Decreased mAKAP, ryanodine receptor, and SERCA2a gene expression in mdx hearts

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【 学位論文題目 】

Decreased mAKAP, Ryanodine Receptor, and SERCA 2a Gene Expression in mdx Hearts
（mAKAP, リアノジン受容体およびＳＥＲＣＡ2a 遺伝子の発現は mdxマウスの心臓において減少を認める）

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INTRODUCTION

The mdx mouse has been the most widely utilized mouse model of DMD, an inherited X-linked neuromuscular disorder that leads to severe skeletal muscle wasting followed by premature death in early adulthood. This model carries a loss of function mutation in dystrophin gene. A previous study showed that mdx mice have profound alterations in contractile properties. How dystophin deficiency affects contractility remains poorly elucidated. We investigated the different gene expressions between the 5-month mdx and control hearts since several studies have suggested that the old mdx might be a better model for DMD-associated cardiac disorders.

METHODS

Suppressive subtractive hybridization and cDNA cloning and screening - Reverse transcription of Poly(A)$^+$ RNA and generation of subtracted libraries were performed using the PCR select cDNA subtraction kit (Clontech) according to the manufacturer s protocol. For arraying subtracted clones, identical blots were prepared using the PCR-Select differential Screening kit (Clontech). Three hundred (300) randomly selected white bacterial colonies of the subtracted cDNA libraries were screened using dot blot analysis.

Northern blot analysis - Total RNA from control and mdx- or hypertrophied hearts were resolved on 1.2% agarose formaldehyde gels. PCR products from inserts of selected clones were used as probes and randomly labeled with [P^{32}] dCTP. The hybridization signals were normalized to a housekeeping gene, GAPDH, to compensate for unequal loading.

3 and 5 RACE, Reverse transcription (RT)-PCR, probe preparation and sequencing - To identify the unkown clone, the cDNA were extended by 3 and 5 RACE using the specific primer and confirmed by RT-PCR. The probes for ryanodine receptor, SERCA2a, FKBP12.6, phospholamban and PDE4D3 were designed based on sequences available in public databases (NCBI). All of the expected PCR products were subcloned and sequenced for confirmation.

Cultured cardiomyocytes - Cultured cardiomyocytes were prepared from 2-3 day old neonatal rat hearts at 4.5 x 10^6 cells per 10 cm dish as described. 100 μM phenylephrine or 10^{-7} M endothelin-1 (ET-1) was added in order to induce a hypertrophic response.

RESULTS

Identification of differentially expressed genes in 5-month mdx hearts - Screening of 290 inserts by dot blot analysis revealed that 30 clones hybridized with control-specific probe but not with mdx-specific probes. The sequence analysis of 30 clones showed that they corresponded to 22 different cDNAs. Northern blot analysis demonstrated that gene expression of 5 clones were significantly lower in mdx heart than in control. In the present study we focused on wt17 clone, which hybridized with a 8.8 kb band. This transcript was 57% lower in mdx than control hearts. Computer database searches of wt17 revealed that no significant similarity was found with any known sequence. In attempt to identify this unknown clone, we performed 5 RACE. We obtained a 900 bp fragment, which revealed 93% identity to a part of the 3 non-coding region of the rat muscle anchoring A-kinase protein (mAKAP) cDNA. Therefore, we conclude that the wt17 clone is the mouse counterpart of the rat mAKAP.

Mouse mAKAP is expressed in the cerebellum, heart and skeletal muscle - Northern blot analysis demonstrated that this gene was expressed exclusively in the cerebellum, heart and skeletal muscles. The transcript in skeletal muscle was slightly lower in the size than those of the cerebellum and the heart.

mAKAP is expressed in differentiated cardiomyocytes - mAKAP was expressed in day 3-cultures when cardiomyocytes showed a differentiated phenotype. However, we did not see any signal in day 1-cultures when the neonatal ventriculocytes reverted to the undifferentiated phenotype. mAKAP signals significantly appeared on 13 day-old rat hearts and were increased by 15 days of age, when differentiating cardiomyocytes of rats declined progressively reaching nearly zero. Thus, these observations suggested that mAKAP was expressed in terminally differentiated cardiomyocytes.

