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The Introduction of Basic Fibroblast Growth Factor Promotes Quadriceps Muscle Regeneration after Damage in Mice

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Abstract

Tissue regeneration is the process of renewal and growth of damaging tissue. It is sometimes done by induction of an external biological factor(s) such as the Basic fibroblast growth factor (bFGF) which carries messages through its cellular receptor to induce biological processes such as cell growth, cell migration, cell survival, and cell differentiation. Due to the great importance of bFGF on cellular regeneration, in this study, we have concentrated on the effect of this factor on the regeneration of damaged muscle. In addition, the tumorigenic effect of this factor during this regenerative process was studied. In this study, we employed inbred mice. The cultured male mouse muscle cells were transplanted into the damaged muscle tissues of HLA_matched female mice. After about one month, selected samples from the transplanted regions of the female mice were analyzed. DNA was extracted from the samples and Sex determination was done to track the potential cell growth and repair by the introduced male cells in the presence and absence of bFGF. The tumorigenic effect of bFGF on this process was also assessed. We found that bFGF had a remarkable effect on damaged muscle regeneration compared to cells without injection of this factor, and more concentration of bFGF was determined to be more effective in muscle regeneration. However, no association was observed with tumorigenesis regarding bFGF injection. According to our findings, bFGF was effective in the regeneration of the injury site and confirmed the results of previous studies. However, no association was found with tumorigenesis.

Keywords: regeneration, bFGF, tumorigenesis, tissue, sex determination.

Introduction

Tissue regeneration is the process by which cells in damaged organs or tissues begin renewing in response to injury. In recent years, there have been significant developments in regenerative medicine for the

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therapeutic purposes of an organ that may be impaired in structure and function due to senescence, disease, injury, or genetic defects (1). This field includes tissue engineering and self-repairing system in the body, which is sometimes done by induction of an external biological factor(s) to accelerate the formation and reconstruction of the desired organ and tissue (1). However, there is little information about the physical processes that regulate regeneration. Although many damaged animal models have been used to study the process of tissue regeneration, especially in damaged skeletal models, the factors involved and how this process is regulated are not well understood (1).

Skeletal muscle makes up 40% of the body mass and contains a large number of differentiated myofibers in which cell division is stopped. Following adult muscle tissue damage, stem cells in the muscle, most of which are in the quiescent phase, re-enter the cell cycle and form myoblasts, which are involved in the construction and repair of myofibers (1). Efficient skeletal muscle regeneration requires the coordinated participation of other cells, such as macrophages, the precursor of adipogenic fibers, connective tissue, and endothelial cells, to form blood vessels. Tissue regeneration and return of tissue cells to normal function and structure in adult mice is done in approximately 21 Days (2, 3). The first inflammatory reaction of macrophages is to stimulate myogenic cell division, while the second one leads to the differentiation of these cells(4), followed by a significant interaction between Endothelial cells, adipogenic fibers, and myogenic cells that are used to coordinate the angiogenesis process, connective tissue formation, and muscle construction process. Thus, the process of muscle regeneration is very efficient (5, 6).

There are important factors involved in the process of tissue regeneration including signaling and regulatory molecules (7). In the process of tissue regeneration, not only one factor such as the number of healthy cells, but the cellular supporting structure called the extracellular matrix (ECM) is important (8). Also, in most cases, factors such as growth factors are required for this process depending on the type of tissues (9). Over the past decade, elevating knowledge about the impact of growth factors on accelerating tissue injury repair, leading to a strong interest in researchers to use various growth factors such as PRP, FGF, IGF, NGF, HGF, and TGFβ (10). Other factors such as PDGF and bFGF have the potential to stimulate the division and differentiation of satellite cells. The bFGF is involved in stimulating cell migration and angiogenesis while PDGF is involved in regulating protein and nucleic acid production (11). Other growth factors, such as VEGF and EGF, are responsible for angiogenesis at the site of injury (12, 13).

