Discussion

Ectopic calcification of skeletal muscle and serum Pi elevation in mdx mice

Ectopic calcification was observed in mdx mouse skeletal muscle, and was determined to be composed of hydroxyapatite (HA). As HA consists of calcium phosphate, it was assumed that the observed ectopic calcification was related to an aberration of calcium or Pi metabolism. The measurement of the serum calcium and Pi concentrations revealed that, while no significant difference was found in the calcium concentration, the Pi level in mdx mice was 1.4-fold higher than in the B10 mice.

It has been demonstrated that an increase in the extracellular Pi level in culture medium promotes mineralization in osteoblasts (Murshed *et al.*, 2005) and vascular smooth muscle cells (Jono *et al.*, 2000). Furthermore, ectopic calcification in soft tissues has been observed in mouse models exhibiting hyperphosphatemia: mice lacking alpha-Klotho (Kuro-o *et al.*, 1997) or FGF-23 (Sitara *et al.*, 2004). We hypothesized that skeletal muscle cells also undergo osteogenic differentiation under high-Pi conditions, leading to ectopic calcification in the skeletal muscle of mdx mice. To test this hypothesis, we cultured murine myoblast C2C12 cells in medium containing various concentrations of Pi.

Pi-induced osteogenesis and inhibited myogenesis in skeletal muscle cells

Skeletal muscle cells by nature maintain multi-lineage potential, and satellite cells are capable of adipogenic and osteogenic differentiation (Asakura *et al.*, 2001; Wada *et al.*, 2002). The C2C12 muscle cell line, derived from adult C3H mouse skeletal muscle, also possesses the properties of satellite cells (Yaffe and Saxel, 1977; Blau *et al.*, 1983). C2C12 cells are capable of adipogenic or osteogenic differentiation under certain inducing conditions, such as supplementing the medium with gamma-linolenic acid to induce adipogenesis, and BMP-2 to induce osteogenesis (Wada *et al.*, 2002; Katagiri *et al.*, 1994).

The present results indicate that C2C12 cells cultured under high-Pi conditions expressed Runx2 and osteocalcin, and generated calcium deposits, which are typical of osteoblastic cells. The Pi-induced osteogenesis in C2C12 cells followed by calcium deposition observed in this study may reflect ectopic calcification in mdx mouse skeletal muscle.

The mechanisms by which extracellular Pi induces calcification in both osteogenic and non-osteogenic cells are not fully understood. However, it has been reported that Pit-1, one of the type-III sodium-dependent phosphate cotransporters, is necessary for increasing Pi uptake and consequently the Pi-induced expression of osteocalcin, osteopontin and other bone-related proteins during calcification in osteoblasts and vascular smooth muscle cells (Li and Giachelli, 2007; Yoshiko *et al.*, 2007). It has been reported that BMP-2 promotes Pit-1 expression, Pi uptake and calcification in vascular smooth muscle cells, suggesting that vascular calcification shares a common mechanism with physiological calcification (Li *et al.*, 2008). Similar regulatory mechanisms may be involved in ectopic calcification in mdx mice.

In this study, the elevation of the medium Pi concentration not only induced osteogenesis but also led to the inhibition of myogenesis in C2C12 cells, which was evidenced by the attenuation of myotube formation and the decrease in the ratio of cells expressing myogenin. Myogenin regulates various genes necessary for skeletal muscle differentiation, including the expression of myosin heavy chain, troponin and another muscle-specific transcription factor, MRF4 (Perry and Rudnick, 2000; Davie *et al.*, 2007). Interestingly, while osteogenic markers started to appear at a Pi concentration of 3~5 mM, the inhibition of myogenesis manifested in a decrease of the fusion index, and myogenin expression was not observed until the Pi concentration reached 7 mM. The difference in susceptibility to the Pi concentration between the inhibition of myogenesis and the facilitation of osteogenesis may explain the status of mdx skeletal muscle; both myogenesis and osteogenesis proceed at 5 mM Pi, the same concentration as the serum Pi in mdx mice. The immunocytochemistry of C2C12 cells indicated that myogenesis and osteogenesis are mutually exclusive; i.e., they do not occur simultaneously in the same cell. When the Pi concentration was elevated to 5 mM, the ratio of Runx2-positive cells increased while that of myogenin-expressing cells remain unchanged. This suggests that it was the reserve cells uncommitted to myogenesis which became Runx2-positive and proceeded with the osteogenic cascade under high-Pi conditions.