Regulation of mAKAP gene expression: decreased in mdx and increased by phenylephrine treatment - ET-1 treated cardiomyocytes showed no significant difference in mAKAP mRNA level, even if we noticed the hypertrophic feature of ET-1-treated cardiomyocytes. Furthermore, in the heart of 16-month old mdx mice, which are known to have the abnormal Ca^{2+} homeostasis, mAKAP mRNA was significantly decreased as compared to the control although there was no observed hypertrophic phenotype. Phenylephrine treated cardiomyocytes resulted in up regulation of mAKAP mRNA as compared to the control.

Decreased mAKAP coincides with down regulation of RyR2 and SERCA2a in 5-month mdx hearts - The RyR2 mRNA level was reduced to 52% in mdx as compared to the control littermate. We detected no difference in FKBP12.6 expression of the 5-month mdx and control hearts. SERCA2a expression exhibited a 75% reduction in the 5-month old mdx, but no difference in the expression of phospholamban. There was no significant changed in the gene expression of PDE4D3 between mdx and control hearts.

DISCUSSION

Our results suggested that decreased mAKAP in mdx hearts is independent of cardiac hypertrophy. On the contrary, the regulation of mAKAP gene expression may be associated with intracellular $Ca^{2+}$and cAMP levels in mdx hearts. However, the molecular mechanism of decreased mAKAP mRNA in mdx mice remains to be elucidated. When we treated cardiomyocytes with a cAMP inducible agent, phenylephrine, mAKAP mRNA was increased significantly as compared to the control. One possible mechanism of increased mAKAP expression, like other AKAPs, is through cAMP signaling. Sequence analysis of the human mAKAP 5 flanking region (GenBank accession no. NT__026437) revealed an existence of TGATGTCA sequences at bp –137 to –130 (relative to atg start site), similar to cAMP-responsive element binding protein (CREB) consensus sequences (TGACGTMA). Therefore, cAMP-induced transcription of mAKAP may be directly or indirectly through the CREB binding site of the mAKAP promoter region.

mAKAP is a member of AKAPs family which provide a molecular framework that orients cAMP-dependent kinase (PKA) and phosphatases through their interaction with the specific targeted substrates. mAKAP regulated RyR function, through PKA-dependent RyR phosphorylation, and result in efflux of Ca^{2+} through the RyR. The presence of AKAP maintains a signal transduction complex that may regulate reversible phosphorylation. Conversely, a loss of function of mAKAP significantly reduced PKA dependent RyR phosphorylation. Therefore, a decreased mAKAP in mdx heart may disturb PKA-dependent RyR2 phosphorylation and Ca^{2+} release from SR. Since PKA-dependent phosphorylation of single RyR2 channels increased mean open probability, open frequency and open time the RyR2 channels. Accordingly, decreased mAKAP may contribute to a reduction in calcium- induced positive inotrophy seen in mdx hearts.

The decreased of SERCA2a mRNA level was more apparent than RyR2 reduction. Another study has revealed decreased SERCA2a mRNA level is accompanied by a decreased in SR Ca^{2+} ATPase activity, whereas Ca^{2+} ATPase activity in the SR contributes to about 70-80% of the Ca^{2+} flux during diastole and a defect in Ca^{2+} removal could also impair relaxation. Accordingly, down regulation of mAKAP, RyR2 and SERCA2a mRNA in mdx hearts may result in altered relaxation, force of contraction and Ca^{2+}pump function observed in mdx myotube and heart.

Taken together, we have shown the decreased mAKAP, RyR2 and SERCA2a gene expression in 5-month mdx hearts which may contribute to impairment of Ca^{2+} handling and excitation-contraction (E-C) coupling in the sarcoplasmic reticulum (SR).

