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Ideal Concentration of Growth Factors in Rabbit's Flexor Tendon Culture

Ho Jung Kang and Eung Shick Kang

Abstract

Growth factors have the ability to stimulate matrix synthesis and cell proliferation in rabbit flexor tendon. Maximal stimulation effects of growth factors have a wide variation. It depends upon the different anatomic sites of the tendon segment, the kinds of growth factor, the concentration of growth factors, and the time sequence. Since proliferation was an early component of intrinsic tendon healing, we investigated the short-term dose response to four different growth factors on in vitro rabbit's tendon culture. We evaluated the effects according to the various concentrations of recombinant human insulin-like growth factor 1 (IGF), recombinant human epidermal growth factor (EGF), fibroblast growth factor (FGF), and recombinant human platelet-derived growth factor-BB (PDGF). Fetal calf serum was the most potent stimulator of cell proliferation and protein synthesis in in vitro rabbit's tendon culture. Matrix synthesis and cell proliferation were stimulated dose-dependently by IGF between the doses of 50 and 150 ng/ml. The maximum mitogenic effect of EGF was observed at the concentration of 100 ng/ml (1.3 times more than the media-only control culture). The rabbit's tendon responded significantly dose-dependently to PDGF, whereas there was no significant response to FGF.

Key Words: Tendon culture, growth factors (IGF, EGF, FGF, PDGF)

INTRODUCTION

Growth factors have the ability to stimulate matrix synthesis and cell proliferation in the rabbit flexor tendon. Maximal stimulation effects of growth factors have a wide variation. It depends upon the different anatomic site of the tendon segment, the kinds of growth factors, the concentration of growth factors and the time sequence. The combination effect of growth factors has already proved to stimulate healing of tendon tissue. Since proliferation is an early component of intrinsic tendon healing, we investigated the short-term dose response to four different growth factors on "*in vitro*" rabbit's tendon culture. We evaluated the effects according to various concentrations of recombinant human insulin-like growth factor I (IGF), recombinant human epidermal growth factor (EGF), fibroblast growth factor (FGF), and recombinant human platelet-derived growth factor-BB (PDGF).

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Department of Orthopedic Surgery, Youngdong Severance Hospital, Yonsei University College of Medicine, Seoul, Korea
Address reprint request to Dr. H.J. Kang, Department of Orthopedic Surgery, Youngdong Severance Hospital, Yonsei University College of Medicine, Youngdong P.O. Box 1217, Seoul 135-720, Korea. Tel: 82-2-3497-3412, Fax: 82-2-573-5393, E-mail: kangho56@yumc.yonsei.ac.kr

MATERIALS AND METHODS

New Zealand White rabbits were sacrificed via a lethal intracardiac injection of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, USA). Intermediate segments of the deep flexor tendons, 5 mm in length, between the metacarpophalangeal and proximal interphalangeal joints were collected from the second, third and fourth fingers of both forepaws. Five tendon segments were cultured in each group. Three repeated procedures were done with the same numbers of tendon. The tendon segments were placed, one segment per well, in 30-multidish plates and 1ml serum-free medium (MCDB 105; Sigma Chemical Co. St. Louis, MO, USA) was supplemented with ascorbic acid (50 μg/ml), transferrin (5 μg/ml), penicillin and streptomycin (50 μg/ml) and bovine serum albumin (1 mg/ml). When cell proliferation was studied, 1.2 μg/ml thymidine was added to the medium. Tendon segments were cultured for 7 days at 37°C in humidified 5% carbon dioxide. The medium was changed every 48 hours. Two control groups were used. The first control group was cultured in serum-free only media, whereas the other historic control had media supplemented with 10% fetal bovine serum (FBS: Sigma Chemical Corp., St. Louis, MO, USA). Platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF) and

recombinant human epidermal growth factor (EGF) (Collaborative Biomedical Products, Bedford, MA, USA) were used in four different concentrations (10 ng/ml, 50 ng/ml, 100 ng/ml, and 150 ng/ml) in serum-free culture media respectively. In every experiment, different concentrations of PDGF, IGF, EGF, FGF were applied every two days (second-day, fourth-day, sixth-day) during the experiment. In every experiment we compared in the two control groups (both serum-free group and medium with FBS group) the effects of different concentrations of growth factors after seven days of short-term tendon culture.

