特定高齢者把握の「基本チェックリスト」回収1083人（回収率88.2%）

介護認定者を除き、基本検診も受けた者583人

上段：18年基準
下段：19年基準

	運動器	閉じこもり	栄養	認知	口腔機能	うつ	のべ数
	11人	12人	5人	24人	16人	24人	
	114人	35人	5人	70人	62人	67人	

実数	18年基準	38人	65歳以上の高齢者の	3.1%	全国平均	1.1%
	19年基準	155人		12.6%		?

＜結果①＞ 運動教室　半数以上参加（30名）

	参加前		参加後		
	平均値	標準偏差	平均値	標準偏差	P値
10M歩行時間（s）	8.45	±1.19	7.79	±0.89	0.031
Up & Go test（s）	9.75	±1.47	9.62	±1.45	0.648
継ぎ足歩行（歩）	6.19	±2.24	8.40	±1.58	0.000
単脚直立時間（s）	33.46	±23.16	53.34	±32.20	0.002
Functional Reach(cm)	24.48	±6.22	24.24	±3.33	0.884
開眼単位面積軌跡長(cm)	23.72	±9.74	35.03	±15.61	0.027
閉眼単位面積軌跡長(cm)	30.47	±12.73	38.75	±15.67	0.091
両手握力（kg）	21.29	±5.12	20.09	±5.37	0.023
腸腰筋筋力(N)	138.24	±38.63	181.31	±36.35	0.003
大腿四頭筋筋力(N)	161.05	±51.19	215.14	±56.02	0.000
BMI	21.14	±3.14	23.96	±3.02	0.271
体脂肪率(%)	32.16	±7.22	32.02	±6.90	0.755

＜結果②＞　運動教室　半数未満参加（18名）

	参加前		参加後		
	平均値	標準偏差	平均値	標準偏差	P値
10M歩行時間(s)	10.73	±3.51	10.49	±3.36	0.508
Up & Go test(s)	12.91	±3.87	13.83	±4.81	0.077
継ぎ足歩行(歩)	4.51	±2.97	5.69	±3.46	0.071
単脚直立時間(s)	20.19	±26.98	18.77	±29.08	0.647
Functional Reach(cm)	24.48	±6.22	24.24	±3.33	0.790
開眼単位面積軌跡長(cm)	23.95	±10.73	29.32	±11.06	0.123
閉眼単位面積軌跡長(cm)	28.92	±12.04	29.70	±13.43	0.840
両手握力(kg)	19.67	±7.69	19.85	±8.88	0.809
腸腰筋筋力(N)	130.11	±39.29	136.30	±44.31	0.531
大腿四頭筋筋力(N)	131.36	±58.11	146.90	±61.52	0.062
BMI	23.86	±2.54	23.76	±2.44	0.166
体脂肪率(%)	33.10	±6.98	32.05	±6.92	0.464

厚生労働科学研究費補助金（長寿科学総合研究事業）
分 担 研 究 報 告 書

高齢者における廃用症候群（生活不活発病）の実態調査と生活機能向上のための運動療法の開発

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研究要旨
神経損傷に起因する運動障害が廃用症候群へと移行することを防止する目的で、積極的に神経機能を回復させるための基礎研究を行った。神経機能修復の鍵となるリン酸化酵素Aktの基質探索をプロテオミクスの手法を用いて行い、中間径フィラメントが新たな標的になることを明らかにした。また、損傷神経の縫合が、細胞死を促進するBH3 onlyプロテインのNOXAの発現を抑制し、修復を促進していることが明らかになった。さらに、神経損傷時に神経で発現する分子の中に、加齢とともに増加する分子が存在することが示唆された。

A. 研究目的

廃用症候群あるいは生活不活発病の研究は臨床的な課題であると考えられがちであるが、そこには基礎医学の分野で解明すべき重要な課題が数多く包含されている。それらは先行する他の研究領域の間にあり、重点的に研究を推進されることはなかった。超高齢化社会を目前に、要介護者の急増など最も身近な且つ医療経済に大きな影響を及ぼす問題に直接かかわる基礎研究の重要性が増している。このような背景のもと、我々は運動神経の障害による運動機能低下から回復する方法や、機能低下を予防する手法の開発に向けて、基礎医学の立場から分子レベルの裏付けを持って新たな分子メカニズムを提言しようと考えている。本研究では、まず神経損傷に起因する運動障害が廃用症候群へと移行することを防止するため、積極的に神経機能を回復させるための基礎研究を行った。特に神経修復にきわめて重要なリン酸化酵素Aktの基質をプロテオミクスの手法で探索した。また、修復を促進すると考えられている神経の縫合の意義について分子レベルで解析した。さらに、運動神経の修復時に発現する分子のうち、加齢により発現が変動する分子の探索を試みた。

B. 研究方法

ラットとマウスの運動神経損傷動物モデルを作成した。舌下神経片側切断モデルを用い、以下の実験を行った。

（1）神経再生の鍵を握るセリン／スレオニンキナーゼAktの基質探索。
神経修復にとって鍵となるセリン／スレオニンキナーゼAktの基質の探索をプロテオミクスの手法で試みた。神経系の細胞株であるPC12細胞を用いた。PC12細胞に活性化Aktを発現する

アデノウイルスとコントロールとしてGFP蛋白を発現するアデノウイルスを感染させた。２日後にPC12細胞を回収し蛋白を精製し二次元電気泳動法にて蛋白を展開した。コントロールから得られた蛋白を二次元に展開したものと、Akt刺激をしたものを二次元電気泳動したものを膜に転写し、Aktがリン酸化する特定のアミノ酸配列を認識する抗体（セルシグナリング社）を用いて、ブロッティングした。これにより得られたAktの基質蛋白のスポットのうち、Akt刺激によりスポットが大きくなったものを選び、その位置の蛋白をゲルから回収した。回収した蛋白は、質量分析機にかけて、アミノ酸配列を同定しデータベースを検索することで、標的蛋白を同定した。

（2）神経修復を促進するための神経縫合の意義に関する分子的解析。

神経損傷時には修復のために神経を縫合することは一般に行われるが、その縫合の意義を分子レベルで検討した。損傷運動神経を縫合した場合としなかった場合での運動神経核における遺伝子発現の変化を解析した。マウス舌下神経損傷モデルを用い、神経切断後そのまま放置したものと、縫合したものを作成し、起始核の舌下神経核で発現する遺伝子をRT-PCR法とin situ ハイブリダイゼイション法により検索した。