Among the growth factors that interfere with the muscle regeneration process, fibroblast growth factor (FGF) is one of the first and most abundant factors studied in living conditions. Fibroblast growth factor stimulates proliferation and suppresses myoblast differentiation. In muscle cells of a healthy skeleton, the amount of this factor (FGF) is very low. This factor is in the extracellular matrix and binds to proteo-heparin sulfate. FGF was first identified in 1973 in extracts from the pituitary gland. This factor is widely expressed in various tissues and cells. FGF expression has been observed in both vertebrates and invertebrates. The FGF family has 23 members, while there are only 18 receptors. 4 members of this family do not attach to the receptor and are classified as homologous FGF. The molecular weight of this factor in vertebrates varies between 18 KD to 38KD. All members of the family have a conserved sequence of 120 amino acids, indicating a similarity of 16 to 65% in the sequence (14). When a skeletal muscle injury occurs fibroblast growth factor is released. Increased FGF levels in dying or regenerating muscle cells have been reported in mice models with Duchene muscular dystrophy (mdx). Also, the amount of these factors increases in other X-linked muscle disorders such as dystrophies in humans and other mammals. Its elevated levels have been observed in both mRNA and protein. Increasing FGF shortly after the inflammation due to tissue damage leads to the induction of chemotaxis and the invocation of muscle progenitor cells to the site of injury. FGF is also thought to stimulate angiogenesis at the site of injury, thereby leading to more efficient muscle regeneration. The amount of fibroblast growth factor is important in regulating the regeneration process. FGF6 is specific to muscle cells and increases dramatically during muscle regeneration. bFGF (FGF2) also leads to an increase in the use of satellite cells at the site of muscle regeneration (15). Doukas and colleagues used the gene transfer system encoding FGF2 and FGF6 to repair skeletal muscle (16). They observed that angiogenesis rapidly occurred in that region, and the myotubes began to regenerate. Although there are many ambiguities regarding the role of FGF in muscle regeneration (17), it is widely accepted that there is a close relationship between the role of this factor in muscle regeneration and the process of angiogenesis (9).

According to the great importance of bFGF in cell regeneration after muscle injuries due to physical or congenital reasons, in this study, we sought to investigate the effect of bFGF on damaged muscle regeneration by injecting muscle cells with and without

bFGF and finally to evaluate tumorigenic effects of bFGF.

Materials and Methods

Subjects & sampling: In this study, we used 16 male/female mice for inbreeding. After about 30 months, while their 30th generation was obtained, the resulting offspring had 75-100% homology according to biological principles and were expected to be HLA-matched. Sampling was done from the quadriceps muscle tissue of the three male mice femur through biopsy (ethics committee approval code is IR.TUMS.MEDICINE.REC.1395.699).

Cell culture & cell tracking: We used DMEM-F12 media with low Glucose and 15% fetal bovine serum (FBS) for culturing muscular tissues. After 7 to 8 days, their culture medium was changed and after one week, when each flask reached a confluence of 80%, each of them was split into two new flasks with a confluency of 50%, and thus each flask was passage four times. At first, two of the flasks were kept in the incubator as the main source of the samples, and the other two were used for transfection by the vector (Aequorea Victoria) carrying the Green Fluorescent Protein (GFP) gene. The cells were transfected in two ways using lipofectamine and electroporation which were observed and confirmed by fluorescent microscopy (Figure 1A). After the specific removal of untransfected cells, the transfected cells remained (Figure 1B). However, the growth of these cells was slow and therefore their number was not sufficient for transplantation. For better cell growth, other vectors (adeno-associated virus (AAV)) carrying the GFP gene were transfected independently into the target cells. Again, untransfected cells were selected against the specific antibiotic, thus only transfected cells remained. However, the cells did not grow well and the number of cells for transplantation was too low. Therefore, we chose another method to track the transplanted cells in the target tissue. Therefore, we used the sex determination technique for cell tracking in this study. In this method, the cultured male mouse cells were transplanted into the tissue of a female mouse that was HLA-matched. Thus, to implement this method, specific primers were designed, and PCR (Polymerase Chain Reaction) Setup was made for male and female mice. After cell culture, when we had 3-4 flasks with 80-90% confluency (about 600,000-700,000 cells), they were transplanted into the female mice muscle. We injected half of the cell suspension with bFGF (in 10ng and 20ng concentrations) and half without bFGF to damage the muscle of female mice. The mice groups are shown in Table 1.

One month after transplantation, 6 mice were sampled, and 2 mice were kept to control for possible complications after transplantation. Samples were taken from 4 mice whose cells were injected with bFGF factor (two with a concentration of 10 ng and two with a concentration of 20 ng) and the other 2 mice were injected with cells without bFGF factor (18). Two mice for investigation of possible tumorigenic effects of growth factor were kept for a longer period. In each female mouse from the region where they received the transplanted cells, different portions (3 to 4) were sampled, and DNA was extracted separately for each portion. Finally, 3 to 4 DNA samples were obtained for each mouse. DNA samples were sex determined by PCR using sequence-specific primers. Table 2 shows the used primers detail and PCR program. The PCR products were resolved on 6% polyacrylamide gel

