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·Original Article·

Permanent myopathy caused by mutation of SCN4A Metl592Val: Observation on myogenesis *in vitro* and on effect of basic fibroblast growth factor on the muscle

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Abstract: Objective The present study is to observe *in vitro* the proliferation ability of the muscle cells from permanent myopathy (PM) patients of nomokalaemic periodic paralysis (normKPP), which is caused by mutations of Met1592Val in the skeletal muscle voltage gated sodium channel (SCN4A) gene on chromosome 17q23.1. We also evaluate the possible effect of the foreign basic fibroblast growth factor (bFGF) in preventing and curing PM. **Methods** The gastrocnemius muscle cells were taken from two male patients with PM of the same Chinese family with Met1592Val mutation of SCN4A, determined by gene screening. Four male patients suffering from the skeletal injury without PM were taken as control. All preparations were protogenerationally cultured *in vitro*. Proliferation of the cultured preparations was measured by MTT. Activities of the lactic dehydrogenase (LDH), creatine kinase (CK), and protein content in these cells were also detected. The effects of bFGF with different doses (10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, 120 ng/mL and 160 ng/mL) on the above mentioned parameters were also evaluated. **Results** Cells from both PM and control subjects were successfully cultured *in vitro*. The cultivation of the muscle cells from PM patients *in vitro* was not yet seen. Results indicated the obvious stimulation of bFGF on cell proliferation, activities of LDH and CK, protein synthesis, in a dose dependent manner. The optimal dose of bFGF was 120 ng/mL ($P<0.05$), beyond which greater dose caused a less effect. The effect of bFGF on 160 ng /mL was stronger than that on 80 ng/mL, but there was no significant difference ($P>0.05$). **Conclusion** Myoblastic cells from patients with PM had a weaker ability of developing into the myotubules, thus they were unable to perform effective regeneration, which resulted in a progressive necrosis. The exogenous bFGF could promote the division and proliferation of the muscle cells *in vitro*. These results shield a light on bFGF's potential role in preventing and treating PM.

Key words: SCN4A; permanent myopathy; cell culture; basic fibroblast growth factor

1 Introduction

Nomokalaemic periodic paralysis (normKPP) is caused by mutation in the skeletal muscle voltage gated sodium channel (SCN4A) gene on chromosome 17q23.1[1, 2], characterized by transient and recurrent attacks of paralysis followed by complete convalescence. However, some patients may develop into permanent myopathy (PM) following repeated attacks. And this condition is usually reported in some patients with mutation of Met1592Val and Thr704Met, which appears in and after middle age, and follows the down phase of the attack frequency of the periodic paralysis with the upper limbs worse than the lower limbs. Although PM is usually independent of paralysis, its severity is correlated with severity of attack and labor intensity of patients in their

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ages. The muscle biopsy demonstrated that there were degenerations in the muscles[3, 4]. So far, for PM there is still no effective therapy. PM may result in disability: patients may be unable to move, or even be kept in bed. So, investigating the cause for PM and finding possible ways defending and treating PM have become the focus in this field. In this paper, the patients who were diagnosed as PM caused by mutation of SCN4A Metl592Val using gene screening, were chosen as subjects and the preparations of the skeletal muscle from the patients were cultured *in vitro*. On this basis, the developing courses of these preparations and effects of the foreign basic fibroblast growth factor (bFGF) on the preparations were observed to reveal the possibility of bFGF in preventing and curing PM.

2 Materials and methods

2.1 Subjects The subjects were divided into 2 groups, the patient group and the control group. The former included 2 male PM patients, who were 42 and 48 years old respectively, coming from the same family with Metl592Val mutation of SCN4A diagnosed by gene screening[5]. The latter included 4 cases of male patients suffering from the skeletal injury without PM, also aged 40-50 years, whose gene screening found no SCN4A mutation (Table 1). All the subjects were volunteers in this study.

2.2 The protogenerative cultivation of the skeletal muscle cells About 0.5g of the pure gastrocnemius muscle preparation was taken respectively from subjects of 2 groups. The preparations were cleaned in D-Hanks solution, and were cut into about 1mm^3 muscle lumps, which were then incubated in the culture flask. The turnover-drying fixing method was used to facilitate the preparations to stick to the flask wall. Three hours later, the culture solution was added into the flask. The preparations were then cultured at 37°C with 5% CO_2. Five to seven days later, half of the first solution was changed, and after each 2-3 days, the whole solution was replaced. The development state of the cultured cells was observed dynamically under the reverted phase microscope.

Table 1. The clinical data of subjects

subjects	Age(years)	Sex	Met1592Val	PM Duration(years)
Patient-1	48	M	+	7
Patient-2	42	M	+	2
control-1	42	M	-	None
control-2	47	M	-	None
control-3	42	M	-	None
control-4	48	M	-	None

PM: permanent myopathy; M: male.