Calcium phosphate deposit-induced osteogenesis in myoblasts

In this study, we demonstrated that the ability of insoluble calcium phosphate to induce osteogenesis is higher than that of soluble Pi, as the expression of Runx2 in C2C12 cells was significantly upregulated when the cells were cultured in the presence of calcium phosphate deposits. This result is consistent with previous studies, in which mesenchymal stem cells and C2C12 cells co-cultured with HA crystals showed osteogenic differentiation (Damien and Parsons, 1991; Tan *et al.*, 2007). The observation that the osteoinductive potential of calcium phosphate deposits is stronger than that of Pi suggests that ectopic calcification in mdx mouse skeletal muscle forms a positive feedback loop; once a calcium phosphate deposit is formed, it induces osteogenesis in adjacent cells and amplifies calcification in the surrounding areas.

Calcification independent of osteogenesis

In addition to osteogenesis-induced calcification, there may be a passive process of calcification formation: the development of calcium phosphate deposition in the presence of high concentrations of Pi and calcium ions. It is well known that calcium regulation is disrupted in dystrophin-deficient muscle, and that calcium ions accumulate in the cytosol of degenerating muscle fibers in mdx mice (Berchtold *et al.*, 2000; Gillis, 1999). The muscle fibers of mdx mice which were alizarin red S-positive but became negative after rinsing in running water were likely rich in calcium ions, as alizarin red S binds not only to calcium salts but also to calcium ions in the soluble state, which form visible precipitates (Lievremont *et al.*, 1982). Though these muscle fibers are assumed to become mineralized, it is unlikely that cells first differentiate into myotubes and then re-differentiate into osteoblastic cells. Likewise, the immunohistochemistry of C2C12 cells revealed that Runx2 was not expressed in the nuclei of myotubes, suggesting that Runx2 is inactivated in these cells. Therefore, ectopic calcification not triggered by osteogenesis can be expected to occur in calcium-rich muscle fibers by the generation of calcium phosphate deposits.

Relationship between serum Pi and ectopic calcification in mdx mouse skeletal muscle

Since hyperphosphatemia and vascular calcification in FGF-23 null mice were corrected by a Pi-deficient diet (Stubbs *et al.*, 2007), ectopic calcification in mdx mouse skeletal muscle may also decrease by feeding mdx mice a low-Pi diet. To investigate the involvement of Pi in ectopic calcification of mdx mouse skeletal muscle, dietary reduction of Pi was performed for 2 months.

A low-Pi diet significantly lowered serum Pi levels in both B10 and mdx mice, and the serum Pi of mdx mice reached the same level as B10 mice fed a normal diet. In the mdx mice fed a low-Pi diet, ectopic calcification of skeletal muscle was markedly inhibited. These results are consistent with our hypothesis that elevated levels of serum Pi induces ectopic calcification in mdx mouse skeletal muscle. However, since raising the serum Pi of normal mice by a high-Pi diet does not promote calcification in any soft tissues (Murshed *et al.*, 2005), factors other than Pi may also be involved in ectopic calcification.

The reason why the serum Pi concentration in mdx mice is higher than in B10 mice is not clear. FGF-23 has recently gained attention as a negative regulator of serum Pi levels, and it was anticipated that a decrease in serum FGF-23 was the cause of serum Pi elevation. Contrary to our expectation, however, mdx mouse serum FGF-23 levels were approximately 1.5 times higher than in B10 mice, suggesting that the serum FGF-23 level was increased to facilitate Pi exhaustion and to reduce the serum Pi level. The involvement of other Pi-regulating factors, such as vitamin D, has yet to be clarified. It is possible that Pi leaks from damaged fibers into the circulation, as the intracellular Pi concentration of mdx mouse skeletal muscle during exercise is elevated compared to B10 mice (Goudemant *et al.*, 1998). In DMD patients and mdx mice, some types of molecules in the skeletal muscle, such as creatine kinase and myoglobin, are reported to be released into the bloodstream through microlesions in the sarcolemma (Ebashi *et al.*, 1959; Hooshmand, 1975; Ando *et al.*, 1978). It is therefore presumed that the leakage of intracellular Pi was likely the source of the elevated serum Pi observed in the mdx mice. Renal failure in mdx mice caused by dystrophin deficiency was unlikely, because the concentrations of serum creatinine and blood urea nitrogen were not significantly different between the mdx and normal mice (Brazeau *et al.*, 1992).