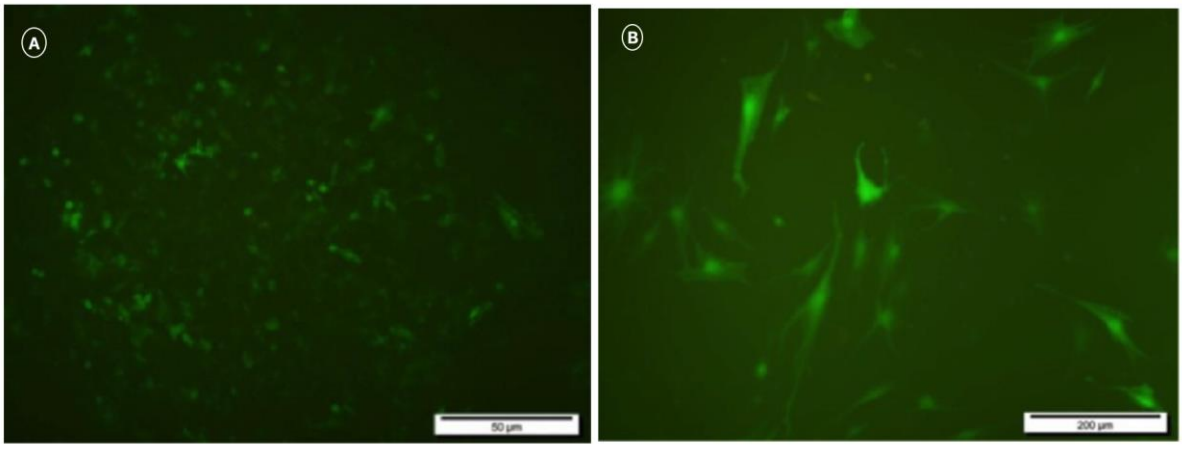

Figure 1. A) Florescent microscopy image of Transfected/untransfected cells, B) Transfected cells after removal of untransfected cells.

Table 1. The inbred mice groups for this study.

Group	Features	Numbers
1	Male mice that used for muscle cell culture and transplantation	10
2	Female mice that were wounded and used for evaluation of bFGf on tissue regeneration	6
2A	Female mice from group 2 that were studied with injection of bFGF	4
2B	Female mice from group 2 that were studied without injection of bFGF	2
3	Female mice from group 2A that were used for evaluation of tumorigenesis effect of bFGF	2

Table 2. Primer sequences for mice sex determination and PCR program.

Type	Sequences	Product size (bp)
Female	Forward 5'- CTGAAGCTTTTGGCTTTGAG	331 bp
	Reverse 5'- CCGCTGCCAAATTCTTTGG	
Male	Forward 5' CTGAAGCTTTTGGCTTTGAG	302 bp
	Reverse 5'- CCGCTGCCAAATTCTTTGG	
PCR Program	94°C for 4 minutes	
	94°C for 30 seconds	
	58°C for 30 seconds	Repeat in 34 cycles
	72°C for 30 seconds	
	72°C for 10 minutes	

electrophoresis alongside a 50 bp DNA ladder and positive controls for male and female mice (Figure 2).

Results

1) The effect of bFGF on the regeneration of damaged muscle: To observe the effect of bFGF on muscle regeneration, we injected the male quadriceps muscle cultured cells with two different concentrations of bFGF (10 and 20 ng/ml) to the corresponding region in female mice and then the rate of replacement and participation of male cells in the repair of injury site were examined by gel electrophoresis technique based on sex determination PCR method (cell tracking by detection of the Y chromosomes). Thus, as shown in Figures 3A&B, the Y chromosome bands were observed in the transplanted mice, so it can be concluded that male cells have been placed in the damaged region. As shown in Figures 3A&B, injection of bFGF at a concentration of 10 ng has led to a higher regeneration and replacement of healthy male cells in the damaged region than the group with no bFGF injection (Column A, Figure 3A).

In this study, two bFGF concentrations were applied. At the concentration of 20 ng, male mouse cell density was greater compared to that at the concentration of 10 ng (Figure 3A, Columns C and A). This difference seemed to be greater in the high-sensitive silver nitrate staining method than in the polyacrylamide gel method (Figure 3B).

