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Is the MLC1/3 promoter / enhancer the right system to generate skeletal muscle specific transgenic animals?

Research Article

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Summary

To document the role of iNOS expression for skeletal muscle alterations as observed in patients with chronic heart failure, the aim of this study was to generate skeletal muscle specific iNOS transgenic animals. The complete coding cDNA was inserted into a vector using the MLC-1/3 promoter/enhancer, which was used for pronuclear microinjection into 223 oocytes. PCR and Southern blot analysis of tail biopsies of 37 founder animals demonstrated that none was positive for the transgene. RT-PCR analysis of bovine oocytes for the expression of myosin light chain-1 revealed a positive amplification product. These results demonstrate for the first time that MLC-1 is already expressed in oocytes thus preventing the generation of transgenic animals using the MLC-1/3 promoter in conjunction with a toxic gene like iNOS.

I. Introduction

Chronic heart failure is not only associated with increased mortality and morbidity but also with exercise intolerance and early fatigue. Recently, we and others were able to demonstrate an increased expression of inducible nitric oxide synthase (iNOS) in skeletal muscle biopsies obtained from patients with chronic heart failure as compared to healthy controls (Adams et al, 1997; Riede et al, 1998). The additional observation that the expression of iNOS correlated inverse with the exercise capacity of these patients (Hambrecht et al, 1999) and the knowledge that the inducible isoform of the nitric oxide synthases generates, once expressed, pathophysiological concentrations of nitric oxide (NO) (Förstermann et al, 1994) implies a relationship between iNOS expression and exercise capacity. To answer the question whether there is a causal relationship between exercise intolerance and iNOS expression in skeletal muscle the generation of a skeletal muscle specific iNOS transgenic animal would be helpful.

To generate skeletal muscle specific expression of the transgene, different promoters like creatine kinase (Manchester et al, 1996) or the myosin light chain (Tsao et al, 1997; Hughes et al, 1999) have been used. Two skeletal muscle myosin light chains, MLC1 and MLC3, are generated from a single gene by transcription from two different promoters and alternate splicing of the pre-mRNA. The myosin light chain 1/3 promoter/enhancer, that drives gene expression in differentiated fast-twitch muscle fibers (Donoghue et al, 1988), was recently used to express insulin like growth factor-1 (IGF-1) in older animals. Direct injection of the MLC1/3-IGF construct into differentiated muscle fibers clearly reduced aging related muscle changes and resulted in increased muscle strength (Barton-Davis et al, 1998). Therefore, the aim of this study was the use of the MLC 1/3 promoter/enhancer to generate skeletal muscle specific iNOS transgenic rats.

II. Materials and methods

A. Generation of the MLC 1/3 iNOS construct

Figure 1 shows a schematic drawing of the iNOS construct. The full-length coding sequence of iNOS was generated by RT-PCR using iNOS-specific primers containing restriction sites for Sal-I or Hind-III (Nos-Sal: 5´-ACGCGTCGACCAGC AGAGTTGGTGCAGAAGCACAAA GTCACAGACATGGCTTGCCCCTGGAAGTTTC-3´; Nos-Hind: 5´-CCCAAGCTTTCAG AGTCTTGTGCCTTTG GGCTC-3´) and a plasmid containing the rat iNOS cDNA (generous gift of Dr. Karlsen, Steno Diabetes Center, Gentofte, Denmark). The PCR product was restriction digested with Sal-I followed by Hind-III, gel purified and cloned into the Sal-1/Hind-III digested and purified pMEX-NMCS2 vector containing the MLC1-promoter and the enhancer element (generous gift of Dr. Craig Neville, Cardiovascular Research Center, Charlestown, MA, USA). The iNOS sequence in the pMEX-NMCS2 was confirmed by sequence analysis using an automated sequencing system (Licor, Lincoln NE, USA).

B. Generation of transgenic animals

The expression construct was excised from the iNOS carrying pMEX-NMCS2 vector by Not-1, gel-purified, and used for pronuclear microinjection of fertilized oocytes from Sprague-Dawley (SD) rats to produce transgenic rats according to the procedure described by Mullins at al. (1990). The identification of transgenic animals in the offspring was achieved by iNOS specific PCR and by Southern blotting with Bam-H1 digested total DNA from tail biopsies (15µg). A 916 bp long ^{32}P-labeled DNA fragment generated by PCR (NOS15 and NOS16 primer [Adams et al, 1998]) was used as probe.