（3）加齢に伴い神経で発現が変化する分子の検索。

加齢ラット（２０ヶ月）を用いて舌下神経を損傷し、発現する遺伝子を損傷部位の神経と、起始核である舌下神経核で検索した。遺伝子発現にはRT-PCR 法を用いた。

（倫理面の配慮）本研究では、ヒトの資料を直接使用することはなかった。また、動物や遺伝子実験については、大阪市立大学の機関承認を得たうえで適切に行った。

C. 研究結果

（1）神経再生の鍵を握るセリン／スレオニンキナーゼAktの基質の探索。

Akt の基質として新たに神経軸索に存在する中間径フィラメントのperipherin を同定した。損傷神経が生存し再生するために重要な役割を果たすAkt の神経での基質を探索するため、神経系の細胞である PC12 細胞にAkt を発現させ、蛋白質を回収後、2次元電気泳動を行った。これをAkt によりリン酸化を受けた部分アミノ酸を認識する抗体でブロッティングし、リン酸化が亢進したスポットを質量解析した。その結果、中間径フィラメントの peripherin が Akt によりリン酸化されることが新たに明らかになった。

（2）神経修復を促進するための神経縫合の意義に関する分子的解析。

切断神経の縫合を行うとアポトーシスを促進する分子 Noxa の発現が運動ニューロンで抑制されることが明らかになった。神経切断後縫合する場合と縫合しない場合では、ほとんどの遺伝子の発現変化は運動神経細胞では変わらないが、p53 依存性にアポトーシスを促進する Noxa という遺伝子の発現が縫合により発現が抑制されることが明らかになった。

（3）加齢に伴い神経で発現が変化する分子の検索。

加齢に伴い神経（シュワン細胞）で発現が増加する候補分子を確認した。

今のところ本分子の遺伝子は同定できていないが、その同定を試みている。このような分子が加齢に因る神経再生の遷延や低下などに関与している可能性がある。

D. 考察

軸索障害時に末梢神経は再生する能力を持つが、その再生の分子メカニズムのなかで、Akt は主要な役割をになう。特に損傷神経の細胞死を防御するための細胞内情報伝達系の鍵となっている。また、Akt には軸索伸展の作用もあることが知られており、細胞死防御と軸索伸展の両者において強力な作用を示す。このような Akt の基質としてはどのようなものが存在するかはまだ十分に知られていなかったが、今回のプロテオミクスによる研究で新たに中間径フィラメントの peripherin が同定された。今のところこのリン酸化が軸索再生などにどのように影響しているか不明であるが、ニューロフィラメントとともに、軸索の骨格形成にかかる分子であることから、再生の促進に何らかの役割を担っていると考えられる。peripherin の一部変異は ALS 等の運動神経の変性と関係していることが示唆されており、Akt による peripherin のリン酸化が運動神経の生存にどのように関与するか興味深い。

また、従来経験的に損傷神経は縫合すると再生が促進されることが知られているが、この分子的なメカニズムについてはあまり知られていなかった。もちろん、縫合することにより軸索再生の足場を提供することや、損傷遠位側のシュワン細胞からの栄養因子を受けやすいなど考えられていたが、今回の結果は全く新しいメカニズムの可能性を示唆した。すなわち縫合することで、細胞死を促進する Noxa の発現が抑制されたことである。他の再生を促進する分子群は縫合の有無により発現変化がなかったことから、縫合により発現する何らかの分子が Noxa の発現を押さえているものと考えられる。このことは神経縫合の意義の一部を初めて分子レベルで示したと考えられる。この Noxa 発現を抑制する分子を投与することにより縫合時の再生をより促進できる可能性が考えられる。本研究によって得られた結果より、peripherin のリン酸化や Noxa の遺伝子発現を制御することが今後の廃用症候群の機能回復や機能維持に向けての治療のターゲットとなりうる可能性が示唆された。

E. 結論

廃用症候群から回復へむけての基礎研究から、加齢により損傷神経で発現が亢進する分子が存在する可能性が明らかになり、加齢と神経修復能の低下をつなぐ新たな分子機序解明の可能性が期待される。また、神経縫合の分子レベルでの意義の解明は、今後の損傷神経修復の促進に向けてヒトでの応用の可能性を示唆する。

F. 健康危険情報

総括研究報告書参照

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

Ⅳ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Ⅴ．研究成果の刊行物・別刷

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Continuous local infusion of fibroblast growth factor-2 enhances consolidation of the bone segment lengthened by distraction osteogenesis in rabbit experiment

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Abstract

Experimental tibial lengthening was achieved in 61 rabbits to examine the effect of continuous local infusion of recombinant human fibroblast growth factor-2 (rhFGF-2) on bone healing of the lengthened segment. The tibial diaphysis was separated by osteotomy and was subjected to slow progressive distraction (rate: 0.35 mm/12 h) using a monolateral external fixator. There were a lag phase for 1 week, a distraction phase for 2 weeks, and a consolidation phase for 5 weeks in this experiment. At various stages of distraction, rhFGF-2 was infused continuously for 2 weeks into the lengthened segment (rate: 14.28 μg/60 μl/day) using an osmotic pump implanted under the skin. Bone healing was significantly accelerated when rhFGF-2 was infused in the beginning of consolidation phase, but not in the distraction phase or in the lag phase. Infusion of normal saline (N/S) using the same osmotic pump had no effect. Dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computerized tomography (pQCT) studies demonstrated that rhFGF-2-treated tibia had increased bone mineral density (BMD), bone mineral content (BMC) and cortical bone thickness (CBT) when compared with N/S-treated tibia. Three-point bending test demonstrated that rhFGF-2-treated bone had significantly stronger mechanical properties than N/S-treated bone. Finally, distribution of the infused materials was checked by using Indian ink or radio-opaque. The dyes distributed widely but exclusively in the lengthened segment. Based on these results, we conclude that direct delivery of rhFGF-2 into the lengthened segment can shorten the consolidation phase of limb lengthening and the method is applicable to the clinical treatment.

Keywords: Local injection; FGF-2; Distraction osteogenesis; Osmotic pump; Limb lengthening

Introduction

Recent advances of external fixation and newer knowledge of distraction osteogenesis have brought a revolution in surgical treatment of congenital or post-traumatic short extremities. Various types of external fixation devices have been developed to achieve limb lengthening and simultaneous correction of the complex bone deformities [1–3]. Lengthening a bone for more than 10 cm is now possible, if the proper technique is used. Principle of distraction osteogenesis has also been applied in treatment of segmental bone loss, infected nonunion and congenital pseudoarthrosis of the bone [4,5].

We have been engaged in studying the basic mechanism of distraction osteogenesis using animal models of limb lengthening [6–10]. Biological events of distraction osteogenesis are understandable if the treatment process is divided into three distinct phases, i.e., a lag phase, a distraction phase, and a consolidation phase. During the lag phase after osteotomy, blood circulation recovers and immature callus is formed around osteotomy site [8]. During distraction phase, new bone regenerate is continuously formed within the lengthened segment. During consolidation phase, the lengthened segment matures and bone union is obtained while the external fixator is still on.

The factors affecting bone healing during distraction osteogenesis may include type of osteotomy, timing and rate of distraction, stability of fixation, age of the patient and underlying disease. Although the efforts to improve osteotomy techniques and stability of fixation [8,11,12], the overall treatment time of limb lengthening still requires a long period. Healing indices, calculated by dividing the treatment time with the amount of lengthening, ranged from 28 to 36 days/cm

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[8,13–15]. The patient has to tolerate wearing a bulky external fixator at least for several months until consolidation of the lengthened segment is obtained.