2) Evaluation of muscle regeneration using an injection of muscle cells without bFGF: The transplanted muscle cells without growth factor could not be replaced at the site of injury. All three columns C, D, and E represent the presence of female mouse cells in the muscle region (only the presence of X chromosomes) and no band was seen for the Y

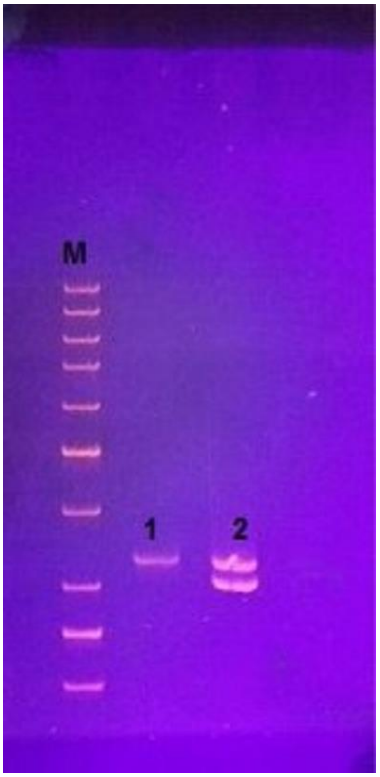

Figure 2. Sex determination in mice by Gel electrophoresis imaging of SSP-PCR; Two sets of X and Y chromosome specific primers were used that one band in the female represent only X-chromosome set of primers has been amplified and two bands shows amplification of two X and Y sets of primers; M= Marker "50bp", 1=Female mice, 2= Male mice.

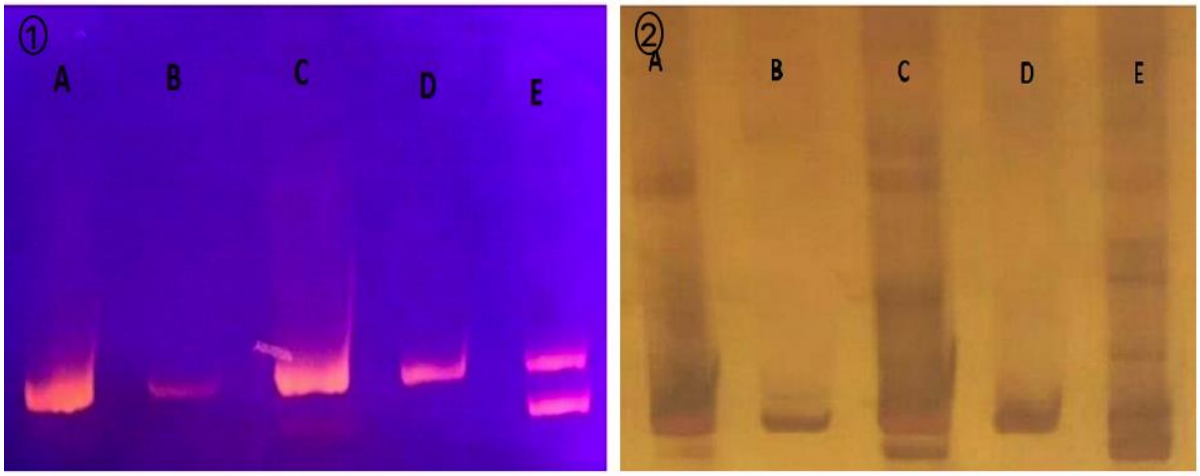

Figure 3. 1) The effect of bFGF on muscle regeneration (visualization by polyacrylamide gel electrophoresis). A& B: Cell injection with bFGF at a concentration of 10 ng, C: Cell injection with bFGF at a concentration of 20 ng, D: female mouse as negative control for Y chromosome & E: male mouse as positive control for Y chromosome. 2) the effect of bFGF on muscle regeneration (visualization by silver staining gel electrophoresis). A& B: Cell injection with bFGF at a concentration of 10 ng, C: Cell injection with bFGF at a concentration of 20 ng, D: female mouse as negaitve control for Y chromosome & E: male mouse as posetive control for Y chromosome (302 bp).

chromosome.

3) Evaluation of tumorigenic effects of bFGF: To investigate the tumorigenic effects of bFGF, two mice that were injected with bFGF were kept for a longer time (over two months). Since no mass and swelling at the injection site were observed, the probability of its tumorigenesis would be less likely.