2.3 The immunochemical staining of the cultured cells The muscle cells which have been cultured for 10 days (1×10^9/L) were incubated on the growth slides for another 24h. The preparations were washed twice using D-Hanks solution at 4°C, and fixed using the cold propylsterone for 15 min. Cells were ready for Desmin staining.

2.4 bFGF intervention The protogeneration-cultured muscle cells from the patients with PM in their logarithmic growth phase were incubated (1×10^5/L) in the culture plate with 96 pores. Cells were divided into 3 groups: the control group (cells plus culture solution); the bFGF group (cells culture solution plus 6 different concentrations of bFGF); the blank control group (only contains the culture solution, which is just for zeroing). Two days later, all the cells were treated with 10% FBS medium for another 48 h to be synchronized to G_0 phase. Then 6 different doses of bFGF (10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, 120 ng/mL, and 160 ng/mL) were respectively used for another 72h of cultivation.

2.4.1 Detection of cell proliferation Following bFGF treatment, 50 μL MTT solution (1 mg/mL) was added into each pore and incubated for 4 h. Then added 150 μL dimethylsulfoxide (DMSO) per pore and shook for 15 min under dim condition. For each single pore, absorbance at 570 nm of wavelength was detected using enzyme immunoassay method. Data from the blank group were used to set zero point.

2.4.2 Detecting activities of the lactic dehydrogenase (LDH) and creatine kinase (CK) DMEM medium without serum was used to culture the preparations of the muscle cells for 72 h, and the culture solution was removed. Then the preparations were cleaned twice using D-Hanks solution. After adding 50 μL PBS solution, cells were then frozen and thawed three times before detecting LDH and CK activities.

2.4.3 Detection of protein Coomassie BB was used to deter-

mine the protein content of the preparations. Detection was also performed after freezing-thawing treatment mentioned above.

2.5 Statistics analysis Data were managed by SPSS 10.0 software. The SNK-q method was used to make comparisons between the data of all groups. $P<0.05$ was considered statistically significant.

3 Results

3.1 *In vitro* comparison of cell development between the control and patient groups Medium solution of the control group seemed much paler than that of the patient group on the third day, which suggested that control cells possessed more vigorous metabolism. On the fourth day, some spindle cells could be seen around the pieces in the control group. On the seventh day or eighth day, 2-3 scattered muscle cells appeared in a parallel way, and tended to mix with each other. Meanwhile, there showed no color change of patient group medium solution, and even no spindle cells could be found until the seventh day.

At about the third week, the cells from both groups showed obvious growth tendency, but with no significant difference in cell number between the two groups. In the control group, the cells possessed more than 3 nuclei, and the long myotubules with chain distributions could be found. In the patient group, although cells grew actively, they were randomly ranged and largely different in size and in spindle shape. Also, there was no myotubulogenesis.

During the fourth to fifth week, the number of the muscle cells in the control group increased sharply, and there were longer, broader, compact, polybranched, and multinuclear myotubules with concentrated myofibriles. At most, the number of the nuclei was more than 10 (Fig. 1). In the patient group, the cells were also productive, but were different in size. Formed myotubules were much fewer, and the arrangement was in a jumble way: one side of some cells inserted into another, and no fusion sign could be found (Fig.2). After fully covering the culture flask, the cells were passed on. The growth duration of each single generation was almost the same in both groups. The vacuoles substances in their cytoplasma did not appear until the eighth generation. The cells decreased in refractivity, their outlines became obvious, and then the cells stopped division, floated and died.

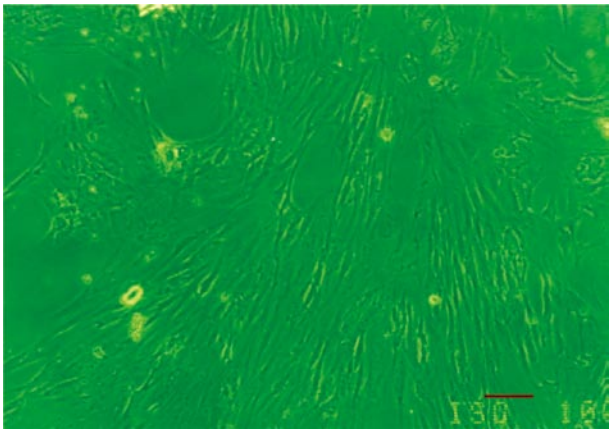

Fig. 1 The control group: the cells grew prosperously, aligned orderly, and showed intensive myotubules. scale bar, 200μm.