C. Myosin-light chain RT-PCR of Oocyte cDNA

RNA was isolated from bovine oocytes following the acid guanidinium thiocyanate-phenol-chloroform method described by Chomzynski and Sacchi (1987). One µg of total RNA was reverse transcribed into cDNA using random hexamer primers (Roche, Mannheim, Germany) and 200 U of reverse transcriptase (SuperScript II, Gibco BRL, Bethseda, USA). The reverse transcription (RT) was carried out in a total volume of 20 µl according the manufacturer's recommendations. To specifically amplify myosin light chain the following primers were used: MLC1-U, 5´-AGGATGAA TTCAAGGAGGCG-3´; MLC1-L, 5´-CTGGATCAGCAGACACTTAG-3´ and the amplification product was separated on an agarose gel and visualized by ethidium bromide staining.

III. Results

A. Generation of transgenic animals

The linearized construct has been injected in 223 oocytes. These manipulated cells were transferred in 9 pseudopregnant foster rats. Thirty-seven pubs resulting from 5 litters have been born. None of the newborns died after birth. All litter mice survived until weaning and juvenile rats have been kept for one additional month to genotype the animals by PCR and Southern blot.

B. Genotyping of possible transgenic offspring

Using PCR technique non of the 37 rats after weaning have been positive whereas the injected fragment as a positive probe gave a signal of expected length (**Data not shown**). To verify the negative PCR results, southern blot analysis has been performed (**Figure 2**). Non of the digested genomic tail DNA samples gave a positive hybridization signal of the expected length. However, the equally digested fragment used for the oocyte manipulations showed a strong signal illustrating that the southern itself worked out. To support the assumption that the failure to receive transgenic founder animals was not due to technical problems other constructs were used to generate transgenic animals. As shown in **Table 1** the general efficiency to obtain positive founders for these other constructs was in the range of 10 to 15%.

C. Detection of MLC1 expression in oocytes

To verify the speculation that the failure to generate iNOS transgenic animals was due to the activity of the MLC1 promoter in oocytes an RT-PCR for MLC1 was performed from oocyte RNA. As depicted in **Figure 3** a clear amplification product could be seen using oocyte RNA. Additionally, a mature bovine skeletal muscle was used to amplify MLC1 as a positive control.

Figure 1: Schematic drawing of the MLC1-iNOS construct. Rat iNOS cDNA was driven by a fast muscle specific MLC 1/3 promoter/enhancer and stabilized by simiam virus 40 polyadenylation sequences (SV40pA).

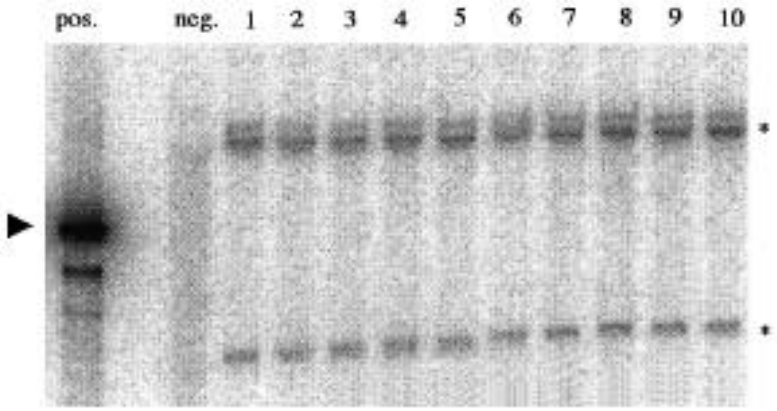

Figure 2: Southern blot analysis of BamHI-digested genomic DNA of founder animals (lane 1-10) as well as of a negative and positive control. The positive hybridization signal is marked with an arrow.

Table 1: The general efficiency of transgenic animal generation in the animal facilities of the MDC, Berlin-Buch, illustrated by two examples in relation to the MLC1-iNOS injection approach. (PMCA- plasma membrane calmodulin–dependent calcium ATPase; AOGEN-AS- angiotensinogen antisense)

Promoter	Gene	Founder all	Founder posit.	Reference
MLC2	PMCA 4	27	4 (14.8%)	Hammes et al.
	AOGEN-AS	26	3 (11.5%)	Schinke et al.
MLC1	iNOS	37	0	