Several authors have attempted to promote bone formation during distraction osteogenesis by local administration of growth factors or cytokines [16–19]. Okazaki et al. [16] reported that a single-shot injection of rhFGF-2 into the regenerating bone was effective to stimulate bone healing in rabbit tibial lengthening. In the present study, we are demonstrating that continuous local infusion of a low-dose rhFGF-2 into the lengthened segment can accelerate bone healing of the lengthened segment in rabbit model.

Materials and methods

Animals

Animal experiment was carried out on 61 male Japanese white rabbits, weighing 1.8 to 2.2 kg, purchased from Oriental Yeast Co. (Tokyo, Japan).

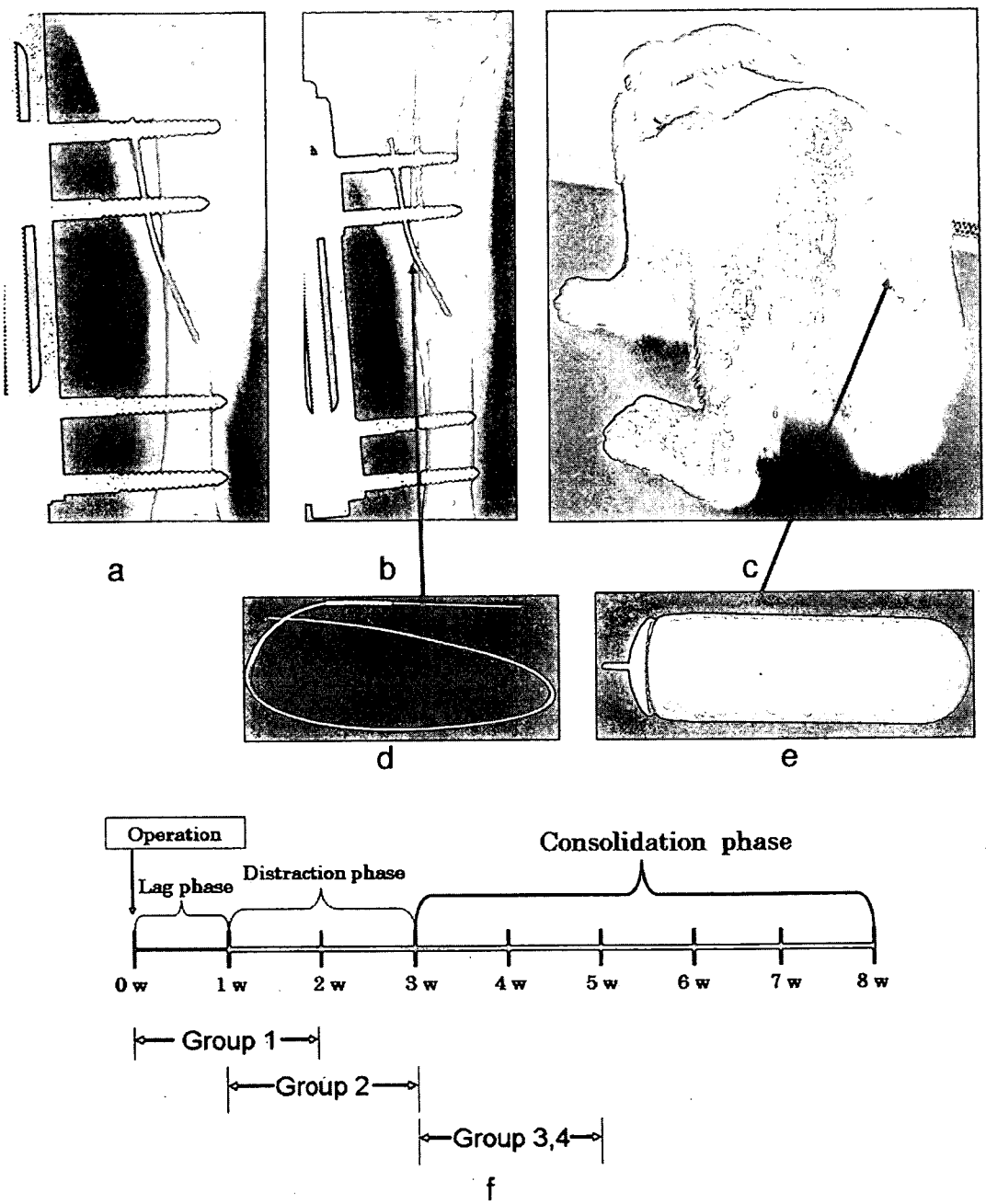

Fig. 1. Experimental design: Radiographs showing the position of the needle at the time of operation (a) and at the end of distraction (b). The osmotic pump (e) was implanted subcutaneously on the back of a young rabbit (c). The pump was connected to a plastic catheter (d) that reached the needle (a, b) inserted into the lengthened segment. Local infusion of rhFGF-2 was achieved for 2 weeks at various stages of the experiment (f). The animals were sacrificed 8 weeks after operation.

The protocol of animal experiment was approved by the local animal protection agency and the ethics committee of the University of Tokushima.

All rabbits were anesthetized by intravenous injection of ketamine hydrochloride and xylazine at doses of 20 mg/kg and 5 mg/kg body weight, respectively. After a unilateral external fixator (Orthofix M-100) was applied to the tibia with four self-taping screws, subperiosteal osteotomy was achieved between the second and third screws using a fine wire saw. A small drill hole was made above the osteotomy and a 23-gauge needle (Fig. 1d) was inserted obliquely into the marrow cavity until it reached the distal bone fragment (Fig. 1a). The needle was connected with a polyvinyl catheter, which was embedded in the subcutaneous tissue until use.

There was a lag phase for 1 week before distraction was started at a rate of 0.35 mm every 12 h (0.7 mm/day). Distraction was continued for 2 weeks so that the actual lengthening of 10±0.1 mm was achieved. After completion of distraction, the animals were kept for 5 weeks until consolidation of the lengthened segment was obtained. At various stages of the experiment, the animals were anesthetized again for subcutaneous implantation of an osmotic pump [(Alzet 2ML4) purchased from Muromachi Kikai Co., Ltd., Osaka, Japan] on their back (Figs. 1c, e). The pump was connected to the polyvinyl catheter which was embedded subcutaneously at the time of primary operation so that the solution in the pump was gradually delivered into the center of the lengthened segment for 14 days (Fig. 1b).

Nine animals were used for preliminary experiment to determine the appropriate dose of the growth factor. Three different doses of rhFGF-2 (7.14 μg/60 μl/day, 14.28 μg/60 μl/day and 35.71 μg/60 μl/day) were infused into the lengthened segments for 14 days in the beginning of the consolidation phase. In the final experiment (43 animals), a constant dose of rhFGF-2 (14.28 μg/60 μl/day) was applied for 14 days at various stages of distraction.