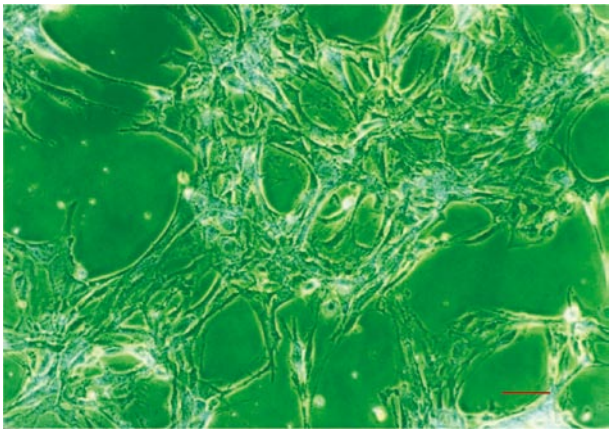

Fig. 2 The test group: the cells also showed productive, but in a jumble arrangement and few fusion sign can be found. scale bar, 200μm.

3.2 Desmin staining More than ninety-seven percent of the cultured cells showed positive responses, both the periphery of the nuclei and the cytoplasma appeared brown, confirming that the cells cultured were the muscle cells.

3.3 Effects of bFGF on the proliferation of the cells The effects of bFGF on muscle cells were obviously dose-dependent. The bFGF dose of 120 ng/mL showed the strongest effect, however, a greater dose (160 ng/mL) caused a decrease in proliferation (Fig. 3). There was no significant difference between the effects of the dose of 160 ng/mL and 80 ng/mL.

3.4 Effects of bFGF on LDH, CK, and protein synthesis

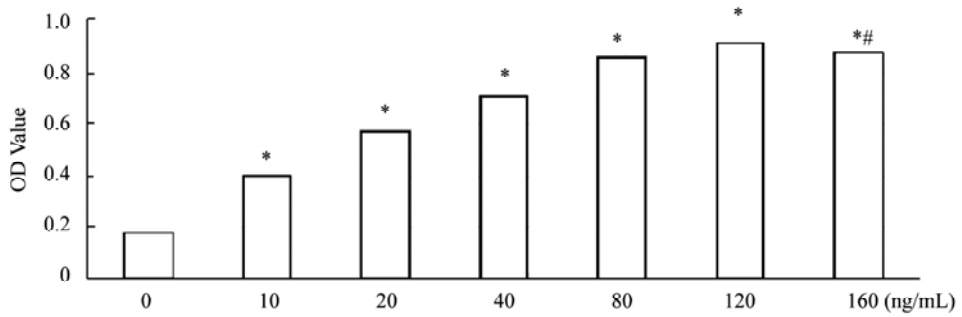

Fig. 3 The effects of different concentrations of bFGF on the proliferation of the cells. *P<0.05 *vs* the dose of 0 ng/mL, # P>0.05 *vs* the dose of 80 ng/mL.

Table 2 The effects of different concentrations of bFGF on the activities of LDH and CK, and on protein synthesis

dose (ng/mL)	Cell freeze thawing		
	LDH (U/L)	CK (U/L)	PROT (mg/L)
0	61±12	7.0±3.6	142±3.5
10	80±28	9.8±1.1	186±42
20	88±36	20.3±1.5	198±32
40	106±35	30.7±1.4	221±26
80	124±38	43.6±3.9	221±26
120	140±28	52.5±4.0	238±30
160	129±32*	47.3±3.6*	229±28*

*P>0.05, compared with the dose of 80 ng/mL.

When the muscle cells had been exposed to bFGF for 72 h, activities of their LDH, CK and the synthesis of protein increased in a dose-dependent way (Table 2).

4 Discussion

The skeletal muscle sodium channelopathy is characterized by transient and recurrent episodes of paralysis or tetanus, followed by complete recovery. The attacks usually begin to occur during the first decade of life, and the frequency and severity of attack declines during the second half of the life. However, the irreversible myasthenia may still appear at later life time in some patients. This myasthenia may develop into atrophy of the muscle. The electromyogram (EMG) showed myopathic changes with the low amplitude and positive sharp waves. The muscle biopsy specimen revealed variability in fiber size, remarkable fibrosis and vascular changes[6]. The cause of myopathy remains unclear so far, and there is still no effective therapy for this disease. In this paper, we mainly investigated the mechanism of this disorder, and found out possible treatment against this disease.