Inflammation and calcification: cause or effect?

In recent studies, inflammation has been proposed as a key factor in the pathogenesis of muscular dystrophy. Though the primary cause of DMD is dystrophin deficiency, the immune response elicited by membrane damage results in the progression of the disease (Tidball and Wehling-Henricks, 2005; Acharyya *et al.*, 2007). The accumulation of macrophages observed around calcifications and muscle fibers undergoing calcification suggests that ectopic calcification is intimately related to inflammation in mdx mouse skeletal muscle. It is probable that calcification triggers inflammation, for it has been reported that calcium phosphate crystals consisting predominantly of HA induce a proinflammatory response in macrophages (Nadra *et al.*, 2005). We also cannot deny the possibility that calcification is regulated by macrophages, as it has been shown that activated monocytes and macrophages enhance the calcification of vascular smooth muscle cells by cell-cell interaction and the secretion of tumor necrosis factor-alpha, a pleiotropic cytokine known to promote vascular calcification (Tintut *et al.*, 2000; Tintut *et al.*, 2002). Whether or not calcification in mdx mouse skeletal muscle induces inflammation has yet to be clarified, and is a topic for future studies.

Conclusion

The goal of this study was to investigate the mechanisms of ectopic calcification operating in mdx mouse skeletal muscle. Given the results of the experiments described above, we conclude that Pi and calcium deposits induce osteogenesis in myoblasts resulting in calcification, while calcification may also be generated passively by the elevation of intracellular Pi and calcium ion levels. A hypothetical model of ectopic calcification in mdx skeletal muscle is presented in Fig. 9.

Ectopic calcification can be observed easily using X-ray

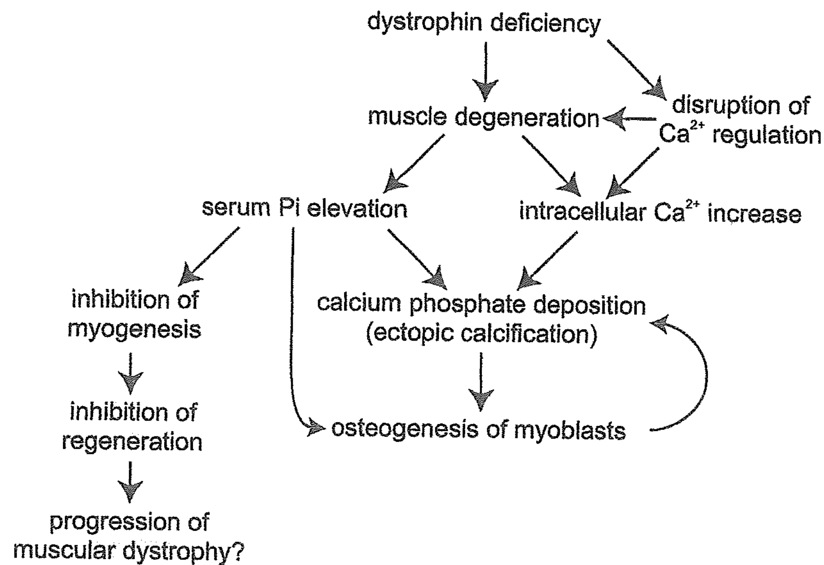

Fig. 9. Hypothetical mechanism of ectopic calcification in mdx mouse skeletal muscle. Leakage of Pi from degenerated fibers elevates the serum Pi, inducing osteogenesis and calcification in myoblasts. Meanwhile, calcium regulation is disrupted in dystrophin-deficient muscle fibers, causing an increase in intracellular calcium. Pi and calcium ions form calcium phosphate precipitates. Calcium depositions induce osteogenesis in myoblasts, thus creating a positive feedback loop of osteogenesis and calcification. In addition, elevated Pi levels inhibit myogenesis, and the decrease of cells committed to myogenesis inhibits muscle regeneration which leads to the progression of DMD.