Group 1 (3 animals)	rhFGF-2 (14.28 μg/60 μl/day) was administered for 7 days in the lag phase and for a subsequent 7 days in the distraction phase.
Group 2 (4 animals)	The same dose of rhFGF-2 as in Group 1 was administered for 14 days in the distraction phase.
Group 3 (16 animals)	The same dose of rhFGF-2 as in Group 1 was administered for 14 days in the beginning of the consolidation phase.
Group 4 (15 animals)	Normal saline (N/S) 60 μl/day was administered for 14 days in the beginning of consolidation phase.
Group 5 (5 animals)	Sham operation; the needle was placed into the center of distraction gap and empty pump was implanted in the back.

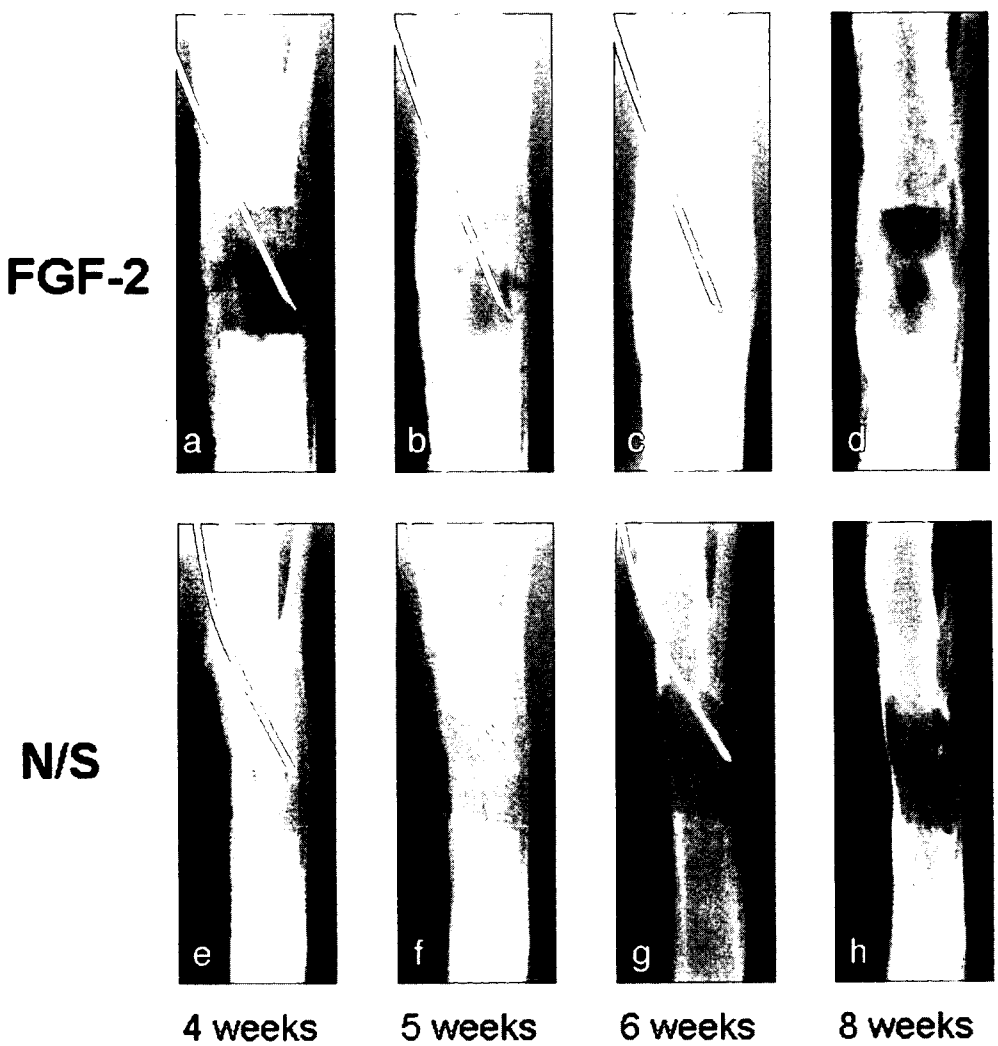

Fig. 2. Radiological changes of the lengthened segment after infusion of rhFGF-2 (a–d, Group 3 animals) and N/S (e–h, Group 4 animals). (a, e) 4 weeks, (b, f) 5 weeks, (c, g) 6 weeks, and (d, h) 8 weeks after operation, respectively.

The animals were housed under standardized environmental condition with 12-h light/dark cycles, and fed standard rabbit chow (RC4, Oriental Yeast Co., Tokyo, Japan). The process of bone formation was monitored every week by soft X-ray (Model CMB-2T Softex Co., Kanagawa, Japan). All rabbits were sacrificed at 8 weeks after operation by injection of sodium pentobarbitone (60 mg/kg body weight) and the external fixator, the polyvinyl catheter and the osmotic pump were removed.

In a subset of the experiment (9 animals), distribution of the infused material was analyzed by using radio-opaque (Urografin) or Indian ink. These dyes were infused into the lengthened segment at various stages of the experiment using the same osmotic pump (60 μl/day).

Densitometry

At 4, 5, 6, and 8 weeks after operation, the animals were anesthetized and DXA analysis was performed using Hologic 2000W device (Waltham, MA, USA). Images of the lengthened tibiae were divided into three distinct regions, namely, the proximal tibia above the osteotomy, the lengthened segment, and the distal tibia below the osteotomy (Fig. 4). Each segment had 10 mm in length. BMC was measured for each region. BMC of the non-operated tibia was also measured as a control.

pQCT studies

At the end of experiment, the animals were sacrificed and the tibiae were prepared for pQCT study. Using the Stratec pQCT (XCT-960A; Norland/Stratec, Fort Atkinson Pforzheim, USA/Germany), a total of 18 slices were analyzed in each tibia. Six slices within the lengthened segment, 6 slices above and 6 slices below the osteotomy, each 1.67 mm in thickness, were analyzed. Six slices of the right tibia (non-operated bone) were used as controls.

Histological analysis

Harvested tibiae were fixed with 10% neutral formalin for 5 days, decalcified in 20% aqueous EDTA for 6 weeks and then embedded in paraffin. Coronal sections of 3 μm in thickness were stained with hematoxylin and eosin.