For the last day of every experiment media were supplemented with 3-H thymidine for measurement of DNA turnover, or 14C-proline (Amersham Corp., Arlington Hts, IL, USA) for measurement of total protein, at concentrations of 10 μ Ci/ml, respectively. Tendons were allowed to be incorporated with labeled materials during the last 24 hours of culturing. Labeled tendons were harvested and washed three times in medium. Dry weight was determined after lyophilization. Dried tendon segments labeled with 3H-thymidine, 14C-proline were hydrolyzed in stoppered test tubes in a 6M HCL at 70 degree Celsius for 24 hours. They were then hydrolyzed, heat evaporated to dryness, and redissolved in liquid scintillant (Budget-Solve Research Products International Corp., Mount Prospect, IL, USA). Results were calculated from the measurement of disintegrations per minute per mg (DPM/mg) of dry mass of tendon segment in a Racbeta liquid scintillation counter. Results of incorporation were expressed as DPM/mg dry weight of tendon. The amounts of labeled thymidine and proline incorporated in samples of tendons were graphed for comparison. Linear regression was calculated by the least squares method. Because some of the curves showed a biphasic tendency and a decreasing response at the highest level, linear regression analysis was limited in all curves to the purely ascending part. E_{max} refers to the estimated maximum response (dpm/mg) of an added factor and is given as a percentage of the response obtained by medium without the factors. Statistical significance of differences between the two groups was tested by Student's t-test. Multivariant analysis of the selected groups against each other was carried out statistically by ANOVA (Schaffé F-test). If the ANOVA of the multiple groups was significant; the test then respectively compared the groups individually; $p<0.05$ was considered statistically significant.

Table 1. 14C-Proline Radioactivity in Four Short-Term GFs Culture with Intermediate Segments of Rabbit's Flexor Tendon

RI activity GFs	RI activity of 14C-proline media only	RI activity of 14C-proline M+10% FCS
IGF-1	707±329	6290±1465
PDGF	731±169	2013±494
FGF	854±98	5401±594
EGF	674±62	1607±62

RI, radioisotope; GF, growth factor; M, media; FCS, fetal calf serum.

RESULTS

Tissue cultured in media with the 10% fetal calf serum (FCS) control group demonstrated statistically significant increases in thymidine and proline uptake, reflecting increased cell proliferation and total protein synthesis, respectively, when compared to the results which were cultured in the media-only control group, and also the mean value to that cultured in each of the various concentrations of growth factor groups. Along with EGF and PDGF of media with the fetal calf serum control group, cell proliferation and protein synthesis increased about three times higher than the media-only control group. Estimated maximal stimulation (E_{max}) of up to 10 times the control value was observed with IGF and FGF with FCS (Table 1). The effect of EGF was dependent on a concentration between 10 ng/ml and 100 ng/ml. However, between the concentration of 100 ng/ml and 150 ng/ml of EGF, we could not find a sig-

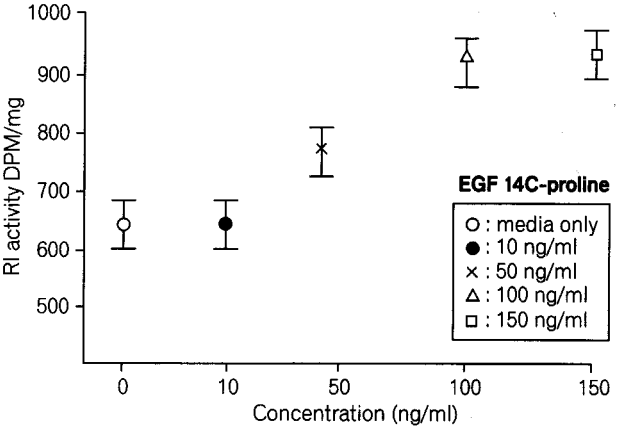

Fig. 1. Dose effect of 14C-proline (protein synthesis) in short-term EGF culture with intermediate segments of rabbit flexor tendon. Values are presented as mean radio uptake+SEM. n=5.

Table 2. Radioactivity in Short-Term PDGF Culture with Intermediate Segments of Rabbit Flexor Tendon

RI activity culture media	RI activity of 3H-thymidine	RI activity of 14C-proline
Media only (M)	494±93	731±169
M+10% FCS	1580±410	2013±494
10 ng/ml PDGF+M	516±27*	783±109*
50 ng/ml PDGF+M	544±96*	772±142
100 ng/ml PDGF+M	574±64*	796±111*
150 ng/ml PDGF+M	594±22*	809±146*

RI, radioisotope; GF, growth factor; M, media; FCS, fetal calf serum.
Radioactive uptake measured as 3H-thymidine and as 14C-proline in each tendon segment in different concentrations.
*A segement with a significantly different value from the serum-free control and the lower concentration segments at $p<0.05$.

nificant dose-dependent response (Fig. 1). Statistical analysis showed that IGF with the media-only group indicated a proliferative response in tendon culture for 7 days, with equivalent effects from 10 ng/ml to 50 ng/ml. The effect of IGF was concentration-dependent between 50 ng/ml and 150 ng/ml. E_{max} of up to two times the control value (media-only) was observed at 150 ng/ml of IGF. However, the Emax of cell proliferation by 100 ng IGF was twice that of PDGF or EGF. Of the other growth factors studied, only PDGF induced dose-dependent increases from 10 ng/ml to 150 ng/ml (Table 2). FGF added separately yielded no statistically significant changes when compared to the media alone.