Discussion

bFGF has been proposed to be important in the cell regeneration process following muscle injuries due to physical or congenital diseases such as different types of muscular dystrophies. The study was launched to evaluate the effect of bFGF on cell regeneration and tumorigenesis (19). As the process of muscle regeneration needs interconnected and coordinated interaction of different cells so that the presence of multiple cells in the culture and injection phase can somewhat accelerate the regeneration. Therefore, in this study, we injected a mixture of cultured cells into the wounded region. One of these include satellite cells that make a small number of muscle cells, they play a key role in muscle regeneration. Fibroblasts which are in the stage of quiescent in the cell cycle, are another set of cells. They can become activated and begin proliferating in the presence of FGF and FBS (20, 21). In the presence of bFGF, the growth rate of fibroblast cells increases dramatically (22). When bFGF is added to the culture medium containing 10% serum, synthesis of deoxynucleic acids is increased, hence the process of cell proliferation is enhanced. Research has shown that FGFs have a higher effect on the proliferation of cells than serum in the culture medium. At a concentration of 10 ng/ml of this factor, thymidine is stimulated to be incorporated into cells and thus it increases the synthesis of DNA. The bFGF increases the participation rate of thymidine (23). On the other hand, if cells remain in the culture medium for a short time, their functional ability will be better after being transplanted into the relevant tissue (24, 25). Therefore, one of the negative effects of the prolongation of culturing is the decreased ability of cell regeneration and it can be due to the reduction of important receptors and enhance the reduced ability of cell involvement into target tissue, such as CXCR4. The latter is a receptor protein that binds to its ligand, a cytokine called stromal cell derivate factor 1(SDF1). When muscle injury occurs due to mechanical damage or disease, several growth factors and cytokines including SDF1 accumulate at the site of injury and thus lead to the recruitment of cells with CXCR4 such as fibroblasts and satellite cells. Thus, by using factors involved in accelerating cell proliferation, such as FGF or FBS, the efficiency of regeneration will be increased. In another phase of this study, cells were cultured in the presence of bFGF in two different concentrations (10 and 20 ng/ml):

1. Cell injection with and without bFGF: In our study, we noted that the 20 ng/ml concentration was more effective in recruiting and replacing cells in the injection site than the 10 ng/ml concentration. In a

similar study, Kasemkijwattana *et al.* injected several factors affecting muscle regeneration such as IGF1 (Insulin Growth Factor-1), FGF, and NGF (Nerve Growth Factor) to the site of damage to the calf muscle in mice and they observed countless muscles fibers had been regenerated in the injury site (26). In another study, Menetrey et al. found that FGF and IGF-1 were up to 5.3 times, and NGF up to 5.1 times more effective than controls (injection of saline instead of growth factor) in regenerating muscle fibers (27).

In our study, for faster, easier, and more cost-effective investigation of the effect of bFGF on the replacement of the cells injected into the damaged region, we decided to take a sample from male mice at the first stage, and after culturing and obtaining a sufficient number (confluency between 80-90% per flask) of these cells, they along with growth factor were injected into female mice. They were traced by sex determination. In a similar study, Menetrey et al. injected FGF, IGH, and NGF factors at the site of injury, and then after one month they used Hematoxylin and Eosin staining and imaging technique to evaluate the rate of regeneration (27). In another study by Bamman et al. direct injection of IGF growth factor at the site of severe muscle injury was found to be more effective in muscle regeneration than through the systemic injection (28). Increased use of growth factors as an accelerator and healer of damaged sites leads to reduced time of regeneration in the muscle after the injury. Our study showed that direct injection of healthy male muscle cells together with adequate FGF growth factor was effective in regeneration and the replacement of cells at the desired site in female mice. We showed that bFGF could accelerate both muscle regeneration and differentiation of satellite cells into muscle fibers (27, 29, 30) Although the sex determination method is fast and cost-effective method, it may not be able to specifically track muscle fibers compared to other conventional methods. In some studies, immunofluorescence and staining methods are commonly used in which the corresponding dye or antibody is specifically attached to the surface protein of muscle cells, and the amount of muscle fibers is determined more specifically (31).

2. Tumorigenesis*:* The fibroblast growth factor (FGF) signaling pathway plays a ubiquitous role in normal cell growth, survival, differentiation, and angiogenesis, but has also been implicated in tumor development (32). Furthermore, FGF receptors have variable activity in promoting angiogenesis, with the FGFR-1 subgroup being associated with tumor progression and the FGFR-2 subgroup with either early tumor development or decreased tumor progression. In this study, to investigate whether the use of bFGF factor can lead to tumorigenesis, two mice were kept longer for over 2 months and no side effects (mass and swelling at the injection site) were observed. There are no reports to challenge our findings (33, 34).

The results of our study may be helpful in the application of bFGF in the regeneration of damaged muscle. Muscle tissue, despite high regenerative capacity, may develop fibrotic scars in response to serious injury but bFGF may prevent this process by increasing the rate of muscle cell growth and regeneration. Further research is needed to evaluate the appropriate concentration of bFGf for regeneration. Also, the synergistic effects of this factor with other growth factors should be sought in different concentrations. The mechanism by which bFGF cells lead to decreased formation of fibrous tissue is to be elucidated.

Conclusion

Our findings show bFGF was effective in the regeneration of the injury site and confirmed the results of previous studies. However, no association was found with tumorigenesis.

Acknowledgments

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Conflicts of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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