Mechanical analysis

The mechanical properties of the tibiae were examined at the end of experiment (8 weeks after operation). The lengthened tibiae were cleaned of soft tissue and three-point bending strength was measured until failure with a support span of 40 mm between the second and third pin holes, using a servohydraulic

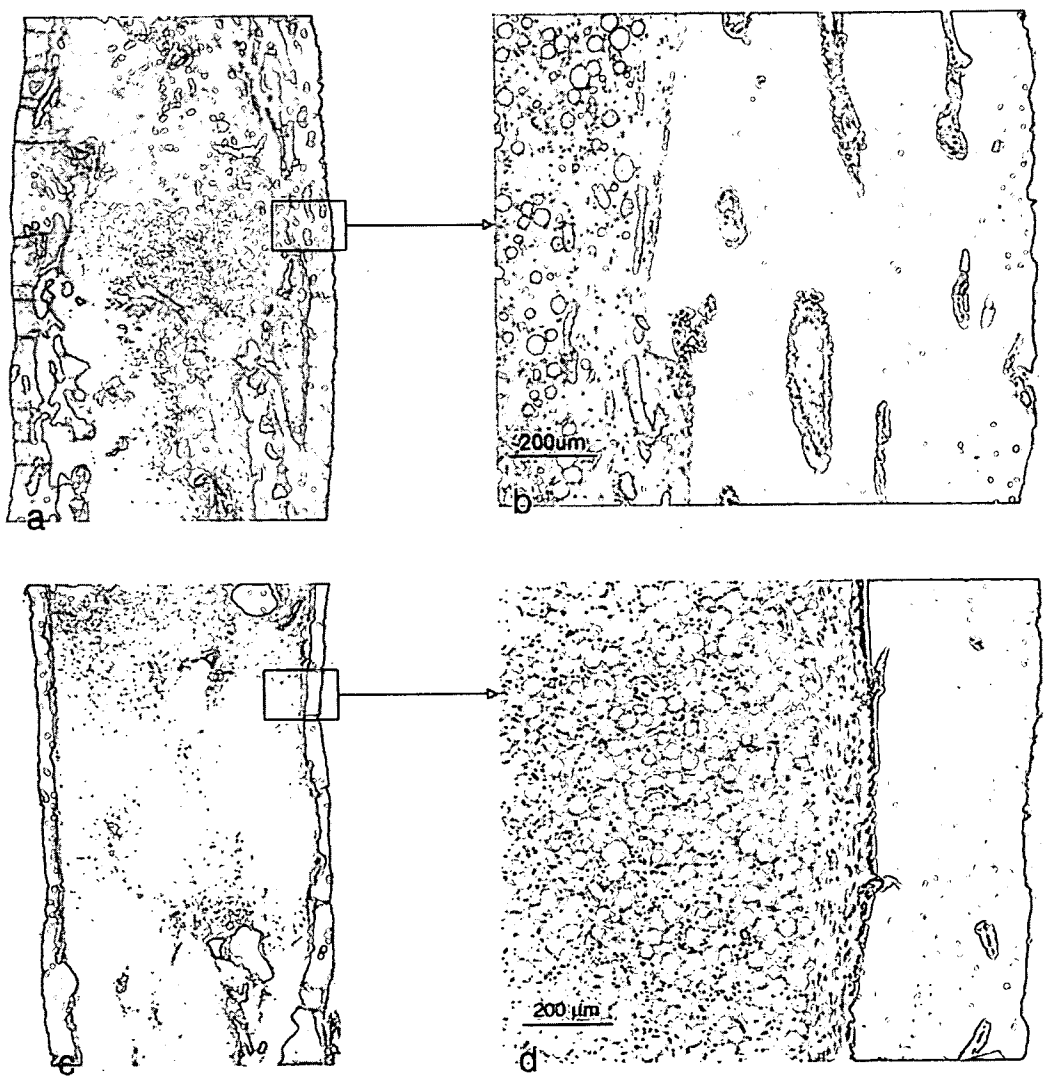

Fig. 3. Longitudinal sections of the lengthened segment prepared at 8 weeks after operation (hematoxylin–eosin staining). (a, b) rhFGF-2 infused tibia in Group 3 animals. (c, d) N/S infused tibia in Group 4 animals.

materials testing system (S-100; Shimazu, Kyoto, Japan) with a 1-kN load cell under displacement control (5 mm/min). The ultimate force, stiffness, and work to failure were determined as described previously [10].

Statistical analysis

Statistical analysis was performed using unpaired Student's *t* test. Each data show the mean±the standard deviation (SD). Values of $p<0.05$ were considered to be significant.

Results

Radiological findings

In all groups of the animals, lengthening of 10±0.1 mm was successfully achieved and bone consolidation was obtained by the end of experiment. In the control animals, the lengthened segment showed a characteristic zone structure consisting of central radiolucent zone and two sclerotic zones (Fig. 1b) as described previously [6]. After completion of distraction, the proximal and distal sclerotic zones became fused, shrank and were eventually absorbed. By the end of experiment, the lengthened segment showed tubular bone structure with a new cortex.

Three different dose of rhFGF-2 were tested in the preliminary experiment. The radiological findings suggested that bone formation was significantly enhanced when rhFGF-2 was infused at the dose of 14.28 μg/60 μl/day or 35.71 μg/60 μl/day for 14 days in the beginning of consolidation phase. Very small effect was detected when rhFGF-2 was applied at the dose of 7.14 μg/60 μl/day (data not shown). Subsequently, a constant dose of rhFGF-2 (14.28 μg/60 μl/day) was used in the final experiment.

Continuous local infusion of rhFGF-2 was achieved at various stages of distraction. There was no significant effect on bone formation when rhFGF-2 was applied in the lag phase (Group 1) or in the distraction phase (Group 2) (data not shown). When the same dose of rhFGF-2 (14.28 μg/60 μl/day) was applied for 2 weeks in the beginning of the consolidation phase (Group 3), however, the periosteal callus dramatically expanded and the outer diameter of the lengthened segment was significantly increased (Figs. 2a–d). In N/S-infused tibiae in Group 4 animals, the periosteal callus was gradually resorbed and replaced by a thin cortical bone by the end of experiment (Figs. 2e–h).

Histological findings

Consistent with the radiological findings, continuous local infusion of rhFGF-2 in the lag phase (Group 1) or in the distraction phase (Group 2) produced no significant effect on