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論文審査の結果の要旨			
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論文題目	Decreased mAKAP, Ryanodine Receptor, and SERCA 2a Gene Expression in mdx Hearts mAKAP, リアノジン受容体およびSERCA 2a 遺伝子の発現はmdxマウスの心臓において減少を認める		
審査委員	主　査　千原和夫 副　査　山村隆 副　査　大西純		
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（要旨は1，０００字～2，０００字程度）

Mdxマウスは、Duchenne型筋ジストロフィーのモデルマウスとして汎用されている。Duchenne型筋ジストロフィーはX染色体劣性遺伝形式をとり、dystrophinの異常により幼少時から筋萎縮、筋力低下をきたし、１０歳前後で起立不能、心電図異常および心筋障害をきたし青年期に死亡に至ることが多い。Dystrophinは、骨格筋、心筋表面膜の内側全体に渡って網目状に存在し、細胞骨格を基底板に結合させる膜蛋白複合体の中の筋サルコレンマに位置する。Dystrophinの正確な役割はまだ十分には明らかにされていないが、この蛋白の欠損や機能異常は膜の不安定化をもたらし異常なCa流入を引き起こすことが知られている。しかしこの異常なCa流入と病気の発症との関連についてはまだほとんど明らかではない。申請者たちはこの点を明らかにする目的で、心機能異常を呈する5ヶ月令のmdxマウスと正常対照マウスの心臓を材料としてsuppressive subtractive hybridizationを行い、両者間で差のある分子を見出そうと試みた。Sbtracted cDNA librariesの300コロニーをdot blot分析でスクリーニングしたところ30クローンに絞り込め、さらにNorthern blot分析でmdxマウス心筋に有意に減少している5つのクローンが特定された。その中で対照に比べてmdxマウスで57%減少しているwt17に注目し、5' RACEでサイズを大きくしhomology検索を行ったところrat muscle anchoring A-kinase protein (mAKAP) であることが判明した。Northern blot分析ではmAKAPは小脳、心筋、骨格筋に特異的に発現していた。mAKAPは13日令のラット心筋に出現し15日令まで増加しその後減少することより、mAKAPは心筋細胞の最終分化時期に発現すると考えられた。mAKAPは16月令mdxマウスで有意に減少していた。培養心筋細胞mAKAPはET-1刺激で変化は見られなかったが、Phenylephrine刺激によって増加した。ET-1は心筋肥大作用を持っているので、mAKAPは心筋肥大とは直接関与しないものと考えられた。何故mdxマウスでmAKAPが減少するのか機序が明らかでないが、Phenylephrine刺激でmAKAPが増加したことより他のAKAPと同様cAMPシグナルの下流にmAKAP発現は位置するのかもいしれない。mAKAP遺伝子プロモーター領域にCREB結合部位と考えれる配列も見出されている。mAKAP

はcAMP-dependent protein kinase(PKA)を介してRyRリン酸化を調節し、その結果sarcoplasmic reticulum(SR)からのCa流出を促進する。5月令mdxマウスではRyR2 mRNAは対照の52%にまで減少していた。また、SERCA2a mRNAの減少はさらに顕著で対照の75%にまで低下していた。一方、FKBP12.6やphospholambanの遺伝子発現にはmdxと対照群で差は認められなかった。SERCA2aの低下はSR CA^{2+}ATPase活性の低下をもたらすが、SRにおける CA^{2+} ATPase活性は心筋拡張期のCa流入の70-80%を支配しており、Ca除去の障害は心筋の弛緩を妨害することになる。

以上、本研究は、Duchenne型筋ジストロフィーの心筋障害の機序について、その動物モデルであるmdxマウスを用い、対照マウスとのsuppressive subtraction hybridization法によりmdxマウスに特異的に減少する遺伝子をクローニングし、少なくともその一つがmuscle anchoring A-kinase protein (mAKAP)であることを明らかにし、さらにmAKAPと関連して細胞内のCa濃度に影響を与え、心筋の興奮・収縮に関与するryanodine receptor およびsarcoplasmic reticulum Ca^{2+} ATPaseもmdxマウスでは減少していることを初めて明らかにした価値ある知見の集積であると認める。よって、本研究者は、博士（医学）の学位を得る資格があると認める。