DISCUSSION

Tissue repair entails a highly regulated sequence of events including chemotaxis of inflammatory cells and fibroblasts, angiogenesis, cellular proliferation and production and remodeling of extracellular matrix.[1,2] Coordination of this process is achieved by autocrine and paracrine actions of growth factors which serve to communicate signals from celll to cell and between cells and matrix.[3,4] Growth factor-like classic peptide hormones bind to specific receptor proteins on the surface of target cells and regulate a variety of cellular functions through the activation of several intracellular signals.[5,6]

Unlike classic hormones, they are important local mediators for cell regulations and they may be expressed in a wide variety of cells and can exhibit activity in a number of different target cells and tissues.[7] The tendon is a living connective tissue composed of cells and matrix with an intrinsic capacity for regeneration and healing.[8] The matrix of tendon is composed predominantly of type 1 collagen, but also of non-collagen components such as proteoglycans, elastin, and fibronectin. The deep flexor tendon shows segmental variations in histologic, vascular, and biochemical composition. Many factors affect the tendon healing process after injury to stimulate matrix synthesis and cell proliferation in the rabbit flexor tendon. Works are now focusing on attempts to better characterize the events which occur on a cellular level during this process. It is hoped that a better understanding of the mediators and effectors of the tendon healing process will allow clinicians to guide and direct healing more successfully in their patients who suffer from tendon injuries. Recent investigations into the mechanisms of tendon healing have indicated the interaction of many substances, including several growth factors.[9] They are expected to be applicable as therapeutics for clinical cases. The complex environment in vivo makes it difficult to determine the specific effects of growth factors on various cells involved in tendon healing. Serum is a complex mixture whose components are as yet poorly defined and whose composition and effect on cultures vary from batch to batch. Becker's study using 10% fetal calf serum showed that active cells were able to proliferate, and that the tendon defects filled with plasmin clot demonstrated acceleration of fibroplasia and collagen synthesis until the defect was reconstituted.[10] The effects of four different growth factors on matrix metabolism in the intermediate segment of deep flexor tendon from the tendon sheath region of the rabbit's forepaw were studied in culture for one week. The synergistic effects of FGF with FCS and IGF with FCS were stronger than EGF with FCS or PDGF with FCS. We confirmed the potent stimulation effect of fetal calf serum in rabbit tendon culture with three different growth factors.

Application of exogenous growth factors can modify the healing process of tendon. The quantitative responses of growth factors vary considerably from one tissue to another. Abrahamsson's study demonstrated that a single addition of IGF to a serum-free medium increased the healing potential of flexor tendon segments.[11] He found that IGF stimulated in short-term explant tendon culture in the interval 10 −250 ng/ml. In a cultured human fibroblast, IGF has the maximal stimulatory effect on collagen production at 500 ng/ml. This study was designed as

a means of determining the interaction between growth factors and concentrations on the process of tendon healing in in vitro rabbit's tendon culture. Matrix synthesis and cell proliferation were stimulated dose dependently by IGF at doses between 50 ng/ml and 150 ng/ml. The maximum mitogenic effect of EGF was observed at the concentration at 100 ng/ml (1.3 times over the media-only control culture). PDGF plays a role in the normal process of wound healing and has been demonstrated to accelerate the rate of healing of various types of wounds. Currently, the assay for PDGF utilizes its mitogenic activity on fibroblasts. Recent studies showed that protein synthesis of human breast cancer cells was stimulated by FGF at 10−100 ng/ml concentration.[12] In our experiment, the rabbit's tendon had a significant dose-dependent response to PDGF, whereas there was no significant response to FGF used alone. This result provides a rationale for further study on the role of growth factor and flexor tendon healing in the rabbit experiment.

In conclusion, fetal calf serum was the most potent stimulator of cell proliferation and protein synthesis in in vitro rabbit's tendon culture. Matrix synthesis and cell proliferation in serum-free media with different growth factors were stimulated dose dependently by IGF at doses between 50−150 ng/ml, by EGF at doses between 10−100 ng/ml, and by PDGF at doses between 10−150 ng/ml. These results may be beneficial in the early stage of rabbit's flexor tendon culture.

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