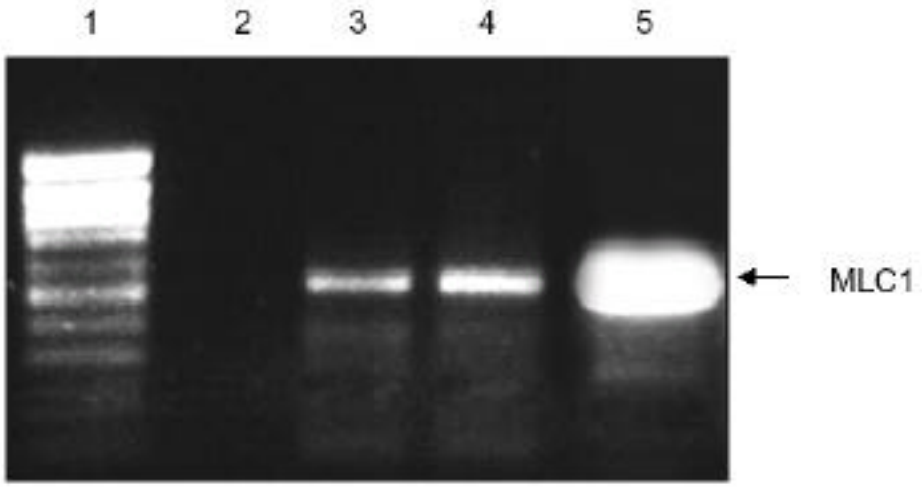

Figure 3: MLC1 RT-PCR of bovine oocytes and differentiated skeletal muscle. RT-PCR for MLC was performed and aliquots were separated on a 1% agarose gel and stained with ethidium bromide. Lane 1: 100 bp molecular size marker; lane 2: negative PCR control; lane 3 and 4: RT-PCR of different oocyte cDNA preparations; lane 5: RT-PCR of differentiated skeletal muscle.

IV. Discussion

The attempt to generate transgenic animals which specifically overexpress the inducible isoform of the nitric oxide synthase (iNOS) would be a helpful model to analyze the contribution of iNOS overexpression in skeletal muscle of patients with chronic heart failure to reduced exercise capacity. Two important findings emerge from this study: 1) the MLC 1/3 promoter/enhancer is not suitable to generate skeletal muscle specific transgenic animals in the case of a toxic transgene like iNOS, 2) the MLC 1 promoter is already active in oocytes. These be useful for the generation of transgenic animals using non-toxic gene products, but is not suitable to generate transgenic animals overexpressing iNOS specifically in the skeletal muscle.

Transgenic animals are valuable tools in understanding the contribution of distinct genes for the pathophysiology of different diseases (Chien, 1996). In the case of iNOS two different transgenic animals were reported in the literature (Takamura et al, 1998; Mungrue et al, 2002), one mouse line expressing iNOS specifically in pancreatic islet cells and one expressing iNOS only in cardiomyocytes. Both iNOS transgenic animal lines

resulting increase of nitric oxide is associated with the death of the respective cells.

V. Generation of transgenic animals with the MLC 1/3 promoter/enhancer

Two skeletal muscle myosin light chains, MLC1 and MLC3, are generated from a single gene by transcription from two different promoters and alternate splicing of the pre-mRNA. A 0.9-kb enhancer element downstream of the MLC1 promoter dramatically enhances the expression in differentiated myotubes but not in undifferentiated myoblast or nonmuscle cells (Powers & Lennon, 1999). Therefore the combination of the MLC1 promoter and the specific enhancer element would be a helpful tool to specifically express genes in differentiated skeletal muscle. An IGF-1 construct containing the MLC1/3 promoter/enhancer was recently used in viral mediated transfection (Barton-Davis et al, 1998). An increased expression of IGF-1 was detectable up to 9 month after viral transfection resulting in an increased cross sectional area of the muscle as well as an increased force generation. Furthermore the MLC1/3 promoter/enhancer construct was successfully used to generate myogenin transgenic mice (Hughes et al, 1999). As reported here the utilization of the MLC1/3 promoter/enhancer to specifically overexpress iNOS in the skeletal muscle of transgenic animals failed, because all 37 founder animals were transgene negative. This failure is possibly not due to technical problems during the injection into the fertilized oocytes, because using other constructs positive founder animals were generated. What may be the reason for this lack of iNOS transgene positive animals? One possible explanation would be that iNOS, which generates once active cytotoxic amounts of nitric oxide (Moncada et al, 1991; Förstermann et al, 1994), is already actively transcribed in the oocyte, thereby generating cytotoxic amounts of nitric oxide which destroys the cell. This would imply that the MLC-1/3 promoter is already active in oocytes. Up to now nothing is known about the expression pattern of MLC-1 in oocytes. Nevertheless, the assumption that MLC-1/3 promoter is already active in oocytes is supported by our PCR results demonstrating a clear positive amplification product of MLC-1 in bovine oocytes. Therefore one may conclude that the positively transfected oocytes die due to an increased expression of iNOS and only the negatively transfected cells are transplanted into the pseudopregnant rat, resulting in transgene negative founder animals. Taken together these results imply that the MLC1/3 promoter/enhancer is helpful to generate skeletal muscle specific transgenic animals if the transgene does not have toxic effects in the oocytes.

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