The skeletal muscle is able to regenerate after damage, mainly accomplished through activating the satellite cells from an inhibitory state under normal conditions[7], making them transform to the myoblastic cells. The myoblastic cells keep dividing, mixing with each other, and at last, develop into myotubules. Under culturing conditions *in vitro*, the formation of the myotubules indicated a mature phase of the muscle cells. So, using culture method *in vitro* makes it convenient to observe the formation, the size of the myotubule and differentiation of the cell for evaluating the myogenesis of the muscle cell[8]. Besides, the muscle cell cultured using progenerative cultivation method kept its original natures, i.e. ensuring a higher fedelity[9]. However, it is hard to culture adult skeletal muscle cells, or to keep the purity of the preparation. Using the method of tissue lump culture, we have successfully cultured the cells of the skeletal muscle *in vitro*, since Desmin staining, a specific dye for the skeletal muscle, demonstrated that the purity of the preparations used in our test was over 97%[10]. Our results showed that at about the second week, the cells from both groups began to grow vigorously, when formation of the myotubules began to occur. This observation indicated that the damaged muscle of the patient with PM was able to regenerate *in vitro*. However, formation of the myotubule was much slower than that of the control group, suggesting a weaker ability of the myoblastic cells from the PM subjects in developing into the myotubule. The muscle cells from the test group grew slowly

during the early culture phase, and the size was different, which further indicated that these damaged cells were unable to perform effective regeneration, resulting in a progressive exhaustion of the necrotic muscle fibers. According to the report[11], some factors can promote the damaged muscle cells to proliferate and differentiate, which in turn, facilitate the regeneration of these cells. bFGF[12,13] , an active peptide composed of 146 amino acids, possesses significant biological effects in promoting the life, growth, proliferation, and differentiation of the muscle cells. There are bFGF receptors on the skeletal muscle cell, the latter synthesizes bFGF itself. In view of weaker ability of proliferation and differentiation of these cells of the PM patients, bFGF was used in this study to detect its role in promoting the regeneration and reparation of the damaged muscle cells. The treatment of repeated freezing and thawing makes cell release LDH, so the content of LDH can reflect the cell number. In this paper, MTT detection demonstrated that bFGF obviously promoted the proliferation of the muscle cells, and it was the same for LDH, which suggested that bFGF not only promoted the proliferation of the myoblastic cells, but also made a positive effect on the survival of the cells.

The activity of CK reflected the differentiating ability of the cultured cells *in vitro*. According to the report, the value of CK could also reflect the level of the myotubules: the more mature the myotubules were, the more complete the differntiation was, the higher CK value was[14]. Our results demonstrated that all the bFGF doses of more than 10ng/ml resulted in the obvious increases of CK content. This result suggested that bFGF vigorously facilitated the proliferation of the myoblastic cells and the maturation of the myotubules. The changes in protein content showed that bFGF strongly increased the ability of the myoblastic cells in synthesizing protein.

These results suggested that the normal skeletal muscle could be cultured *in vitro*, and like cells *in vivo*, the whole development course could be divided into 3 phases: the static phase, the differentiating and proliferating phase, and the myotubule formation phase. Meanwhile, the regeneration ability of the skeletal muscle cells from the patients was obviously weak. But not all the muscles of PM patients were damaged. According to our observations, it is feasible to graft the normal muscle tissue to the patient to treat PM.

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SCN4A Met1592Val突变所致的永久性肌病体外成肌过程观察及碱性成纤维细胞生长因子的作用

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摘要：目的 正常血钾性周期麻痹是由位于17号染色体上编码人类骨骼肌钠通道基因(SCN4A)发生突变所致，以肢体无力短暂、反复发作并完全恢复为特点，部分患者可发展为永久性肌病(permanent myopathy, PM),该病的发病机制尚不清楚，至今也无有效的治疗方法。本文观察SCN4A Met1592Val突变所致PM患者骨骼肌细胞体外生长过程，同时探讨外源性碱性成纤维细胞生长因子(basic fibroblast growth factor, bFGF)对PM防治作用的可能性。**方法** 对两名来自一个由基因诊断确诊为SCN4A Met1592Val突变家系的患者和四名正常对照者的腓肠肌细胞进行体外培养，观察其生长过程；同时给予6个浓度(10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, 120 ng/mL和160 ng/mL)的bFGF刺激，用MTT法、考马斯亮蓝比色法、反复冻融细胞，对骨骼肌细胞增殖进行检测。**结果** 腓肠肌细胞可体外培养，与在体发生过程相似,但PM组肌细胞发育迟滞；bFGF对肌细胞生长的增殖作用、促LDH、CK活性及蛋白质合成作用呈明显的浓度依赖性，浓度为120 ng/mL时作用最显著（$P<0.05$），此时浓度再增大其效应反而下降，在浓度为160 ng/mL时的作用大于80 ng/mL,但无统计学意义（$P>0.05$）。**结论** PM患者受损腓肠肌在体外有再生能力，但融合成肌管能力极弱，存在再生障碍，不能对变性、坏死的肌纤维形成有效再生，故呈进行性肌纤维耗竭；外源性bFGF可促进PM患者体外培养骨骼肌细胞增殖分裂，并可促进肌管成熟，对进展性肌病应具有防治作用。

关键词： 正常血钾性周期麻痹；永久性肌病；细胞培养；碱性成纤维细胞生长因子