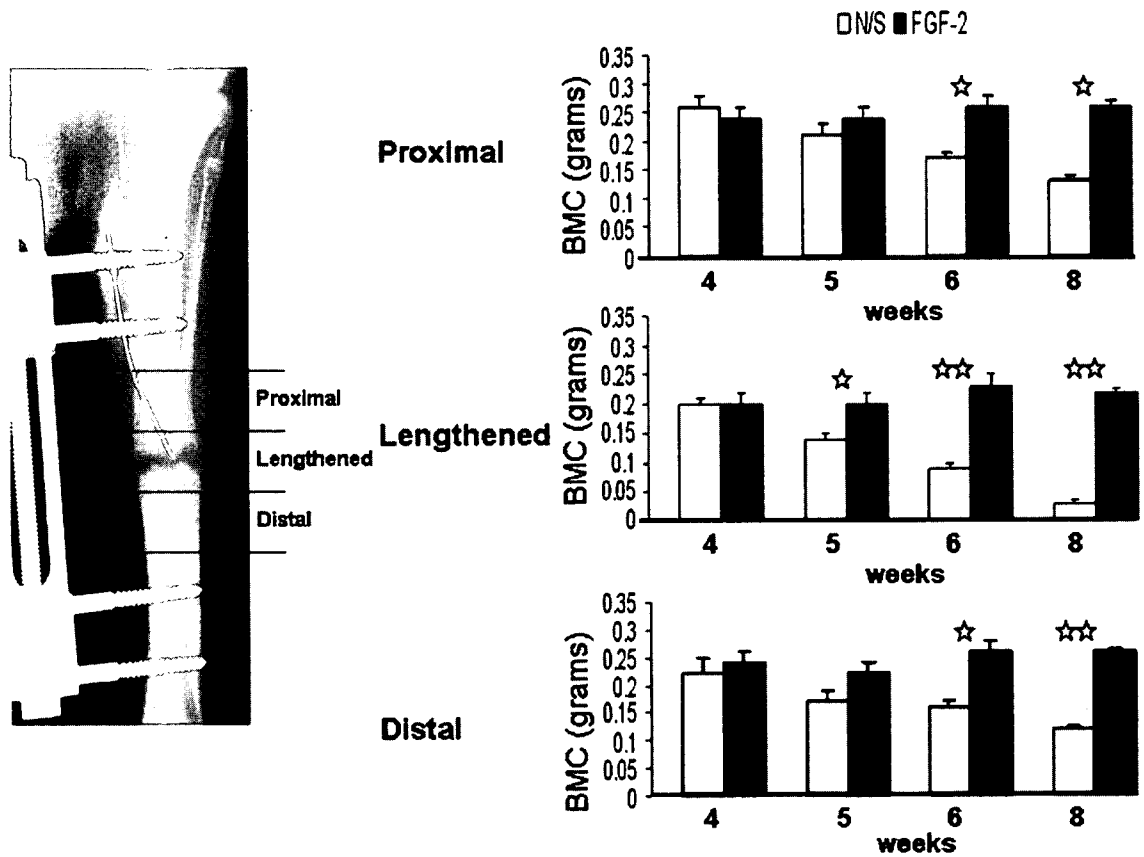

Fig. 4. The time course of bone mineral content (BMC) in the proximal, lengthened, and distal parts of the tibia measured by DXA (4, 5, 6 and 8 weeks post-operation). *$p<0.05$, **$p<0.001$.

histological structures of the lengthened segments (data not shown). Infusion of rhFGF-2 in the beginning of consolidation phase, however, produced dramatic increases in cortical bone thickness and overall diameter of the lengthened segment (Fig. 3).

DXA studies

BMC and BMD were measured exclusively in Group 3 and Group 4 animals. Three distinct areas of the lengthened tibia were analyzed by DXA (Fig. 4). The proximal and distal segments contained the original tibia above and below osteotomy, each 10 mm in length, respectively. The middle segment was the lengthened segment consisting of new bone regenerate.

In control animals (Group 4), consistent with radiological findings, BMC of the lengthened segment declined gradually during consolidation phase (Fig. 4). Not only the lengthened segment but also the proximal and distal segment gradually lost BMC. Continuous local infusion of rhFGF-2 into the lengthened segment in the beginning of the consolidation phase (Group 3) perfectly prevented the declination of BMC (Fig. 4). Consequently, by the end of experiment, BMC of the new bone regenerate in Group 3 animals showed seven times higher than that in Group 4 animals ($p<0.001$).

The BMD measured by DXA showed similar patterns as BMC (data not shown).

pQCT studies

Fig. 5 shows cross sections of the tibia made by pQCT at the end of experiment. Interestingly, the lengthened segment in Group 3 animals had double-layer bone cortices, whereas those in Group 4 animals had a single layer bone cortex. Consequently, CBT of the lengthened segment in Group 3 animals was two times larger than that in Group 4 animals ($p<0.01$) (Figs. 5 and 6b). There was no significant difference in cross-sectional morphology of the proximal and distal segment among Group 3 and 4 animals. There was no significant change in CBT of the contralateral tibia in both groups.

Fig. 6 shows volumetric BMD calculated by pQCT data. Consistent with DXA studies, continuous local infusion of rhFGF-2 into the lengthened segment (Group 3) had a significant effect to prevent decrease in BMD of all three distinct areas. Consequently, BMD of the lengthened segment in Group 3 animals was 162% of those in Group 4 animals ($p<0.001$).

Mechanical analysis

Three-point bending test demonstrated that the ultimate force of the lengthened tibia in Group 3 animals was 72% larger than that in Group 4 animals ($p<0.008$, Fig. 6c). There were no significant differences of the ultimate force in contralateral tibia between two groups.

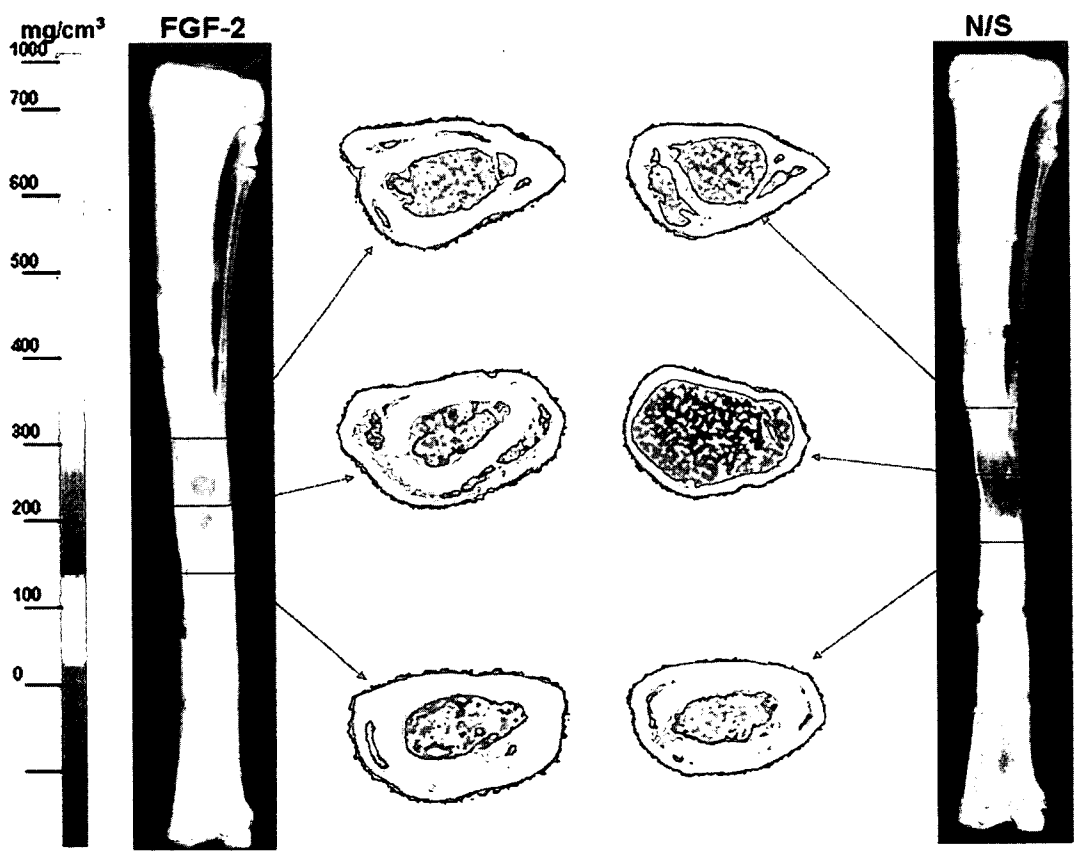

Fig. 5. The pQCT images of the proximal, lengthened and distal of the distracted tibia at 8 weeks post-operation.

