to a sequence of the brain-specific first exon (**b**) (5'-CTTTCAGGAAGATGACAGAAT-3') (15,16) and an oligonucleotide identical to a sequence of the muscle-specific first exon (**m**) (5'-ATGCTTTGGTGGGAAGAAGTAGAG-3') were used for amplification with a primer complementary to nucleotides 265-285 of the dystrophin cDNA. Arrows indicate the identity of the amplified products. Ex1b-2: fragment corresponding to brain-specific exon 1 - exon 2; Ex1m-2: fragment corresponding to muscular exon 1- exon 2.

exon 3 to 4, exon 48 to 49 and exon 62 to 79 were also successful, although with different efficiencies in different fibroblast cultures (data not shown).

The promoter which initiates the synthesis of the dystrophin mRNA in fibroblasts was determined by cDNA-PCR using a 5' primer placed in the muscle (15) or brain-specific (16,17) first exon and a 3' primer in the second common exon. Detection of an amplified product using the muscle-specific primer, but not the brain-specific primer (Fig. 2), indicates that the promoter used in muscular tissues, and not the brain-specific promoter, is responsible for the transcription observed in fibroblasts.

The splicing of exon 78, reported to be more or less tissue specific and generating dystrophins with different COOH termini (16, 18, 19), was also examined on dystrophin mRNA by cDNA-PCR. The results

indicate that both spliced and unspliced forms of transcript are present in fibroblasts cultures (data not shown).

Using a monoclonal antibody directed against amino-acids 1181-1388, dystrophin was detected on a western blot of proteins extracted from cultured skin fibroblasts (Fig. 3). However dystrophin was not detectable in all fibroblast samples (lane 5). Dystrophin was also detected in the murine 3T6 cell line. The greatest amount of protein was observed in L809 amniotic human cells where the quantity is similar to that detected in brain (compare lane 1 and 2). In contrast mouse LTK$^-$ cells and several amniotic cell cultures were negative for dystrophin expression (data not shown).

Discussion

The dystrophin protein and mRNA have been detected in various cultures of fibroblastic cells but in variable amount according to the culture. This variability could explain previous reports indicating that dystrophin mRNA was in very low amount in human fibroblasts (14). The reason of this variation remains unclear. Theoretical considerations predict that the huge dystrophin gene (2.3 Mb) would be unable to be transcribed in its entirety in rapidly proliferating cells because DNA replication is expected to interfere with the prolonged transcriptional process and to stop it prematurely (20). However, we observed only small variations of dystrophin mRNA amounts between proliferating and confluent L809 cells (data not shown). The hypothesis that this variability of dystrophin expression is related to the culture passage number, therefore to an *ex vivo* aging process, is under investigation.

The promoter responsible for this transcription is the so-called muscle-specific promoter. This observation is in agreement with our previous data showing that various fragments of this promoter exhibit some transcriptional activity when transfected into fibroblasts (21). In addition, transcripts synthesized under the control of this promoter

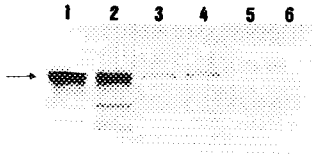

Figure 3. Western blot of protein extracted from: brain rat (1), L809 ammiotic human cells (2), human cultured skin fibroblasts (3,4,5), cultured skin fibroblasts from a Duchenne muscular dystrophy patient (6) . The blot was probed with the anti-dystrophin monoclonal antibody NCL-DYS1 directed against aa 1181-1388.

have also been described in glial cells (22,23), emphasizing the fact that the muscular promoter is not strictly muscle-specific and is more or less leaky in some non-muscular tissues. It can be proposed that *in vivo* specificity of dystrophin gene expression is increased by its size, requiring that cells actively transcribing the gene are not actively proliferating, as explained above and as demonstrated for the Drosophila Ubx gene (20). Indeed, active transcription of the dystrophin gene is only observed in post-mitotic cells, neurons and myotubes.

Fibroblasts from patients with Duchenne muscular dystrophy exhibit abnormal properties *in vitro*: decrease of intercellular adhesiveness (24), increased rate of cell-substratum detachment (25), abnormal spreading (26) and growth kinetics (27), alteration of protein synthesis (28) and of some enzymatic activities (27, 29). The biochemical defect responsible for these abnormalities is unknown. As dystrophin and the 70-75 kDa protein (data not shown) have been detected in fibroblasts, both are good candidates for accounting for these cell disorders. However, the frequency and pattern of deletions observed in Duchenne muscular dystrophy patients predict that the expression of the 70-75 kDa protein would not be affected in the vast majority of them. Thus, it seems more likely that a defect of dystrophin itself or other products of the huge dystrophin gene is involved in the generation of these abnormalities.

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