CT and other techniques, without the need for biopsy or near-infrared fluorescence imaging methods (Zaheer *et al.*, 2001). The possibility of monitoring ectopic calcification non-invasively in mdx mice provides a novel means of diagnosis and evaluation of therapeutic treatments for DMD.

To our knowledge, this study is the first attempt to comprehensively describe ectopic calcification in mdx mouse skeletal muscle and elucidate the mechanisms underlying the phenomenon (Fig. 9). In addition, although growth factor-mediated osteoinduction is already known, this is the first report to suggest that the calcification of skeletal muscle cells is caused by elevated Pi levels. We expect that our findings will offer novel insight into ectopic calcification in skeletal muscle and lead to an improved understanding of the pathology and therapy of muscular dystrophy.

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引き続きアスコルビン酸内服治療を開始したが副作用発現はなく，現在経過観察中である．今後1カ月，3カ月，6カ月時点でのCMT scoreと電気生理検査評価を行い，本治療法の最終的な効果判定を行う．

結　論　アスコルビン酸内服当初は副作用の発現などの有害事象なく，20 mg/kg/日では安全に投与することができる．今後，経時的な評価を行い適正内服量を確立する．

第50回日本小児神経学会総会（平成20年，東京）P-008．脳と発達40巻（総会号）S-276にて発表

第三部：新たな筋ジストロフィー治療戦略

鍼通電刺激によるミオスタチン発現抑制と筋ジストロフィー治療の可能性

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鍼治療とは，わが国の伝統医学である東洋医学の治療法であり，生薬を用いた漢方治療と一対をなしている．その特徴は，「氣」という単一概念で総体的に生命現象をとらえることである．他方，ゲノム解析の進展は，分子レベルで総体的に生命現象を俯瞰可能にした．人間が作った概念という点で「氣」と「遺伝子」は類似しているが，「氣」は実態が不明確な認識の産物である．

そこでわれわれは，鍼治療の科学的根拠を明らかにするために，鍼治療法のうち鍼通電刺激療法を対象に，マウスを用いた動物実験系で解析してきた．本講演では，鍼通電刺激した骨格筋のトランスクリプトーム解析結果から，①骨格筋に及ぼす鍼通電刺激の影響，②ミオスタチン遺伝子発現の抑制効果，③鍼通電刺激の作用機序の仮説，を紹介する．そして，④廃用性筋萎縮マウスモデルでの鍼通電刺激の筋萎縮抑制効果を報告し，疾患治療などへの応用の可能性について考察したい．

筋ジストロフィーの薬物治療 ―リードスルー薬物による挑戦―

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われわれはナンセンス変異型遺伝性筋疾患を克服するために，未熟終止コドンを読み飛ばす「リードスルー」薬物候補および，その薬物送達について検討している．独自に開発したリードスルー検出系のトランスジェニックマウスやDuchenne型筋ジストロフィー（DMD）の疾患モデルであるmdxマウスを用い，これまでにジペプチド系抗生物質ネガマイシンやネガマイシンの三次元構造類似分子，ネガマイシン誘導体，未承認アミノグリコシドから新規リードスルー惹起薬物候補を数種特定している．その中には，ゲンタマイシンやネガマイシンよりもその効率が高く濃度依存的な活性をもち，経口投与可能であるうえ，安全性も高く，mdxマウスやDMD患者様由来培養細胞を用いた生化学的・免疫組織化学的解析結果も良好で薬物候補として有望な物質が含まれている．また，経皮吸収促進剤を用いることで皮下投与と同等のリードスルー活性を認める経皮吸収型薬物送達法を開発した．