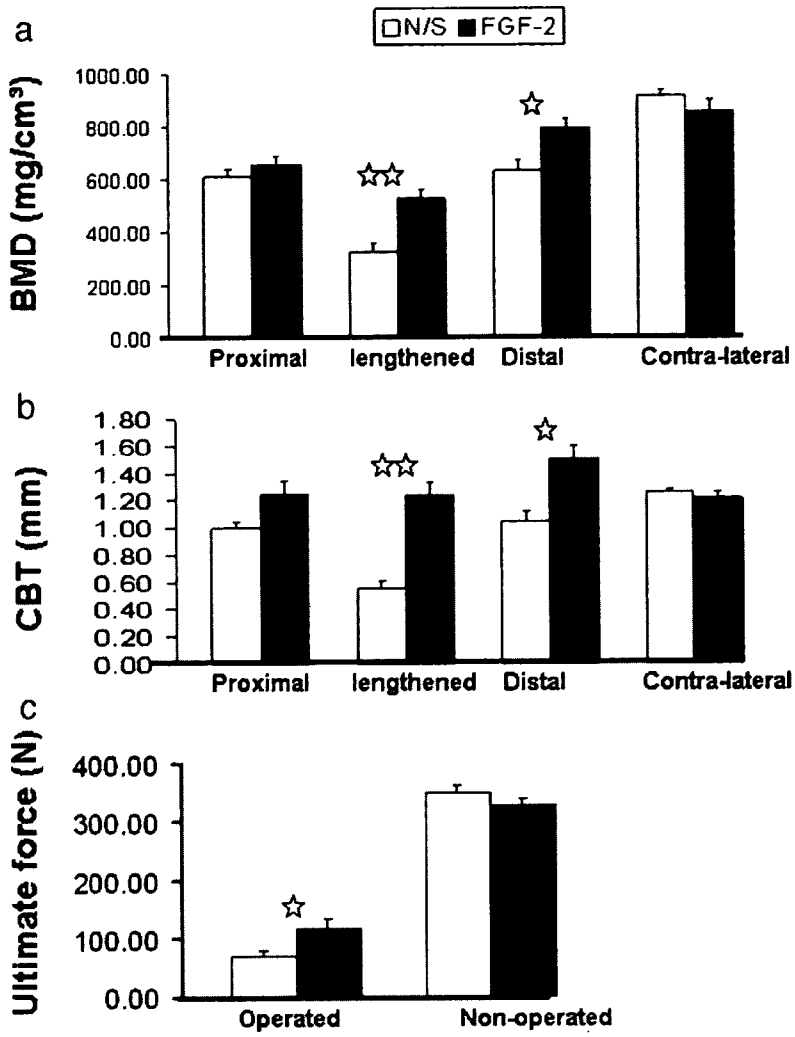

Fig. 6. BMD (a) and CBT (b) of the proximal, lengthened and distal parts of the distracted tibia at 8 weeks after operation measured by pQCT. Ultimate force of the lengthened segments (c) was measured by three-point bending test. $^{*}p<0.05$, $^{**}p<0.001$.

Distribution of infused materials

In order to examine the distribution of locally infused materials, osmotic pump was filled with radio-opaque marker (Urografin) or Indian ink. Figs. 7a and b demonstrate the accumulation of Urografin in the center of the lengthened segment after 24 h infusion in the beginning of consolidation phase. Fig. 7c demonstrates the accumulation of Indian ink (blue) within the lengthened segment in the same condition. When these marker dyes were infused either in the lag phase or in the distraction phase, they rapidly leaked into the surrounding tissue and only a limited accumulation of the dye in the lengthened segment was detected (data not shown).

Discussion

FGF-2 is a wide-spectrum mitogenic, angiogenic, and neurotrophic factor being expressed at low levels in many tissues and cell types. The molecule consists of 154 amino acids and has a relatively short half life [20].

The effect of FGF-2 on bone formation has been examined in animal experiments. Systemic administration of FGF-2 stimulated endosteal bone formation, resulting in an increase in the cortical bone thickness of the rat tibia [18,21,22]. Systemic administration of high-dose FGF-2, however, is not recommended for clinical use because it may produce undesirable side effects. Cooper et al. [23] reported that intravenous injection of FGF-2 was associated with a high rate of proteinuria in humans.

The effect of local administration of FGF on bone formation has also been examined in animal experiments. Kawaguchi et al. [24] applied the recombinant human FGF-2 on fracture healing model of non-human primates. Bone union was obtained by 6 weeks in all 10 animals treated with FGF-2, while 4 of 10 animals treated with the vehicle alone remained in a nonunion state even after 10 weeks. They proposed rhFGF-2 as a potent bone anabolic agent for clinical use, since it had

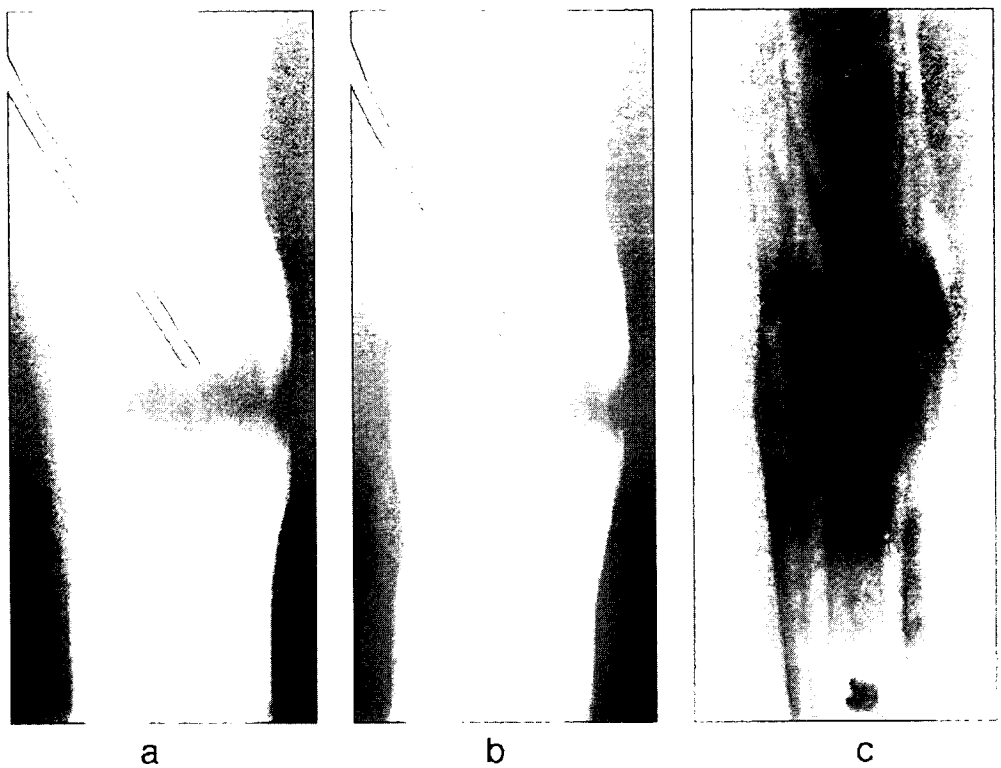

Fig. 7. Radiographs of the lengthened tibia before (a) and after (b) infusion of Urografin (60 μl/24 h) at the beginning of the consolidation phase. Distribution of Indian ink is shown in the frontal section of the lengthened segment (c).

improved the mechanical properties of the bone. Nakamura T. et al. [25] reported in a dog experiment that local administration of rhFGF-2 accelerates fracture healing and remodeling of the callus.

Nakamura K. et al. [26] examined the effect of intraosseous injection of rhFGF-2 at various dose (4–1600 μg). A relatively high dose (>400 μg) of rhFGF-2 was required to induce new bone formation around the injection site. Okazaki et al. [16] demonstrated that a single-shot injection of FGF-2 (200 μg in 150 μl N/S) into the distraction osteogenesis stimulated bone formation in rabbits.

In the present study, we established a novel method to deliver the growth factors directly into the regenerating bone in limb lengthening. An overall dose of rhFGF-2 used in the present study was 10 times less than those in the previous studies with systemic or local injection [18]. In addition, application of rhFGF-2 into the regenerating bone segment resulted in expansion of the periosteal callus. By the end of the experiment, CBT of the lengthened segment reached the same level as that of the normal bone. In the control animals, severe bone atrophy was observed during the consolidation phase not only in the lengthened segment but also in the proximal and distal segments. Continuous infusion of rhFGF-2 in the beginning of the consolidation phase dramatically prevented those bone atrophy, resulting in an improvement of mechanical properties of the lengthened tibiae at the end of the experiment. There was no significant effect on the contralateral tibia, suggesting that systemic effect is negligibly small in this system.

It is generally known that significant osteopenia occurs during limb lengthening especially in the distal segment below osteotomy [27]. Indian ink injection in the present study suggested that locally infused rhFGF-2 should mostly have stayed in the regenerated part of the lengthened segment. Some of them, however, may infiltrate into the periosteum and also into the proximal and distal parts of the regenerated part and stimulate the local bone formation. This phenomenon should explain the increases in BMD not only in the lengthened segment but also in the proximal and distal segments.

It is not known why infusion of rhFGF-2 in the lag phase or in the distraction phase resulted in a small effect on bone healing. Indian ink study suggested that a low-dose of rhFGF-2 infused at this stage might have leaked into the surrounding tissue.

Various growth factors besides FGF-2 have been used to stimulate bone healing during distraction osteogenesis. Li et al. [17] reported that rhBMP-2 had positive effects when it was injected subcutaneously or applied with absorbable collagen sponge. The BMP-2 may stimulate osteogenic differentiation of the periosteum-derived mesenchymal cells, while FGF-2 may stimulate proliferation of those mesenchymal cells [28,29].

In conclusion, the present study has demonstrated that continuous local injection of FGF-2 into the lengthened segment during the consolidation phase definitely stimulates bone healing during distraction osteogenesis. Increased BMC, BMD and CBT of the lengthened segment as well as the proximal and distal segments should produce increased mechanical properties

of the whole tibia. The potential risk of the present method is infection of the implanted pump. By using an extracorpal pump, instead of an osmotic pump, this method is applicable to clinical treatment and will definitely contribute to shorten the treatment time of limb lengthening by distraction osteogenesis.

Acknowledgments

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ORIGINAL PAPER

Continuous infusion of insulin-like growth factor-I into the epiphysis of the tibia

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Abstract We have developed a method to promote longitudinal bone growth at the level of a specific growth-plate (GP) in young rabbits. Insulin-like growth factor-I (IGF-I) was continuously infused by means of an osmotic pump into the bone marrow cavity of the proximal epiphysis of the tibia. Radiological measurement showed a 2-mm overgrowth of the tibia after 4 weeks of treatment, while histological analysis demonstrated a 15% increase in the thickness of the selected GP. The local infusion of IGF-I increased the numbers of both proliferative and hypertrophic chondrocytes and promoted hyperplasia of bony trabeculae within the epiphysis. The distribution of material infused locally into the epiphysis was simulated by the infusion of Indian ink using the same methodology (osmotic pump) as that for IGF-I. Most of the dye remained within the bone marrow cavity of the epiphysis, but a portion infiltrated into the GP, reaching the deep layer of the physeal chondrocytes and primary spongiosa of the metaphysis. These results suggest that the method reported here is a valid one for delivering cytokines or growth factors to the selected GP and for controlling the growth and differentiation of physeal chondrocytes.

Résumé Nous avons mis au point une méthode pour évaluer la croissance longitudinale du cartilage de croissance chez les jeunes lapins. Nous avons utilisé pour cela une pompe osmotique avec utilisation d'un facteur de croissance (IGF1) insuline like growth factor en injection continue au niveau de la cavité médullaire à proximité de l'épiphyse du tibia. Radiologiquement, les mesures ont montré 2 mm de croissance supplémentaire sur le tibia après 4 semaines de traitement. L'analyse histologique a également montré que le cartilage s'était épaissi de 15%, le nombre de chaque type de chondrocyte étant augmenté. Par ailleurs cette perfusion d'IGF1 entraîne une hyperplasie de l'os trabéculaire au voisinage de l'épiphyse. La diffusion du produit a été visualisée à l'aide d'un marqueur à base d'encre de chine injecté en même temps dans la pompe osmotique. La plupart du liquide reste au niveau de la cavité médullaire de l'épiphyse et une partie de ce liquide s'infiltre au niveau de la plaque de croissance atteignant les couches profondes et l'os spongieux primaire de la métaphyse. Ces résultats nous permettent de penser que cette méthode est utile pour apporter des cytokines et des facteurs de croissance de façon sélective au niveau de la plaque de croissance et permet également de contrôler la croissance et la différenciation cellulaire des chondrocytes.

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Introduction

In childhood, the ends of the long bones, called the epiphyses, are separated from the diaphysis by the cartilaginous growth-plates (GPs). These GPs are exclusively responsible for the longitudinal growth of bone. Traumatic injury [16], infection [1] or tumour [5] involving the GP may cause impairment of the longitudinal growth of the bone, resulting in various deformities and inequality in the length of the affected limb. Fractures of the diaphysis in children, on the other hand, and inflammatory lesions